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Research Article

Genetic Markers Indicate that 1,25-dihydroxyvitamin D Treatment may not Protect Against Hepatocellular Carcinoma

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Abstract

Objectives: The impact of 1,25-dihydroxyvitamin D on hepatocellular carcinoma (HCC) cells is a complicated area. In this study, we aimed to clarify the effect of 1,25-dihydroxyvitamin D on HCC cells according to genetic markers.

Methods: The optimal concentration of 1,25-dihydroxyvitamin D is treated to HepG2 cells (250 nM at the 48th hour). From treated HepG2 cells, total Ribonucleic Acid (RNA) was isolated, and Ki-67, MMP-2, MMP-9, HIF-1α, hTERT, and piR-823 gene expressions were determined by SYBR Green-based real-time polymerase chain reaction.

Results: : Increased expressions of Ki-67, hTERT, and piR-823 were determined compared with the control group at the 48th hour after treatment ($p<0.001$), while decreased gene expressions of MMP-2, MMP-9, and HIF-1α were observed compared with the control group ($p<0.001$).

Conclusion: Currently, there are several different opinions about the usage of vitamin D, especially in HCC. In addition to researchers who argue that vitamin D has anticarcinogenic and protective properties, an increasing number of researchers argue that tumor cells can become aggressive after HCC occurs. According to our results, it was determined that vitamin D causes HepG2 HCC cells to become aggressive in terms of gene expression in the parameters used as a marker for proliferation, adhesion, and differentiation.

Keywords: 1.25-Dihydroxyvitamin D, hepatocellular carcinoma, motility, PIWI interacting RNA, proliferation

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Hepatocellular carcinoma (HCC) is the fourth lethal cancer type worldwide. It can be treated easily if it is diagnosed at early stages. Nowadays, some natural compounds, synthetic essential molecules, and vitamins are used to protect against cancer.[1-5] Vitamin D is a lipophilic molecule, and some forms can be used directly in the liver and kidney.[6] 1,25-Dihydroxyvitamin D is the active form of vitamin D and acts on the molecular mechanisms of cells. Some previous studies suggested that 1,25-dihydroxyvitamin D is used to protect against cancer, especially HCC. However, recent studies indicated that 1,25-dihydroxyvitamin D may have negative effects on cells and suggested that 1,25-dihydroxyvitamin D usage cannot be effective for treatment during carcinogenesis.[3,7-11]

Ki-67 is a transcription factor, and its expression increases in cancer cases. *Ki-67* expression has been known as a biomarker of proliferation. High *Ki-67* expression, as well as hypoxia-inducible factor-1 alpha *(HIF-1α)* expression, results in low differentiation and poor prognosis in HCC.[12] HIF-1α is another transcription factor and prognostic marker that is correlated with a higher rate in lymph node metastasis for HCC.[13] Matrix metalloproteinases (MMPs)

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are special enzymes that degrade the extracellular matrix and promote angiogenesis and tumor invasion and migration. When the extracellular matrix is disrupted, cancer cells begin to invade adjacent tissues, and metastasis occurs.[14] *MMP-2* and *MMP-9* are functional MMP members that have high gene expression patterns during metastasis and invasion of cancer cells.[15] The human telomerase reverse transcriptase (*hTERT*) gene region is another proliferation marker of cancer cells. By determining the expression changes of *hTERT*, researchers can have an idea of cancer cell proliferation and survival. *hTERT* is a catalytic enzyme that regulates telomerase activity. In cancer cells, approximately 90% of tumors have high *hTERT* expression. *hTERT* indicates not only telomerase activity but also proliferation rates of cancer cells.[16] PIWI-interacting RNAs (piRNAs; piRs) are small noncoding RNAs that are 24-31 nucleotides in length. piRNAs act to regulate target genes by transposon silencing and reduce proliferation and proangiogenic activity of cancer cells or promote apoptosis. They can be classified as tumor-suppressive or oncogenic piRNAs according to their functions. *piR-823* is a member of the piRNA family and has an oncogenic activity in cancer cells.[17]

In this study, we aimed to determine the effect of 1,25-dihydroxyvitamin D on HepG2 HCC cells according to gene expressions of various markers. We observed the proliferation changes by detecting *Ki-67*, *hTERT*, and *HIF-1α* gene expressions, while the adhesion of cells was detected by *MMP-2* and *MMP-9* gene expressions. Moreover, we identified that 1,25-dihydroxyvitamin D causes the upregulation of an oncogenic piRNA expression, piR-823. These data are significant for HCC researchers to understand what 1,25-dihydroxyvitamin D treatment causes cellular behaviors in a genetic perspective.

Methods

Cell Culture and 1,25-Dihydroxyvitamin D Treatment: HepG2 HCC cell line (ATCC, Washington D.C., USA) was maintained in a humidified incubator with 5% CO_2 at 37°C. Dulbecco's Modified Eagle's Medium with phenol red (DMEM; Gibco, USA) with 10% fetal bovine serum (FBS, Capricorn, Germany) and 1% penicillin/streptomycin (Capricorn, Germany) was used to culture HepG2 cells.

Before treatment of 4-hydroxycoumarin (Sigma, USA) to HepG2 cells, 7×10^3 cells were plated into each well of 96-well plate (SPL, Korea). According to our previous study, the detected IC50 concentration of 1,25-dihydroxyvitamin D was treated to HepG2 HCC cells.[18] IC50 concentration of 1,25-dihydroxyvitamin D was 250 nM at the 48th hour.

Total RNA Isolation and Real-Time Polymerase Chain Reaction: Total RNA was isolated according to the manufacturer's instructions of NucleoSpin RNA Kit (Macherey-Nagel, Germany). Total RNA was converted to cDNA using a reverse transcription kit (Bioneer AccuPower, Korea). In the obtained cDNA, *piR-823*, *Ki-67*, *MMP-2*, *MMP-9*, *HIF-1α*, and *hTERT* expressions were determined using real-time polymerase chain reaction (RT-PCR) (LighCycler96, Roche, USA). Glyceraldehyde-3-phosphate was used as an internal control. SYBR Green-based primers were designed and supplied by Oligomer (Ankara, Turkey). RT-PCR was conducted at the following conditions: pre-denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing/extension at 60°C for 30 s. Gene expressions were calculated using the delta-delta CT (ΔΔCT) formula. The primer sequences are shown in Table 1.

Statistical Analysis

Normal distribution of continuous variables was determined using the Shapiro-Wilk suitability test. Comparisons between groups of normally distributed variables were evaluated using one-way analysis of variance. Multiple comparisons of gene expressions were performed using Student's t-test. All statistical analysis was performed using IBM SPSS Statistics 21.0 software package at Eskisehir Osmangazi University, Department of Statistics, Turkey. Data are presented as mean±standard deviation. In the figures, only the mean values have been shown.

Table 1. The primer sequences used in real-time polymerase chain reaction

Gene name	Forward sequence	Reverse sequence
piR-823	5′-AGCGTTGGTGGTATAGTGGT-3′	5′-CTTATGGAGCCTGGGACTCTGACC-3′
Ki-67	5′-TCCTTTGGTGGGCACCTAAGACCTG-3′	5′-TGATGGTTGAGGTCGTTCCTTGATG-3′
MMP-2	5′-TCTCCTGACATTGACCTTGGC-3′	5′-CAAGGTGCTGGCTGAGTAGATC-3′
MMP-9	5′-TTGACAGCGACAAGAAGTGG-3′	5′-GCCATTCACGTCGTCCTTAT-3′
HIF-1α	5′-GGCGCGAACGACAAGAAAAAG-3′	5′-CCTTATCAAGATGCGAACTCACA-3′
hTERT	5′-TGACACCTCACCTCACCCAC-3′	5′-CACTGTCTTCCGCAAGTTCAC-3′
GAPDH	5′-CGAGGGGGGAGCCAAAAGGG-′3	5′-TGCCAGCCCCAGCGTCAAAG-3′

piR-823: PIWI Interacting RNA-823; MMP-2: Matrix Metalloproteinase-2; MMP-9: Matrix Metalloproteinase-9; HIF-1α: Hypoxia-inducible factor-1 alpha; hTERT: The human telomerase reverse transcriptase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Results

According to our data, *Ki-67* gene expression was upregulated in the 1,25-dihydroxyvitamin D-treated group (0.73±0.013) compared with the control (-2.6±0.089; Fig. 1a; p<0.001). Furthermore, the upregulation of *hTERT* gene expression was observed in the 1,25-dihydroxyvitamin D-treated group (0.547±0.006) compared with the control (-0.203±0.01; Fig. 1b; p<0.001). High *piR-823* expression was detected after 1,25-dihydroxyvitamin D treatment (1.169±0.003) compared with the control (0.797±0.003; Fig. 1c; p<0.001).

MMP-2 and *MMP-9* gene expressions were downregulated in the 1,25-dihydroxyvitamin D-treated group (0.447±0.014 and -0.35±0.007) compared with the control statistically (0.68±0.017 and 0.18±0.008; Fig. 2a and 2b; p<0.001). Moreover, the downregulation of *HIF-1α* gene expression was detected in the 1,25-dihydroxyvitamin D-treated HepG2 cells (0.06±0.005) compared with the control (0.207±0.008; Fig. 2c; p<0.001).

Discussion

Vitamin D is a lipophilic vitamin, and its 1,25-dihydroxyvitamin D form is the active form that can be used in essential molecular mechanisms of cells. Vitamin D has a significant role in immune system regulation, proliferation, differentiation, and apoptosis of cells under physiological and pathological conditions.[19] 1,25-Dihydroxyvitamin D enforces molecular mechanisms through vitamin D receptor (VDR) activation.[20] 1,25-Dihydroxyvitamin D binding to VDR makes conformational changes that facilitate $25(OH)_2D_3$ with several transcriptional factors in the nucleus. The interaction of $25(OH)_2D_3$ with transcriptional factors activates gene transcription.[21] This gene region may be a region that is related to apoptosis, proliferation, differentiation, or adhesion characteristics of cells. In recent studies, it has been argued that vitamin D cannot have any antiproliferative therapeutic effect in liver cancer according to its characteristic features and cellular mechanism.[22,23] Lappe et al.[24] indicated that vitamin D and Ca supplements cause cancer in older women. Pivonello et al.[25] (2016) reported that vitamin D administration may cause increased proliferation of JHH6 HCC cells and suggested that vitamin D is a mitogen in HCC cells. 1,25-Dihydroxyvitamin D have an impact on different gene regions. In a study, HIF-1α induced colon cancer cells, and 1,25-dihydroxyvitamin D treatment reduced cellular proliferation through HIF-1α and VEGF.[25] 1,25-Dihydroxyvitamin D modulated cytokine-induced MMP synthesis and collagen degradation by human lung fibroblasts.[26] Moreover, 1,25-dihydroxyvitamin D promoted cell differentiation and decreased telomerase activity in different healthy cell lines.[27,28]

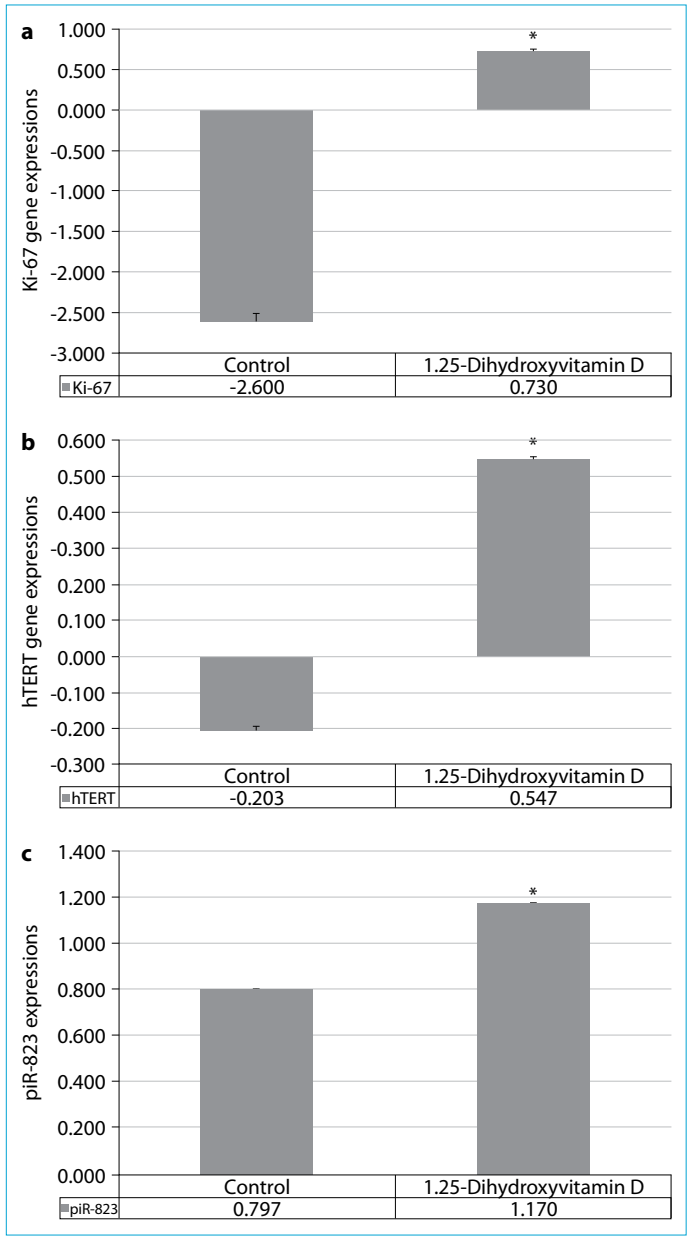

Figure 1. The effect of 1,25-dihydroxyvitamin D treatment on **(a)** Ki-67, **(b)** hTERT, and **(c)** piR-823 expressions of HepG2 hepatocellular carcinoma cells (p<0.001).

hTERT: The human telomerase reverse transcriptase; piR-823: PIWI Interacting RNA-823.

Generally, Ki-67, a cell proliferation antigen, is used in cancer histopathology.[29] In a study, Ki-67 expression varies due to cell-cycle regulation via CDK4/CDK6 on cellular proliferation.[29] Antibodies raised against the Ki-67 protein which is important for evaluating cell proliferation immunohistologically, particularly useful on the prognostic value of cell growth in clinical specimens of human neoplasms.[30] To identify the proliferation of HepG2 cells, especially, the upregulation of *Ki-67* gene expression showed that 1,25-di-

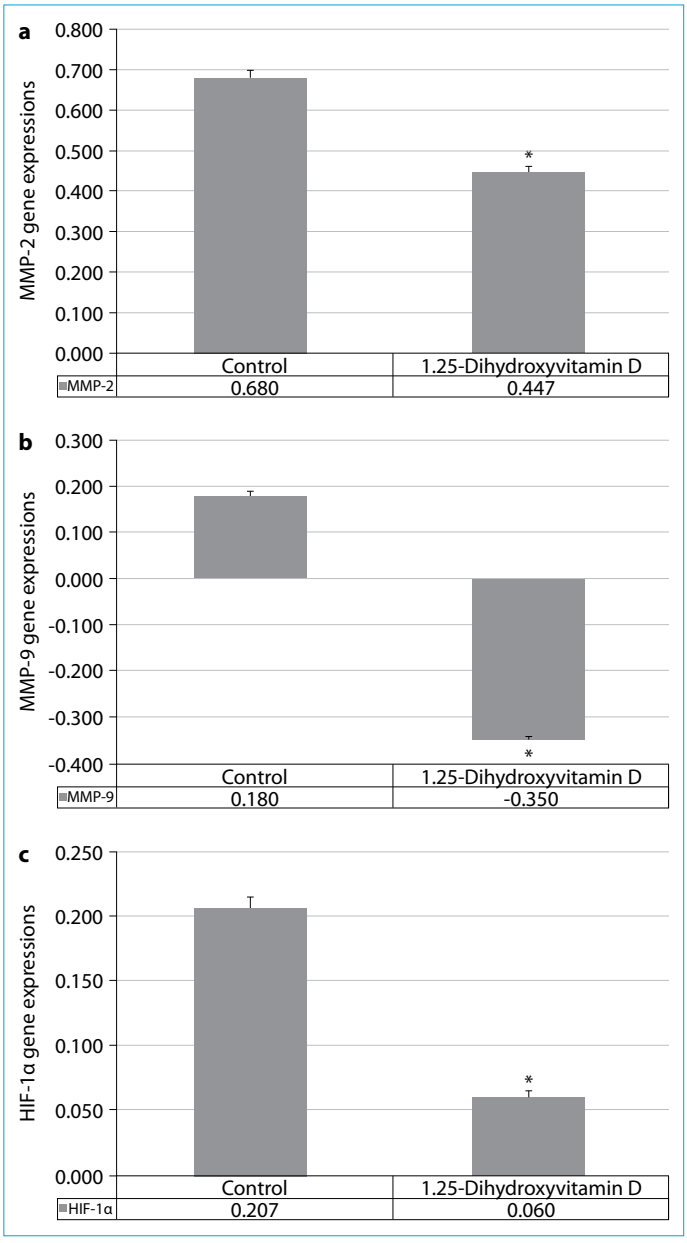

Figure 2. The effect of 1,25-dihydroxyvitamin D treatment on **(a)** MMP-2, **(b)** MMP-9, and **(c)** HIF-1α gene expressions of HepG2 hepatocellular carcinoma cells ($p<0.001$).

MMP-2: Matrix Metalloproteinase-2; MMP-9: Matrix Metalloproteinase-9; HIF-1α: Hypoxia-inducible factor-1 alpha.

hydroxyvitamin D treatment increases proliferation. *hTERT* induced the activity of telomerase. In cancer cells, high telomerase activity and *hTERT* expression were observed.[31-32] 1,25-Dihydroxyvitamin D treatment caused HepG2 cells to increase *hTERT* expression. High *hTERT* gene expression supported the increase in the proliferation of HepG2 cells. Generally, high *hTERT* expression caused HepG2 cells to survive and proliferate. To determine the adhesion changes of cells, *MMP-2* and *MMP-9* gene expressions were observed. As a result of 1,25-dihydroxyvitamin D treatment, the adhesion functions were increased due to low *MMP-2* and *MMP-9* expressions. *HIF-1α* gene expression was used to determine the differentiation of cells after 1,25-dihydroxyvitamin D treatment. Low HIF-1α expression supplied high survival of HepG2 cells. HIF-1α is widely used to identify the transition of epithelial cells to mesenchymal cells.[13,25] Decreased *HIF-1α* expressions suggested that 1,25-dihydroxyvitamin D treatment caused HepG2 cells to increase their adhesive properties. This high adhesive property was also supported by observing low *MMP-2* and *MMP-9* gene expressions.

Epigenetic mechanisms can be classified as DNA methylation, histone modifications, and small noncoding RNAs, which cannot change the nucleotide sequences but can change the expression patterns of genes. piRNAs are the novel members of small noncoding RNAs and are members of epigenetic mechanisms. piRNAs especially target for expressing or repressing methylated or acetylated regions of DNA.[34] In vitamin D intracellular signaling, *VDR* and *CYP* genes have large CpG islands where suitable places for DNA methylation are in their promoter regions. Furthermore, histone modifications (acetylation, phosphorylation, etc.) are altered by VDR and its target genes. This is evidence that increased levels of methylation in the VDR gene can lead to altered transcription and disruption of vitamin D synthesis associated with various diseases.[19,35] The relationship between *VDR* genes, DNA methylation, and histone modifications is also evidence that these epigenetically variable gene regions may effect piRNA expressions. According to this perspective, we wanted to observe *piR-823* expression changes after 1,25-dihydroxyvitamin D treatment. *piR-823* is one of the first investigated piRNA region that has an oncogenic activity in various cancer cells. piR-823 is effective on the expression of *HSP27*, *HSP60*, and *HSP70* simultaneously to promote proliferation and inhibit apoptosis by binding to common transcription factors HSF1.[36] *piR-823* is a predetermined type of piRNA in gastric cancer. Studies on *piR-823* reported that the level of expression of *piR-823* was positively associated with lymph node metastasis and distant metastasis.[37,38] *piR-823* inhibition could effectively inhibit tumor growth in therapeutic xenograft models and offer an encouraging strategy for the treatment of multiple myeloma.[39] Moreover, *piR-823* expression is a significant point of our research. *piR-823* acted as an oncogene after 1,25-dihydroxyvitamin D treatment. 1,25-Dihydroxyvitamin D treatment resulted in detecting high *piR-823* expression.

These findings are preliminary *in vitro* results of active vitamin D application for HCC. According to our results, 1,25-dihydroxyvitamin D form may not be beneficial for

anticancer usage after being HCC. Moreover, increase in proliferation, adhesion, and telomerase activity indicated that this treatment made HCC cells to become more adhesive instead of healing. These results are significant for understanding the impact of the active form of vitamin D on genetic biomarkers of cellular carcinogenesis mechanisms as proliferation, adhesion, differentiation, and telomerase activity. Furthermore, it is indicated that the active form of vitamin D may affect the epigenetic mechanisms of cells via piR-823 expression, which is oncogenic in several cancers. This result is preliminary, which presents the relationship between 1,25-dihydroxyvitamin D and piRNA in HCC cells. We suggest that vitamin D usage after being HCC is harmful for individuals in the treatment of cancer according to *in vitro* results.

Disclosures

Ethics Committee Approval: It was a cell culture study.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – O.C., E.C.; Design – O.C.; Supervision – O.C.; Materials – O.C.; Data collection &/or processing – O.C., E.C.; Analysis and/or interpretation – O.C., E.C.; Literature search – O.C.; Writing – O.C., E.C.; Critical review – O.C., E.C.

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