

Research Article

DICKKOPF-1 (*DKK-1*) Gene Associations in Human Cancers by Vitamin D and Sulforaphane

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Abstract

Dickkopf Wnt Signaling Pathway Inhibitor-1 gene (*DKK-1*) encodes a member of the Dickkopf family of proteins and is involved primarily in the embryonic development and bone formation in adults. Based on its known relationship to Wnt signaling pathway, we set out to determine how this gene is affected by dietary agents- vitamin D and sulforaphane (SFN). Cells were exposed for 24 hours to vitamin D3 [1,25(OH)2D3] (100nM) either alone or in combination with L-sulforaphane and TSA (20µM and 1µM respectively) at 70% confluency. We hypothesized that *DKK-1* expression will be increased in both cancer cells using our select treatments. In proliferating Caco-2 cells, treatment with 1,25(OH)2D3 had nearly a 3-fold increase in *DKK-1* (p<0.05) expression

while in MCF-7 cells saw no effect of the treatments. Co-treating the cells with both compounds seemed to attenuate the D effect, implying competing needs along the signaling pathway and require further investigation.

Keywords: Sulforaphane; Trichostatin A; Vitamin-D receptor; DICKKOPF-1 (*DKK-1*) gene; Colon cancer; Breast cancer

Abbreviations: 1,25(OH)2D3- 1,25 di-hydroxyvitamin D; BC- Breast Cancer; CRC- Colorectal Cancer; SEM- Standard Error of Means; SFN- Sulforaphane; TSA- Trichostatin A; VDR- Vitamin-D receptor

1. Introduction

Dickkopf Wnt Signaling Pathway Inhibitor-1 gene (*DKK-1*) encodes a member of the Dickkopf family of proteins [1] and has been reported to be involved primarily in the embryonic development and bone formation in adults. Much of the *DKK-1* literature, therefore, reflect research on primary [2] and secondary hyperparathyroidism [3], rheumatoid and psoriatic arthritis [4-7], non-alcoholic fatty liver disease [8], osteoporosis [9], ankylosing spondylitis [10], systemic sclerosis [11] and alopecia [12]. The role of *DKK-1* in cancer revolves primarily around components of Wnt-signaling pathway. *DKK-1* binds to the low-density lipoprotein-6 or LRP6 co-receptor and inhibits β -catenin-dependent (canonical) Wnt signaling, serving as a potent Wnt antagonist. Although linked quite frequently to Wnt-signaling in cancer pathology, very little is known about *DKK-1* as a gene and its possible linkages to other systems functions in humans. Aberrant activation of Wnt- β catenin pathway in most human cancers has been well-documented assuming its role in cell proliferation and invasion [13]. Overexpression of *DKK-1* has been associated with uncontrolled Wnt signaling in hepatoblastomas [14] and human endothelial colony-forming cells [15] demonstrating its role in tumoral angiogenesis.

The family of Wnt glycoproteins regulates development and homeostasis under normal conditions. However, the signaling pathways are severely disrupted in cancer. The evidence pool on *DKK-1* in breast cancer (BC) is rather small, and non-existent with vitamin D as a treatment. In an animal study of metastatic BC to the bones, the researchers found *DKK-1* to be expressed 6 times more in cells with osteolytic lesions in animals [16]. Studies on human subjects with and without BC and bone metastasis reported significantly high levels of *DKK-1* in the serum samples of patients with breast cancer ($P=0.016$) compared to controls

[16, 17] and poor BC prognosis [18]. Uncontrolled *DKK-1* expression and/or downregulation have been reported in different BC cell lines [19, 20] suggesting potential targets in reversing the effect in BC [21, 22]. One study investigating the inhibitory effect of human mesenchymal stem cells (Z3 cells) on the growth of human MCF-7 breast cancer cells demonstrated that β -catenin was down-regulated followed by the over expression of *DKK-1* in Z3 cells compared to MCF-7. The inhibitory effect of Z3 cells on MCF-7 cells were attenuated after neutralizing *DKK-1*. Therefore, *DKK-1* secreted by Z3 cells involves the inhibition via the Wnt pathway. [23] In MCF-7 cells, the downregulation of *DKK-1* has been linked with higher cell proliferation by losing control of Wnt/ β -catenin signaling pathway, giving a new mechanistic insight. [20].

Traditionally the main recognized function of vitamin D has been calcium and phosphate homeostasis. Nevertheless, the importance of vitamin D3 as a protective agent against various cancers is quite significant. The active form of vitamin D, (1,25-dihydroxyvitamin D3) or vitamin D3 and the nuclear vitamin D receptor (VDR) are responsible for regulating several genes involved in cell differentiation and cell proliferation [24]. However, there is only 10 studies investigating *DKK-1* with vitamin D and only three of them focused on colon cancer. Our current study adds to existing but rather limited research base of *DKK-1* in relation to vitamin D response in two different human cancer cell lines.

DKK-1 is expressed at high level in colon cancer cell lines such as Caco-2 or HT-29. The association between CRC and vitamin D3 was first suggested in ecologic studies, but further was confirmed by observational studies in humans and experimental studies in both animal and cell culture studies [25]. In human SW480-ADH colon cancer cells,

vitamin D₃ increased *DKK-1* mRNA and protein expressions in a dose dependent manner and induced certain histone modification patterns, further influencing the expressions [26]. Studies in humans, on the other hand, have looked at DNA methylation and risk of colorectal cancer, to elucidate any associated epigenetic mechanisms. In a Canadian cohort of colorectal cancer patients, dietary vitamin D intake was strongly negatively associated with *DKK-1* methylation [27]. Results from laboratory and human study findings contradict each other, which further emphasizes the need to explore these associations.

In addition to mainstream dietary macro and micronutrients, recent research has been focused on investigating the role of phytochemicals in cancer prevention. Phytochemicals are naturally present compounds that have demonstrated anti-inflammatory, anti-hypertensive, antioxidant as well as anti-carcinogenic properties, and some example phytochemical groups are lycopene, genistein, sulforaphane, (-)-epigallocatechin-3-gallate (EGCG), curcumin, etc. Such compounds have been shown to modify self-renewal properties of cancer stem cells, but no studies to date have investigated specific genes in relation to individual phytochemicals by their specific beneficial properties [28]. Sulforaphane (SFN) is classified as a histone deacetylase (HDAC) inhibitor [29-31] present abundantly in food sources like broccoli, Brussel sprouts, cabbages, cauliflowers etc. HDACs are a class of enzyme responsible for regulating gene transcription [32]. HDAC inhibitors such as SFN increase transcription of key genes and play roles in regulatory pathways that ultimately discourage oncogenesis from taking place [29-31]. No studies to date have looked at the effect of vitamin D and SFN on *DKK-1* in human breast or colon cancer cells.

This study is the first to investigate the importance of *DKK-1* regulation by vitamin D and SFN, separately and in combination in breast and colon cancer cells in human. Based on our previous findings, we anticipate that treating cancer cells with vitamin D will increase *DKK-1* expression and further increase in the presence of SFN. Comparing two invasive cancers side-by-side would also provide valuable understanding of any regulatory differences by cell types and possibly lead to the development therapies targeting *DKK-1* pathways, which could improve the prognosis of innumerable cancer sufferers.

2. Materials and Methods

2.1 Cell culture

Both cells lines- breast cancer (MCF-7) and colorectal cancer (Caco-2) were obtained from American Type Culture Collection (ATCC). Cells were treated on day 7 from the date of seeding into 6-well plates. The experiment included six different treatment groups namely- DMSO control (0.1%), 1,25 (OH)₂D₃ (100nM), SFN (20 μM), TSA (1μM), 1,25 (OH)₂D₃ + SFN and 1,25 (OH)₂D₃ + TSA. After treatment, the cells were incubated for 24 hours before harvesting. MCF-7 cells were obtained from Arcaro lab. 1, 25 (OH)₂D₃ was purchased from Enzo Life Sciences, Plymouth Meeting, PA. Trichostatin A and SFN were purchased from Sigma-Aldrich, St. Louis, MO. Real-time PCR materials, including all primers and master mix were purchased from Applied Biosystems, Foster City, CA. Cell culture dishes were bought from Costar, Cambridge, MA. All other cell culture materials and materials for RNA isolation and cDNA synthesis were purchased from Life Technologies, Grand Island, NY.

2.2 RNA isolation and reverse transcription

At the completion of the treatment period, cells were washed with saline, then treated with 1 mL Trizol per well

of a 6-well plate, and cells were scraped to be harvested for subsequent RNA extraction. Cells were frozen at -80°C until RNA was ready to be isolated in collection tubes. To isolate the RNA, 200 μL chloroform was added to each homogenate for phase separation. Isopropanol (500 μL) was then used to precipitate the RNA. Washing three times with 1 mL of 75% ethanol was followed by the addition of 40 μL of Diethyl pyrocarbonate (DEPC) water. The samples were then incubated for 10 minutes at 56°C to ensure maximum RNA solubility. RNA samples were then quantified using a Nanodrop machine ($\lambda 260/280$). cDNA was prepared using the SuperScript III First Strand Synthesis System for RT-PCR (Life Technologies, Grand Island, NY).

2.3 Real time PCR

For all experiments, mRNA expressions of *GAPDH*, *DKK-1* were measured by ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions consisted of 10 μL of SYBR GREEN Gene Expression Master Mix (Applied Biosystems, Foster City, CA) with forward and reverse primers (10nM each) and 10 μL of DNase/RNase Free Water with 100 ng of cDNA. Duplicates were set up for each sample. The levels of *DKK-1* were measured by the comparative $\Delta\Delta\text{CT}$ method (ThermoFisher Scientific, 2016). For each sample, the relative abundance of *GAPDH* and the target genes were obtained. The difference between those two abundances (target gene- *GAPDH*) is the ΔCT for that sample which were the values used for statistical analysis. The mean ΔCT values were then normalized against the mean ΔCT values of the control sample, Dimethyl Sulfoxide (DMSO), resulting in a $\Delta\Delta\text{CT}$ value. 2 was raised to the $-\Delta\Delta\text{CT}$ to produce a relative quotient (RQ) for each sample, with the RQ of the control being one. RQ values were used to create the mRNA expression curves for each gene. We used the same sequences as

reported by Zhou et.al, for *DKK-1* [20]. *GAPDH* primer sequences are as below:

GAPDH-F CATGGGTGTGAACCATGAGA
GAPDH-R GGGTGCTAAGCAGTTGGT

2.4 Statistical analyses

Results from real-time PCR were analyzed using one-way ANOVA with a p-value of <0.05 used as the cut-off for statistical significance. The analysis was setup as a randomized block design with each experiment as a block. Brown-Forsythe Tests was also used to assess significant differences among the test standard deviations from the control (DMSO), also using a P-value of <0.05 as a cut-off. All statistical analyses and figures were generated using GraphPad Prism (v 8.4.0) and results are presented as fold-increase with corresponding p-values.

3. Results

We hypothesized that the expression of *DKK-1* will be increased by 1,25(OH)2D3 alone, and further enhanced by the addition of SFN because of its known HDAC inhibitory function, in both cell lines. In proliferating Caco-2 cells, ANOVA ($p<0.0001$) and Brown-Forsythe tests ($p=0.03$) reveal significant differences across the treatment groups compared to control. *DKK-1* expression (N=6) was increased by 1,25(OH)2D3 as hypothesized (3.30- fold, $p<0.05$). However, the addition of SFN did not make a significant change in *DKK-1* expression (2.875- fold, $p=0.0012$) presumably because SFN alone had no effect on *DKK-1* (0.91- fold, $p=0.56$). The expression followed similar patterns as 1,25(OH)2D3 in D+TSA group (2.885- fold, $p=0.0012$) and TSA alone (5.80-fold, $p<0.0001$) with nearly twice as much the expression in *DKK-1*, compared to 1,25(OH)2D3 alone. The results were quite different in MCF-7 cells as seen from the overall results of the ANOVA ($p=0.73$) and Brown-Forsythe ($p=0.65$) tests. Not only were

there no effect of any of the treatment groups, but the addition of SFN to 1,25(OH)2D3 seemed to inhibit *DKK-1* expression. In MCF-7 cells, the results were- 1,25(OH)2D3 alone (0.40 fold, p= 1.00), SFN alone (1.64 fold, p=0.95),

D+SFN (-0.66 fold, p=1.00), D+TSA (2.5- fold, p=0.81) and TSA alone (2.69-fold, p=0.76) group without statistical significance. The results for RT-PCR experiments are presented in **Figure 1**.

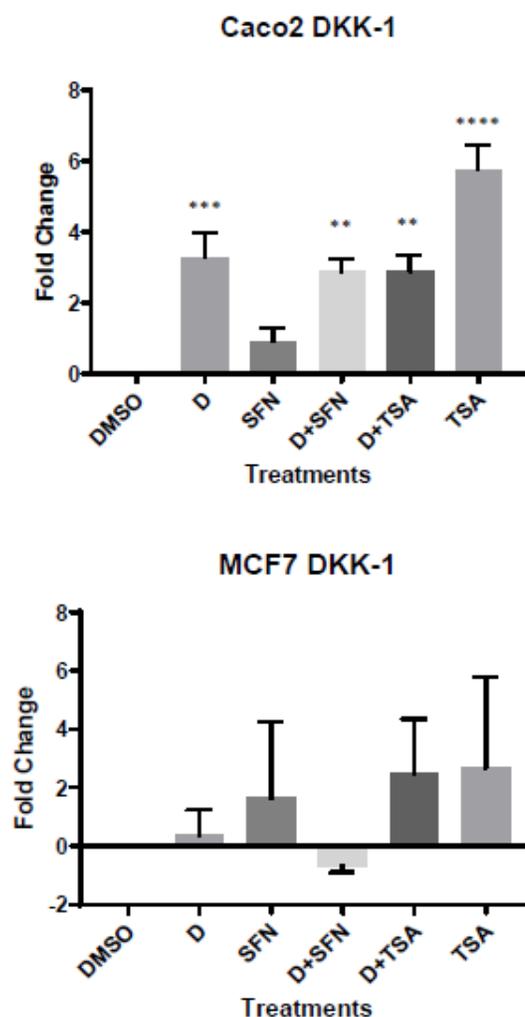


Figure 1: mRNA expressions of *DKK-1* in proliferating Caco-2 and MCF-7 cells. A) *DKK-1* expression was significantly increased by D alone in proliferating Caco-2 cells (>3-fold increase) and the effect remained similar in D+SFN and D+TSA groups. While TSA alone showed the maximum increase in *DKK-1* expression, our target HDAC inhibitor SFN had no effect; B) *DKK-1* expressions were not affected by any of the treatment groups in MCF-7 cells. Results are from N>3 experiments for both genes and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 represent significance.

4. Discussion

The study findings suggest that vitamin D might be attenuating effects of SFN in *DKK-1* expression in two different human cancer cell lines. This is the first study to use proliferating colorectal cancer cells, Caco-2, as a model- which is not usual for its research application. In a separate study, we have shown that Caco-2 cells behave differently under different conditions- proliferating vs. differentiated (Revise and resubmit: Journal of Food Science and Agriculture JSFA-19-3273). We presumed Caco-2 proliferating cells retain properties of any invasive, metastatic cancers of other human organs, and hence used as a comparison cell line to MCF-7. Our HDAC inhibitor findings are similar to what we have demonstrated before, gene expressions related to HDAC inhibition could be cell-specific[33] and requires further investigation.

The vitamin D receptor (VDR) antagonizes β -catenin signaling. In vitro, VDR activation in breast cancer cells reduced β -catenin activation and transcriptional activity leading to elevated expression of the extracellular Wnt inhibitor *DKK-1* protein, and a reduction in the interaction of β -catenin with the cyclin D1 promoter. Expression of a stabilized form or β -catenin ablated the protective effects of VDR activation. Collectively, these findings delineate a protective role for VDR signaling through disruption of β -catenin activation[34].

The ability of various bioactive food components to influence the balance between proliferative and quiescent cells by regulating *DKK-1* may account for their biological response [28]. There is no data on the effect of SFN alone on *DKK-1* to elucidate some of the underlying mechanisms. Undoubtedly, additional studies are needed to clarify the

physiological role of such bioactive components in cancer management.

In the study of colonic SHZ-88 cells, the growth, migration, and invasion were significantly inhibited with upregulation of *DKK-1* and down regulation of Wnt/ β -catenin target genes. Mesenchymal stem cells derived from the perichondrium inhibit the growth of breast cancer cells through the Wnt/ β -catenin signaling pathway in vitro and in vivo via regulation of *DKK-1* [35]. Studies on colorectal cancer cells suggest that vitamin D3 activates *DKK-1* transcription in an indirect manner, suggesting a novel mechanism of Wnt inhibition [13]. In an immunodeficient mouse study, exogenous expression of E-cadherin resulted in a 17-fold increase in *DKK-1* mRNA expression. Vitamin D3 is also known to inhibit β -catenin transcriptional activity by promoting vitamin D receptor (VDR) binding to β -catenin and the induction of E-cadherin expression. The increase in the expression of *DKK-1* mRNA and protein, however, acts as a tumor suppressor in human colon cancer cells. In contrast, vitamin D3 represses *DKK-4* transcription by inducing direct VDR binding to its promoter. *DKK-4* as a novel target of the Wnt/ β -catenin pathway has been shown to be up-regulated in colorectal tumors and increase cell migration and invasion. Together, these results show that vitamin D3 exerts a complex set of regulatory actions leading to the inhibition of the Wnt/ β -catenin pathway in colon cancer cells [26]. This report demonstrates that *DKK-1* expression varies by cancer cell types with same nutrient interventions- highlighting a more heterogeneous nature of *DKK-1* responses. This also points to the need for comparative studies that simultaneously measure the potential pro- and anti-carcinogenic effects of *DKK-1* activation.

Future studies might investigate whether vitamin D and other bioactive compounds influence *DKK-1* stimulation. Such research would further elucidate the relationship between *DKK-1* and natural bioactive compounds in cancer prognosis.

5. Conclusion

Insights into the biology of breast and colon cancers have been gained from the investigation of genes commonly overexpressed in these cells in cancer and have led to paradigms that have informed the study of epigenetic alterations. These insights are important in developing new diagnostic and prognostic assays and potential therapies for cancer.

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Conflict of Interest

None.

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