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ORIGINAL ARTICLE

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Expression of VDR and CYP24A1 mRNA in human tumors

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Abstract 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and its analogues have been shown to inhibit proliferation of human cancer cells mediated by vitamin D receptor (VDR). The over-expression of 25-hydroxyvitamin D-24-hydroxylase (CYP24A1), an enzyme involved in the metabolism of $1,25(OH)_2D_3$ and its analogues, is associated with poor prognosis of some human cancers. In this study, we employed real-time reverse transcription PCR to examine the expression of VDR and CYP24A1 mRNA in a cohort of human breast, lung, colon and ovary tumor samples. We found that CYP24A1 mRNA was significantly up-regulated in colon, ovary and lung tumors, but down-regulated in breast tumor relative to the analogous normal tissues. As a comparison, VDR mRNA was modestly down-regulated in colon, breast and lung tumors, but highly up-regulated in ovarian tumors. Treatment of two breast cancer cell lines, SW-620 and MCF-7, and one colon cancer cell line, HT-29, by $1,25(OH)_2D_3$ for 48 h profoundly stimulated CYP24A1 mRNA expression ($EC_{50} = 0.6$, 0.8 and 29.5 nM in SW-620, HT-29 and MCF-7, respectively), but did not significantly affect VDR mRNA expression. Growth as assessed by DNA synthesis was modestly arrested by $1,25(OH)_2D_3$ after 72 h of incubation, but was not altered after a 5-day incubation period. These data suggest that the VDR signaling pathway may be compromised via the modulation of CYP24A1 and VDR in human tumors.

Keywords Vitamin D analogues · VDR · CYP24A1 · Chronic renal disease · Cancer · Real-time polymerase chain reaction

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Introduction

Vitamin D_3 is modified by 25-hydroxylase in the liver and 25-hydroxyvitamin D 1α-hydroxylase in the kidney to form the active metabolite, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which is then metabolized by 25-hydroxyvitamin D-24-hydroxylase (24-OHase, CYP24A1) [1, 2] to yield the biliary excretory product calcitroic acid. The binding of $1,25(OH)_2D_3$ or its analogues to the vitamin D receptor (VDR), a nuclear receptor, activates VDR to interact with retinoid X receptor (RXR) and recruit cofactors to form the VDR/RXR/cofactor complex, which binds to vitamin D response elements in the promoter region of target genes to regulate gene transcription [3].

It is well documented that $1,25(OH)_2D_3$ (calcitriol, the endogenous VDR activator) regulates the homeostasis of calcium and phosphorus, and also controls the expression of many genes. Chronic renal disease, characterized by reduced synthesis of $1,25(OH)_2D_3$, inadequate renal phosphate clearance and calcium imbalance, and over-stimulation of the parathyroid gland and parathyroid hormone synthesis, often results in secondary hyperparathyroidism (SHPT). Vitamin D analogues that activate VDR such as calcitriol and paricalcitol (an analogue of calcitriol) are now routinely used to manage SHPT. In addition, VDR has been found in more than 30 tissues including intestine, colon, breast, lung, ovary, bone, kidney, parathyroid gland, pancreatic β-cells, monocytes, keratinocytes, and many cancer cells, suggesting that the vitamin D endocrine system may also be involved in regulating the immune systems, cellular growth, differentiation and apoptosis [4].

The role of VDR in modulating cell proliferation and differentiation is well documented. In addition, several studies have demonstrated that $1,25(OH)_2D_3$ and its analogues could inhibit proliferation and induce differentiation in various cancer cell types [5]. However, the anti-proliferative effects of vitamin D analogues in

cancer cells are variable, ranging from no effect in some cells to profound inhibition in other cell lines [6–9]. Also, the outcome from clinical trials testing the anti-neoplastic effects of vitamin D analogues has thus far been less exciting than expected [10]. Emerging evidence shows that CYP24A1, the gene that encodes the enzyme 25-hydroxyvitamin D-24-hydroxylase, which is responsible for metabolizing 1α-25$(OH)_2D_3$, is elevated in various human tumors [11–13], and its over-expression is linked to poor prognosis of esophageal cancer [14]. Although there are previous reports examining either VDR or CYP24A1 in colon or breast tumors [4, 15], a detailed comparison of VDR and CYP24A1 expression status in different tumor types has not been reported. In this study, we employed real-time reverse transcription PCR to examine expression of VDR and CYP24A1 mRNA in a cohort of human breast, lung, colon and ovary tumor samples. We also examined the effect of 1,25$(OH)_2D_3$ on VDR and CYP24A1 mRNA expression and growth inhibition in two breast cancer cell lines, SW-620 and MCF-7, and one colon cancer cell line, HT-29. Our data show that the expression of CYP24A1 and VDR mRNA is altered in human tumors.

Materials and methods

Materials

1α,25-dihydroxyvitamin D_3 (1α, 25-$(OH)_2D_3$, calcitriol) was from Abbott Laboratories. Other reagents were of analytical grade.

Cell culture

MCF-7, HT-29 and SW620 cells (American Type Culture Collection, Manassas, VA) were cultured at 37°C in a humid atmosphere consisting of 5% CO_2 and 95% air. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 U/ml streptomycin, 4 mM glutamine, 4 mg/l pyridoxine hydrochloride, and 10% fetal bovine serum (Life Technologies).

Human tissue RNA samples

RNA was isolated from tumor biopsies from lung, breast, colon and ovary, and non-matched normal biopsies from lung, breast, colon, and ovary (obtained from the NCI sponsored Cooperative Human Tissue Network) using Trizol (Invitrogen, Carlsbad, CA), chloroform extraction, and Qiagen RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was converted to cDNA using SuperScript III (Invitrogen). The number of samples for each tissue type and relevant information is shown in Table 1.

Primers and probes for Real-time Reverse Transcription-PCR (RT-PCR)

For real-time RT-PCR (qPCR), primers were synthesized along with TaqMan™ probes that were 5′ labeled with the reporter 6-carboxyfluorescein (FAM) and 3′ labeled with the quencher tetramethylrhodamine (TAMRA). The specific oligonucleotide primers and probes for the VDR gene are as follows: 5′-CGACCCCACCTACTCCGACTT-3′ (forward), 5′-GGCTCCCTCCACCATCATTC-3′ (reverse), and 5′-CCAGTTCCGGCCTCCAGTTCGTG-3′ (probe); for the GAPDH gene: 5′-AAATTCCATGGCACCGTC-3′ (forward), 5′- GATGGTGATGGGATTTCCA-3′ (reverse), and 5′-CAAGCTTCCCGTTCTCAGCC-3′ (probe); for the actin gene: 5′-CTGGAACGGTGAAGGTGACA-3′ (forward), 5′-CGGCCACATTGTGAACTTTG-3′ (reverse), and 5′-CAGTCGGTTGGAGCGAGCATCCC-3′ (probe); and for the 28S rRNA gene: 5′-TTCACCAAGCGTTGGATTGTT-3′(forward), 5′-TGTCTGAACCTGCGGTTCCT-3′(reverse), and 5′-TCACGACGGTCTAAACCCAGCTCACG-3′(probe). The 28S-specific probe was 5′ labeled with hexachlorofluorescein (HEX) and 3′ labeled with the quencher TAMRA. These primers and probes were

Table 1 Number of samples

Tissue type	Normal	Tumor	Sample information
Breast	16	23	Tumor: average age, 55 (range 28–79 year); all carcinomas Normal: average age, 35 (range 20–50 year); reduction mammoplasty primary source
Colon	15	24	Tumor: average age, 66 (range 25–97 year); adenocarcionoma, leiomyosarcoma; equal number of male and female samples Normal: average age, 61 (range 32–77 year); uninvolved normal tissue
Lung	20	20	Tumor: average age, 65 (range 43–79 year); adenocarcionoma, SCLC, and leiomyosarcoma; equal number of male and female samples Normal: average age, 63 (range 38–85 year); some samples were from patients with mild emphysema
Ovary	13	24	Tumor: average age, 54 (range 28–74 year); adenocarcionoma, granulosa cell Normal: average age, 44 (range 33–63 year); uninvolved normal tissue

purchased from Integrated DNA Technologies, Inc (Coralville, IA). Analysis of the CYP24A1 gene utilized a primer and probe set obtained from the Assay-on-Demand collection (assay Hs00167999_m1, Applied Biosytsems, Inc., Foster City, CA).

Real-time RT-PCR

PCR was performed with a 7900HT sequence detector (Applied Biosystems). Each sample has a final volume of 25 μl containing 10 ng of cDNA, 0.4 mM each of the forward and reverse PCR primers and 0.1 mM of the TaqMan probe. Temperature conditions consisted of a step of 5 minutes at 95 °C, followed by 40 cycles of 60 °C for 1 min and 95 °C for 15 s for total of 40 cycles. Data was collected during each extension phase of the PCR reaction and analyzed with the SDS software package (Applied Biosystems). Threshold cycles were determined for each gene. A parallel standard curve (in duplicate) using 10, 3.3, 1.1, and 0.37 ng of cDNA synthesized from total RNA from a cell line (HEK-293) was generated for each gene.

This standard curve was used to determine the relative concentration of RNA in each sample by comparison using methods described in the ABI Prism User Bulletin Number 2. The level of expression of VDR or CYP24A1 mRNA was given as relative copy numbers normalized against the geometric mean of 28S, GAPDH, and beta actin gene expression and shown as mean ± the standard deviation. The non-parametric Mann-Whitney test was used to determine a *P*-value for differential expression between the tumor and normal sample groups because these groups did not fit a normal distribution.

Proliferation assays

Cells were plated at 1×10^6 cells/ml, 200 μl/well into 96-well plates (Corning, Corning, NY). One day after plating, cells were treated with test agents for 24 h and then labeled with 0.3 μCi/well of ^{3}H-thymidine for another 48 h. Each well was washed with 0.3 ml/well of PBS, incubated with 0.2 ml/well of ice-cold 10% trichloroacetic acid (TCA) for 30 min at 4°C, and then followed by another wash of 0.2 ml/well of TCA. Samples were dissolved in MICROSCINT 20 (Packard Instrument Company, Meriden, CT) before counting. In some studies, cells, 1 day after plating, were treated with $1,25(OH)_2D_3$ for 2 days, then changed into fresh medium plus drug for another 3 days before subjected to 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay [15].

Semi-quantitative RT-PCR

Cells were harvested and RNA was isolated and analyzed for mRNA levels for the genes of interest and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Briefly, total RNA was first reverse transcribed to cDNA using an oligo dT as a primer (Invitrogen). The cDNA samples were then amplified by PCR at 94°C 30 s/55°C 30 s/72°C 1 min for 30 cycles using specific PCR primers as shown in Table 2.

The PCR products were analyzed by a 1.8% agarose gel. The images of the PCR products on the gel were digitally captured and the density of each band was measured by ChemicDoc XRS (Bio-Rad, Hercules, CA). The GADPH band was used to normalize the density of the PCR product bands.

Results

First we determined the relative differences in the VDR and CYP24A1 mRNA levels in normal human tissues using real-time RT-PCR (Fig. 1). Normal colon and lung expressed a relatively high level of VDR mRNA. The VDR mRNA level was much lower in normal ovary and breast tissues (2% and 9% of colon, respectively). As a comparison, the CYP24A1 mRNA expression was relatively low in the four normal tissue types; the CYP24A1 mRNA level was not detectable in many colon (7 out of 15) and ovary (10 out of 13) tissue samples.

When the CYP24A1 mRNA level in various tumor tissues was compared with that in normal tissues, we found that CYP24A1 mRNA was significantly up-regulated in human colon, ovary and lung tumors, but down-regulated in breast tumors relative to their respective normal tissue samples (Fig. 2). As an example, among the 20 lung tumor samples examined, 9 samples exhibited a more than fivefold increase in CYP24A1 mRNA when compared with the mean from the 20 normal lung tissue samples.

A comparison of VDR mRNA expression in these same samples (Fig. 3) demonstrated that VDR mRNA

Table 2 RT-PCR primers

Name	Sequence#	Product size (bp)	Primers
Human VDR	NM000376	227	5′-GACTTTGACCGGAACGTGCCC-3′(forward); 5′-CATCATGCCGATGTCCACACA-3′(reverse)
Human 24-Ohase	L13286	316	5′-CGGGTGTACCATTTACAACTCGG-3′(Frward); 5′-CTCAACAGGCTCATTGTCTGTGG-3′(reverse)
GAPDH	Bc014085	450	5′-ACCACAGTCCATGCCATCAC-3′(forward); 5′-TCCACCACCCTGTTGCTGTA-3′(reverse)

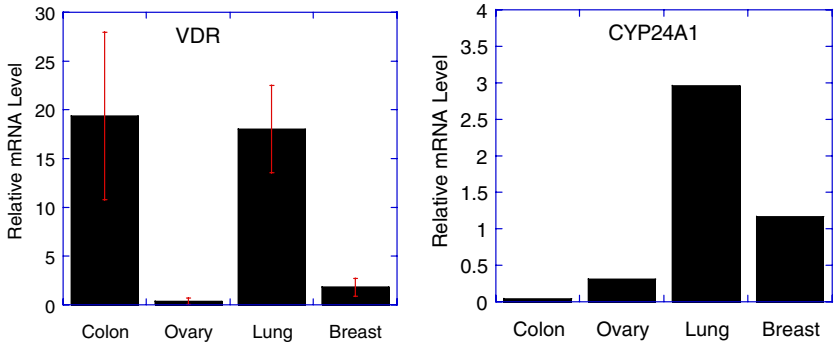

Fig. 1 VDR and CYP24A1 mRNA expression in normal tissues as evaluated by real-time PCR. Data are normalized to 28S, GAPDH, and beta actin gene expression, and then expressed as relative levels versus cDNA from HEK-293 cells. The VDR mRNA levels are expressed as mean ± the standard deviation (number of samples indicated in "Materials and Methods"). Because the CYP24A1 mRNA levels were below detection in 7 out of 15 colon and 10 out of 13 ovary tissue samples, data shown are means of theoretical maximal expression values without the standard deviation

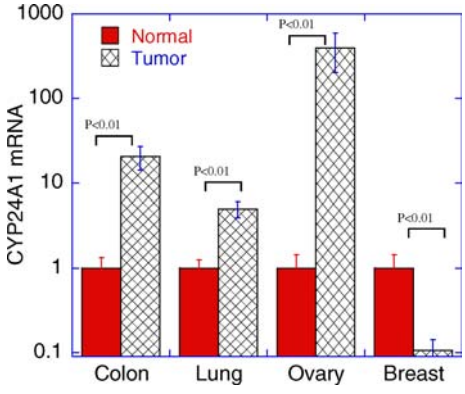

Fig. 2 CYP24A1 mRNA expression in normal versus tumor tissues as evaluated by real-time PCR. Data were normalized using the mean from normal tissues (as shown in Fig. 1), and expressed as mean ± the standard deviation (number of samples and statistical analysis indicated in "Materials and Methods")

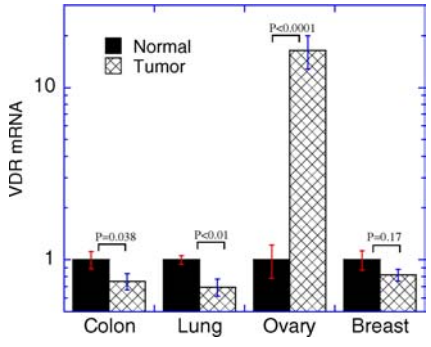

Fig. 3 VDR mRNA expression in normal versus tumor tissues as evaluated by real-time PCR. Data were normalized using the mean from normal tissues (as shown in Fig. 1), and expressed as mean ± the standard deviation (number of samples and statistical analysis indicated in "Materials and Methods")

was modestly down-regulated in human colon, breast and lung tumor samples, but highly up-regulated in ovarian tumor samples relative to their respective normal tissue samples.

To investigate if any correlation existed between the expression of VDR and CYP24A1 mRNA, the VDR mRNA level for each individual tumor sample was plotted against its CYP24A1 mRNA counterpart. Figure 4 shows that there is no clear correlation between VDR and CYP24A1 mRNA levels in colon and lung tumors other than that CYP24A1 mRNA is significantly up-regulated, while VDR mRNA is generally down-regulated in the tumor samples compared to the normal samples. In breast tumors, there is no obvious trend regarding the expression of VDR and CYP24A1 mRNA. However, in ovarian tumors, both VDR and CYP24A1 mRNA levels are highly up-regulated relative to normal ovary samples and VDR mRNA seems inversely proportional to CYP24A1 mRNA.

In order to further investigate if altered expression of CYP24A1 and/or VDR might compromise the anti-proliferative effect of $1,25(OH)_2D_3$, we examined the effects of $1,25(OH)_2D_3$ on two breast cancer cell lines, SW-620 and MCF-7, and one colon cancer cell line, HT-29. Fig. 5 shows that these cancer cell lines express a low level of VDR (3.0, 13.2 and 20.8% of GAPDH in HT-29, MCF-7, and SW620, respectively). In the absence of $1,25(OH)_2D_3$, the expression of CYP24A1 is also low (1.0, 7.9, and 8.3% of GAPDH in MCF-7, HT-29, and SW620, respectively). Although $1,25(OH)_2D_3$ treatment for 48 hr did not have a significant effect on the expression of VDR mRNA, it profoundly stimulated CYP24A1 mRNA expression with EC_{50} (the concentration that produces 50% of the maximum response) values at 0.6, 0.8 and 29.5 nM for SW-620, HT-29, and MCF-7, respectively. $1,25(OH)_2D_3$ at 1 μM stimulated CYP24A1 mRNA expression by 136, 14, and 9-fold to 130%, 112% and 71% of GAPDH in MCF-7, SW-620, and HT-29, respectively. When the effect of $1,25(OH)_2D_3$ on cell proliferation was examined after 72 hr of incubation (Fig. 6), thymidine incorporation at 1 μM $1,25(OH)_2D_3$ was 83 ± 7%, 78 ± 7%, 32 ± 7% of control for MCF-7, HT-29 and SW-620, respectively. As a comparison, thymidine incorporation in cells treated with serum free medium for 72 h were 89 ± 10%, 45 ± 1%, 92 ± 24% of control for MCF-7, HT-29 and

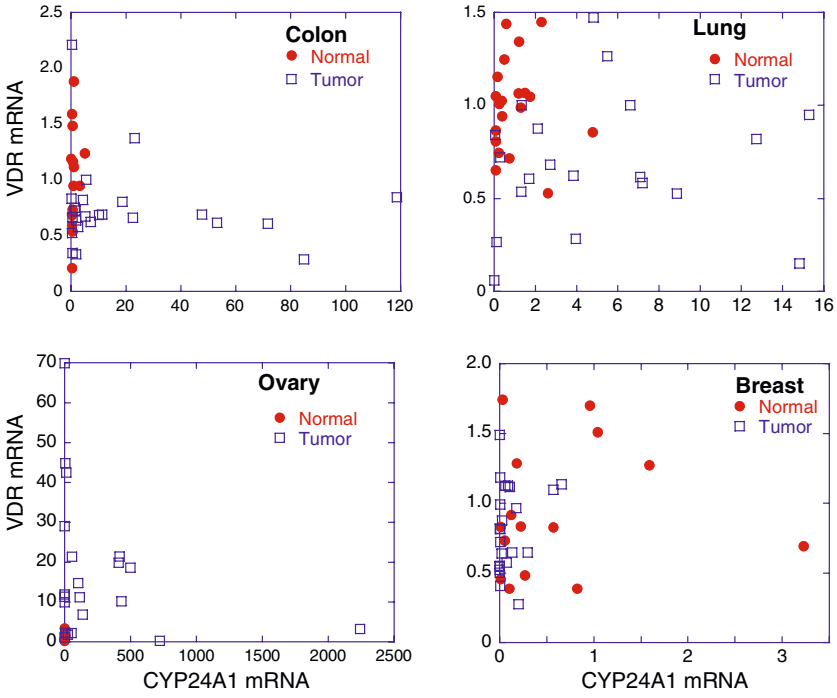

Fig. 4 Data analysis on VDR and CYP24A1 mRNA expression in individual normal and tumor tissue samples. The VDR mRNA level for each individual normal or tumor sample was plotted against its CYP24A1 mRNA counterpart. Data expressed were normalized using the mean from normal tissues (as shown in Fig. 1)

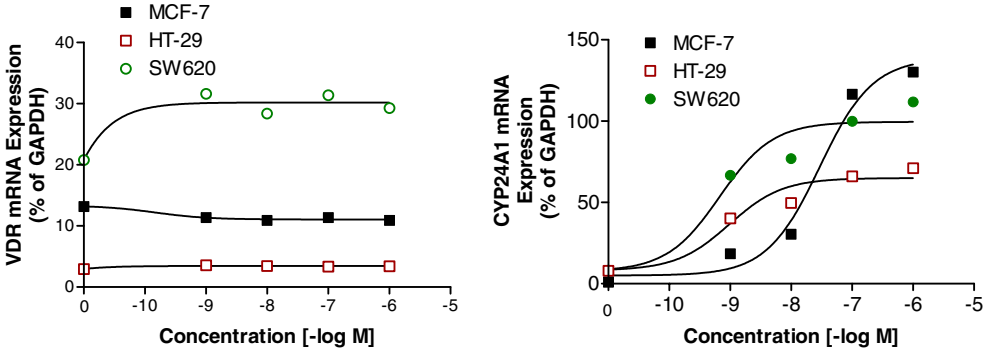

Fig. 5 Effect of $1,25(OH)_2D_3$ on the expression of CYP24A1 and VDR mRNA in cancer cells. Cells were treated with increasing concentrations of $1,25(OH)_2D_3$ for 2 days. The density of each RT-PCR product was measured and expressed as % of GAPDH. The results represent two independent experiments

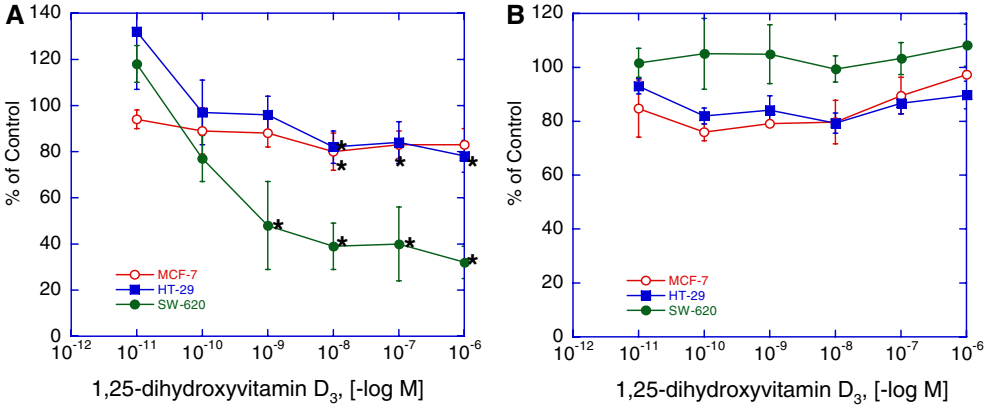

Fig. 6 Effect of $1,25(OH)_2D_3$ on cell proliferation. Cells were treated with increasing concentrations of $1,25(OH)_2D_3$ for 72 h (**a**) or 5 days (**b**) as described in "Materials and Methods". Data were expressed as % of control (no drug treatment, 100%). Each value shown is mean ± the standard deviation from $n=4$. Statistical comparisons were performed by unpaired t-test. $^*P<0.01$ vs. control

SW-620, respectively. The SW-620 cells were more responsive to $1,25(OH)_2D_3$, with an IC_{50} value of 0.1 nM. Interestingly, when cells were treated with $1,25(OH)_2D_3$ for 5 days with one change of medium plus fresh drug on Day 3, the growth inhibitory effect on the three cell lines was no longer observed (Fig. 6). These

results suggest that induction of CYP24A1 may allow the cancer cells to elude the anti-proliferative effects of $1,25(OH)_2D_3$.

Discussion

The main purpose of this study is to conduct a simultaneous examination of the expression pattern of VDR and CYP24A1 mRNA in a cohort of human breast, lung, colon and ovary tumor samples. Our results demonstrate that the expression of VDR and CYP24A1 mRNA is altered in tumor tissues. Since $1,25(OH)_2D_3$ is involved in regulating cell differentiation and proliferation, our results suggest that the VDR signaling pathway is compromised during tumorigenesis.

In both colon and lung tumors, CYP24A1 mRNA is significantly up-regulated, while VDR mRNA is generally down-regulated when compared to normal tissues. While there is no previous report on lung tumors, Shabahang et al. [11] showed that, when the level of VDR in 12 malignant colonic tumors was compared with that of adjacent normal tissue, in 9 cases out of 12, the tumor expressed a lower VDR level. However, in that study the expression of CYP24A1 was not assessed. They also showed that, at least in human colon cancer cell lines, the level of VDR correlates with the degree of differentiation. Although the biopsies of tumor and normal tissues in our study were not matched to the same patients, our observation in colon tumors is consistent with results reported by Shabahang et al. We have also examined the diagnosis information on each colon tumor sample, but were unable to correlate the expression level of either VDR or CYP24A1 mRNA with the degree of differentiation in our colon tumor samples.

Previously Bortman et al. [13] reported that, in breast cancer and adjacent normal tissue from 50 Brazilian patients, VDR mRNA was detected, but no significant difference was observed between normal and tumor tissues, which is similar to our observation that, unlike in the other three tumors, there was no clear separation of VDR expression levels in tumor and normal breast tissues. Regarding CYP24A1 expression in breast tumors, we found no previous reports other than one study showing that a region of amplification within chromosomal region 20q13.2 was mapped to the CYP24A1 - gene [12]. When 3 breast tumor samples were examined, relative levels of CYP24A1 mRNA were higher in tumors with higher amplification at the CYP24 locus [12], suggesting that CYP24A1 may be up-regulated in breast cancer. However, in our current study, we observed that the mean value of CYP24A1 mRNA in the breast tumor samples was significantly lower than that in the normal samples. More studies comparing CYP24A1 expression in breast tumor will be needed to understand the role of CYP24A1 in breast tumor pathogenesis.

It is interesting to see that VDR mRNA, like CYP24A1 mRNA, is highly up-regulated in ovarian tumors, which is different from the observation made in the other three tumors. Amplification of chromosomal region 20q12q13 containing the CYP24A1 gene has been reported in ovarian cancer [16], but the up-regulation of CYP24A1 in ovarian tumor has not been reported. The VDR expression pattern in ovarian tumors has also not been reported previously. Although inhibition of ovarian cancer cell growth by $1,25(OH)_2D_3$ has been shown [17, 18], a clinical trial testing the efficacy of $1,25(OH)_2D_3$ combined with isotretinoin in treating 22 epithelial ovarian cancer patients for 74 weeks has not produced positive results [10]. It requires more studies to understand why VDR is selectively up-regulated in ovarian cancer.

It is known that cancer cell lines display a range of sensitivities to the anti-proliferative effects of $1,25(OH)_2D_3$. The reason for $1,25(OH)_2D_3$ insensitivity is largely unknown, and could result from defects in any of the components in the VDR signaling pathway including VDR and CYP24A1. Previously it has been shown that different batches of MCF-7 cells may express different levels of VDR and there is an inverse correlation between the VDR level and inhibition of cell growth by a vitamin D analogue [7]. In another study testing a breast cancer cell line, HBL100, it was found that the lack of anti-proliferative effect of vitamin D analogues was due to a functional defect in VDR [8]. Moreover, it has been shown that, in THP-1/DR which is resistant to vitamin D analogues, the resistance was due to a failure in VDR translocation to the nucleus, again suggesting a defect in VDR function. Most recently, Banwell et al. [19, 20] reported that the aggressive cancer cell lines insensitive to $1,25(OH)_2D_3$ have elevated nuclear co-repressor levels with associated histone deacetylase (HDAC) activity. Combining a low dose of the HDAC inhibitor Trichostatin A with $1,25(OH)_2D_3$ synergistically inhibits the proliferation of PC-3 prostate and MDA-MB-231 breast cancer cell lines. These studies, together with our results, suggest that the VDR signaling pathway may be compromised during tumor pathogenesis.

All three cancer cell lines tested in this study responded robustly to $1,25(OH)_2D_3$ during a 48 h treatment period when CYP24A1 mRNA induction was assessed. The cells responded modestly to $1,25(OH)_2D_3$ during a 72 h treatment period in terms of inhibition of thymidine incorporation. Interestingly, the modest effects of $1,25(OH)_2D_3$ on growth inhibition were no longer observed when cells were incubated with the drug over a 5 day period. Taken together, the results suggest that induction of CYP24A1 may result in metabolism and inactivation of $1,25(OH)_2D_3$, resulting in insensitivity of cells to the growth inhibitory effect of the drug. Our results are consistent with previous reports that, in prostate cancer (PCa) cells, the degree of growth inhibition by $1,25(OH)_2D_3$ appears to be inversely proportional to the CYP24A1 activity in the cells. Among the human PCa cell lines, DU 145 cells exhibit a high level of CYP24A1 induction and are least responsive to

$1,25(OH)_2D_3$. As a comparison, the basal and induced expression of CYP24A1 in LNCaP cells is very low and growth inhibition by $1,25(OH)_2D_3$ is substantially higher [21]. Ly et al. [22] have shown that inhibition of CYP24A1 activity by the P450 inhibitor liarozole in DU 145 cells increased the half-life of $1,25(OH)_2D_3$ and significantly improved growth inhibition in these cells. Another study [23] showed that the use of the P450 inhibitor ketoconazole inhibited the CYP24A1 activity and potentiated the growth inhibitory effects of $1,25(OH)_2D_3$ in primary human PCa cells.

In conclusion, our results suggest that the expression of CYP24A1 mRNA, and to a lesser degree, VDR mRNA, is altered in human tumors. In the pathogenesis of solid tumors, the function and metabolism of $1,25(OH)_2D_3$ and its growth regulatory effect may be compromised as a way to favor tumorigenesis.

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