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ORIGINAL PAPER

Serum Vitamin D Metabolites in Colorectal Cancer Patients Receiving Cholecalciferol Supplementation: Correlation with Polymorphisms in the Vitamin D Genes

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Abstract Cholecalciferol (D_3) supplementation results in variable increases in serum 25(OH)D_3 levels, however, the influence of genetic polymorphisms on these variable responses is unclear. We measured serum 25(OH)D_3, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_3$ and VDBP levels in 50 colorectal cancer (CRC) patients before and during 2,000 IU daily oral D_3 supplementation for six months and in 263 archived CRC serum samples. Serum PTH levels and PBMC 24-OHase activity were also measured during D_3 supplementation. TagSNPs in *CYP2R1*, *CYP27A1*, *CYP27B1*, *CYP24A1*, *VDR*, and *GC* genes were genotyped in all patients, and the association between these SNPs and serum vitamin D_3 metabolites levels before and after D_3 supplementation was analyzed. The mean baseline serum 25(OH)D_3 level was less than 32 ng/mL in 65 % of the 313 CRC patients. In the 50 patients receiving D_3 supplementation, serum levels of 25(OH)D_3 increased (p=0.008), PTH decreased (p=0.036) and 24,25$(OH)_2D_3$, 1,25$(OH)_2D_3$, VDBP levels and PBMC 24-OHase activity were unchanged. *GC* SNP rs222016 was associated with high 25(OH)D_3 and 1,25$(OH)_2D_3$ levels at baseline while rs4588 and rs2282679 were associated with lower 25(OH)D_3 and 1,25$(OH)_2D_3$ levels both before and after D_3 supplementation. *CYP2R1* rs12794714 and rs10500804 SNPs were significantly associated with low 25(OH)D_3 levels after supplementation but not with baseline 25(OH)D_3. Our results show that D_3 supplementation increased 25(OH)D_3 levels in all patients. *GC* rs4588 and rs2283679 SNPs were associated with increased risk of vitamin D_3 insufficiency and suboptimal increase in 25(OH)D_3 levels after D_3 supplementation. Individuals with these genotypes may require higher D_3 supplementation doses to achieve vitamin D_3 sufficiency.

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Introduction

Serum 25(OH)D_3 level is the accepted indicator of vitamin D_3 sufficiency in humans. Since dietary sources of vitamin D_3 are limited, the major cause of human vitamin D_3 insufficiency (defined as serum 25(OH)D_3<32 ng/mL) is inadequate exposure to the sun [1, 2]. Several factors including living at higher latitudes, cultural practices limiting skin exposure to sunlight, advanced age, obesity, and dark skin pigmentation are associated with low serum 25(OH)D_3 levels [3–5]. We have reported that recent cancer chemotherapy may be a predisposing factor for vitamin D_3 deficiency in CRC patients [6].

The clinical significance of vitamin D insufficiency in patients with established colorectal cancer, especially those with metastatic disease, is unclear. However, the association between vitamin D_3 insufficiency and an increased risk of developing colorectal cancer is supported by several case–control studies [7, 8]. In a Norwegian study, higher colorectal cancer mortality was observed when the diagnosis of colon cancer was made during winter or spring (lower $25(OH)D_3$) compared to summer and autumn (higher $25(OH)D_3$) [9]. Furthermore, the Nurse's Health Study (NHS) and Health Professionals follow-up study showed that the lowest risk of death (adjusted for other variables) from CRC disease was seen in patients in the highest quartile of serum $25(OH)D_3$ levels [10].

Polymorphisms in several vitamin D genes (*CYP2R1*, *CYP27A1*, *CYP27B1*, *CYP24A1*, *VDBP* and *VDR*) have been associated several human diseases, including CRC [11]. The proteins encoded by these genes include CYP2R1 and CYP27A1 which convert vitamin D to $25(OH)D_3$. $25(OH)D_3$ is then converted by CYP27B1 to $1,25(OH)_2D_3$, the biologically most active form of vitamin D_3. CYP24A1 converts $25(OH)D_3$ and $1,25(OH)_2D_3$ to 24, $25(OH)_2D_3$ and 1, 24, 25 $(OH)_2D_3$, respectively, which are less active. Vitamin D binding protein (VDBP), encoded by *GC* (group-specific component), is the transporter of vitamin D_3 metabolites in circulation while VDR, a nuclear hormone receptor, mediates the action of vitamin D_3 by transcriptionally controlling the expression of hormone sensitive genes. Studies show significant association between single nucleotide polymorphisms (SNP) in the *GC* (rs2282679), *DHCR7* (rs12785878), and *CYP2R1* (rs10741657) genes and serum $25(OH)D_3$ levels [12, 13]. There are reported associations between $25(OH)D_3$ levels and SNPs in *GC* (rs4588 and rs7041), *VDR* (rs10735810) and *CYP27B1* (rs10877012) genes [14, 15]. The AA genotype of *VDR* rs1544410 has been associated with higher serum $1,25(OH)_2D_3$ levels compared to the GG genotype [16]. Reports on the effect of D_3 supplementation on the associations between polymorphisms in the vitamin D_3 pathway genes and serum $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$levels are few. A suboptimal response to D_3 supplementation was associated with *GC* rs4588 SNP in healthy adults [17]. Comprehensive studies to identify SNPs in the vitamin D_3 metabolizing and signaling pathway genes that are associated with response to D_3 supplementation will provide insight into factors influencing vitamin D health and information on the planning of prospective cancer chemoprevention and therapeutic clinical trials.

In this study, we measured serum vitamin D metabolite levels ($25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_3$) together with vitamin D binding protein (VDBP) levels in 313 CRC patient samples and correlated these measures with SNPs in the major vitamin D_3 metabolizing and signaling pathway genes at baseline and with vitamin D sufficiency. We also tested for associations between these SNPs and changes in the serum vitamin D_3 metabolites levels in a subset of CRC patients receiving a fixed dose of 2,000 IU of oral cholecalciferol daily for 6 months.

Materials and Methods

Research Blood Sample Collections and Storage

Archived blood samples of 263 CRC patients were obtained from Roswell Park Cancer Institute (RPCI) Data Bank and BioRepository. Blood samples were also collected prospectively from RPCI CRC patients enrolled in a study of fixed oral dose of 2000 IU of cholecalciferol (D_3) once daily for 6 months. Eligibility criteria, baseline and follow-up clinical evaluation of the patients enrolled in the D_3 supplementation protocol has been published [18]. Protocols for these studies were both approved by the RPCI Institutional Review Board (IRB) prior to starting these studies.

Serum samples for vitamin D_3 metabolite and PTH level measurements were collected before treatment and on days 14, 30, 60, 90, and 180 of D_3 supplementation and stored at −80 °C until assayed. VDBP was measured in samples collected at baseline and on day 90 only. Baseline blood samples collected in EDTA tubes and stored at −80 °C was used for DNA isolation. Serum samples for PTH assay were stored at −80 °C and were assayed within 12 months of samples collection. PTH levels in archived serum samples were not measured.

Analytical Assays

Serum $25(OH)D_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$ Assays

A high-performance liquid chromatographic assay with tandem mass spectrometric detection (LC/MS/MS) was used to simultaneously measure $24,25(OH)_2D_3$ and $25(OH)D_3$ in 0.25 mL of human serum sample. Stable-isotope labeled 25-Hydroxyvitamin D_3-(26,26,27,27,27-*d*6) was the internal standard [19, 20]. Sample preparation for LC/MS/MS analysis included liquid–liquid extraction using methanol and chloroform (1:3 *v*/*v*). The dried organic layer containing $24,25(OH)_2D_3$ and $25(OH)D_3$ was reconstituted in 60 μL of methanol (MeOH)/water (60/40 %) and 25 μL volumes were injected and separated on reverse phase dC-18 column (Waters Corp) using MeOH/water gradient (0 min 80 % MeOH, 3 min 99 % MeOH, 10 min 99 % MeOH,10.1 min 80 % MeOH, 15 min 80 % MeOH at flow rate of 220 μL/min). TSQ Quantum ULTRA mass spectrometer with positive atmospheric pressure chemical ionization (APCI) source was used to monitor $24,25(OH)_2D_3$, $25(OH)D_3$ and the internal standard at transitions of *m*/*z* 399>127, 383>365 and 389>371 respectively. The assay

lower limit of detection were 0.25 ng/mL for both $24,25(OH)_2D_3$ and $25(OH)D_3$. Serum $25(OH)D_3$ levels measured by our LC/MS/MS method were 10–17 % higher than the values obtained using DiaSorin 25-hydroxyvitamin D RIA kits. Serum $1,25(OH)_2D_3$ levels were measured using DiaSorin RIA kits as previously described [21].

Plasma PTH and VDBP Assays

Serum PTH levels were measured using biologically intact hPTH 1–84 immunoradiometric Kits from DiaSorin (Stilwell, MN). Quantitative determination of total serum vitamin D binding proteins (VDBP) was performed using K2314 VDBP ELISA kits from ALPCO immunoassays (Salem, NH). Both assays were performed according to the instructions supplied with kits.

24-Hydroxylase Activity Assay

PBMC 24-OHase activity was assayed as previously described [22, 23]. The assay consisted of 2×10^6 PBMC (as the 24-OHase enzyme source), and 0.5 μCi (110,000 dpm) [^{3}H]-$25(OH)D_3$ as the substrate; total assay volume was 0.5 ml of buffer (0.19 M sucrose, 25 mM sodium succinate, 2 mM $MgCl_2$, 1 mM EDTA and 20 mM HEPES), pH 7.4. The reaction was initiated by the addition of substrate followed by 30 min incubation at 37 °C. [^{3}H]-$25(OH)D_3$ oxidative metabolites were extracted by liquid–liquid partition, separated by HPLC and measured by liquid scintillation counting of collected HPLC fractions. Radioactivity in the fractions containing all 24-oxidative metabolites of $25(OH)D_3$ were pooled and 24-OHase activity, normalized for variations in extraction efficiency, was calculated using the radioactivity ratio of pooled 24-oxidative metabolites/total radioactivity recovered and expressed as femtomoles per hour per mg of protein.

Genotyping Assays

Genomic DNA was isolated from whole blood samples using Flexi Gene DNA kits (Qiagen Inc, Valencio, CA). The quality and quantity of DNA isolated was determined using both Nanodrop (Thermo Scientific, Waltham, MA) and PicoGreen (Molecular Probes Inc., Eugene, OR) methods.

TagSNPs in *CYP2R1*, *CYP27A1*, *CYP27B1*, *CYP24A1*, *VDR* and *GC* genes were selected from databases (NCBI; HapMap) and from *CYP24A1* re-sequenced data generated in our laboratory from Caucasian DNA samples from the Coreil Cell Repository (unpublished). Briefly, SNP genotype datasets for Caucasians were loaded into the Haploview program (version 4.0) (Broad Institute, MIT and Harvard, Cambridge, MA, USA) and polymorphisms with frequencies greater than 5 % were selected for haplotype analysis at an r^2 threshold of 0.8. Haplotypes close to or greater than 2 % were organized into single blocks from which haplotype tagSNPs were derived. The tagger software was also used to generate tagSNPs. Other websites used for tagSNPs selection included NIEHS (http://snpinfo.niehs.nih.gov/snptag.htm) and GVS (http://gvs.gs.washington.edu/GVS/). A total of 80 tagSNPs were genotyped using the Sequenom MassArray platform (Sequenon, San Diego, CA) in accordance with the manufacturer's instructions (http://www.sequenom.com/seq-genotyping). Controls were included to ensure genotyping accuracy in addition to genotyping approximately 10 % of the samples in duplicates.

Data Analysis

Patients with baseline serum $25(OH)D_3$ levels <20 ng/mL, 20–31.9 ng/mL and ≥32 ng/mL were considered to be vitamin D_3 deficient, insufficient and sufficient, respectively. Free index (a measure of non-protein bound) $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were calculated as a molar ratio of each vitamin D_3 metabolite/VDBP. Frequencies and relative frequencies were computed for categorical variables and numeric variables were summarized using mean, standard deviation, standard error of mean, median and range as appropriate. Fisher's exact test was used to study the association between categorical variables and the Wilcoxon rank sum test was used to compare the groups' numeric variables.

The generalized linear models with corresponding link function and distribution of different response variables were used to investigate the association between study variables and a set of variables for multivariate analysis. $P<0.05$ nominal significance level was used in all testing. All statistical analyses were done using SAS (version 9.1).

Association analysis of serum vitamin D_3 metabolite concentrations with SNPs were performed using generalized linear models. Logistic regression models were used to measure SNP association analyses with sufficiency status (insufficient/deficient vs. sufficient). For the D_3 supplementation population, vitamin D_3 metabolite levels were log transformed and genotypes were coded as 0, 1, and 2 for homozygous major, heterozygous and homozygous minor genotypes, respectively. Due to small sample size of D_3 supplementation group and rarity of minor genotypes, the minor and heterozygous genotypes were pooled and compared to major genotype (reference group) for all SNPS. Models were adjusted for age, sex, BMI, tumor stage, chemotherapy status and plasma VDBP levels. The β-coefficients from the linear models represent the quantitative change in plasma measures with the addition of the minor allele. The β coefficients from the logistic regression models reflect the increase in

odds of deficiency (or sufficiency) with each additional copy of the variant allele.

Results

Patient Characteristics, Baseline Serum Vitamin D_3 Metabolite and VDBP Levels

Characteristics, including self-reported racial background, of all 313 CRC patients, stratified into three vitamin status categories (deficient, insufficient, and sufficient, based on baseline serum 25(OH)D_3 levels), are shown in Table 1; 69.1 % of the vitamin D_3 sufficient patients had blood samples collected in summer/fall compared to 52.2 % and 39.8 % for the insufficient and deficient patients, respectively. Neither BMI nor other patient demographic characteristics (gender, age, race, and tumor stage) showed significant association with vitamin D_3 status. Serum 24,25$(OH)_2D_3$ levels were significantly associated with baseline serum 25(OH)D_3 levels ($p<0.0001$), whereas serum 1,25$(OH)_2D_3$ and VDBP levels were not.

Effect of D_3 Supplementation on Serum Vitamin D_3 Metabolites, PTH, VDBP Levels and PBMC 24-OHase Activity

Time course of the changes in serum 25(OH)D_3, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_3$, PTH and VDBP levels and PBMC 24-OHase activity during D_3 supplementation are shown in Fig. 1. The increase in 25$(OH)_2D_3$ attained equilibrium levels by day 90; the largest percentage increase in 25(OH)D_3 levels was in the vitamin D_3 deficient category (Fig. 1a). Similar increases in 24,25$(OH)_2D_3$ levels were observed (Fig. 1b). The 1,25$(OH)_2D_3$ levels also increased but remained within the physiologic range of 20–100 pg/mL (Fig. 1c). Serum 25(OH)D_3 and 24,25$(OH)_2D_3$ levels at baseline and during D_3 supplementation were linearly correlated (Fig. 1d). The correlation between 25(OH)D_3 and 1,25$(OH)_2D_3$, and between 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ serum levels are shown in Fig. 1e and f, respectively.

A decrease in serum PTH levels was observed within 30 days of starting D_3 supplementation and no further decrease in PTH levels was observed from day 60 onwards (Fig. 2a). No significant changes in PBMC 24-OHase activity and serum

Table 1 Demographic and serum vitamin D_3 variables in CRC patients stratified by baseline serum vitamin D_3 status

Characteristics/variables		Baseline vitamin D_3 status*			*p* value
		Deficient (*N*=88)	Insufficient (*N*=115)	Sufficient (*N*=110)	
Age, years <40		4	4	9	0.44
40–49		13	24	12	
50–59		26	26	32	
60–69		29	37	34	
≥70		15	25	23	
Gender	F	44 (50 %)	48 (41.7 %)	55 (50 %)	0.37
	M	44 (50 %)	67 (58.3 %)	55 (50 %)	
Race	White	76 (86.4 %)	101 (87.8 %)	100 (90.9 %)	0.52
	Black	8 (9.1 %)	12 (10.4 %)	6 (5.5 %)	
	Others	4 (4.5 %)	2 (1.7 %)	4 (3.6 %)	
Tumor stage	Early (I/II)	8 (9.1 %)	18 (15.8 %)	16 (14.7 %)	0.35
	Advanced (III/IV)	80 (90.9 %)	96 (84.2 %)	93 (85.3 %)	
Sample collection season	Summer–Fall	35 (39.8 %)	60 (52.2 %)	76 (69.1 %)	0.004
	Winter–Spring	53 (60.2 %)	55 (47.8 %)	34 (30.9 %)	
BMI (kg/m^2)	<25	27 (30.7 %)	23 (21.3 %)	34 (33.0 %)	0.065
	25–30	25 (28.4 %)	43 (39.8 %)	42 (40.8 %)	
	>30	36 (40.9 %)	42 (38.9 %)	27 (26.2 %)	
Vitamin D binding protein (VDBP) (μg/mL)		354.1±77.6	357.8±75.0	387.3±102.4	0.079
D_3 metabolites	25(OH)D_3 (ng/mL)	14.4±4.0	25.7±3.4	38.8±5.9	Inference
	24,25$(OH)_2D_3$(ng/ml)	0.4±0.3	1.0±0.7	2.2±2.1	<0.0001
	1,25 $(OH)_2D_3$ (pg/mL)	53.0±16.2	53.5.4±15.1	54.2±15.4	0.46

For numerical variables statistics represented as mean±SD

For categorical variables statistics represented as frequency (% relative frequency)

*Definitions of vitamin D_3 status categories are based on serum 25(OH)D_3 levels

<20ng/mL=deficient, 20–31.9 ng/mL=insufficient and ≥32ng/mL=sufficient

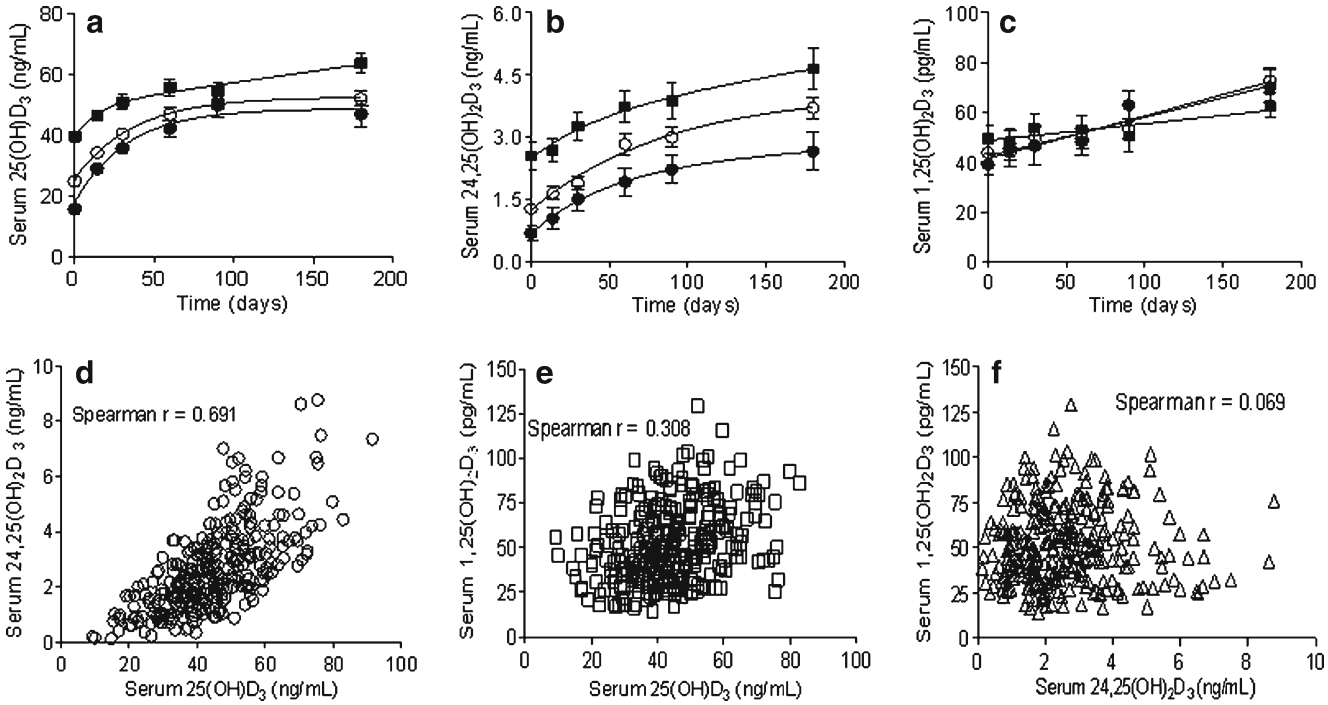

Fig. 1 Time course of the changes in serum 25(OH)D_3 (panel **a**), 24,25$(OH)_2D_3$ (panel **b**), and 1,25$(OH)_2D_3$ (panel **c**) levels in vitamin D_3 deficient (*filled circle*), insufficient (*open circle*), and sufficient (*filled square*) CRC patients receiving 2000IU of oral cholecalciferol daily. Vitamin D_3 status stratification based on baseline serum 25(OH)D_3 levels: deficient<20 ng/mL (N=9), insufficient 20 to 31.9 ng/mL (N=24) and sufficient≥32ng/mL (N=17). Panels **d**, **e**, and **f** show plots (and correlation coefficients) of serum 25(OH)D_3 versus 24,25$(OH)_2D_3$, 25(OH)D_3 versus 1,25(OH)D_3, and 24,25$(OH)_2D_3$ versus 1,25(OH)D_3 levels at baseline and during D_3 supplementation. Caption in panel **a**, also refers to panels **b** and **c**

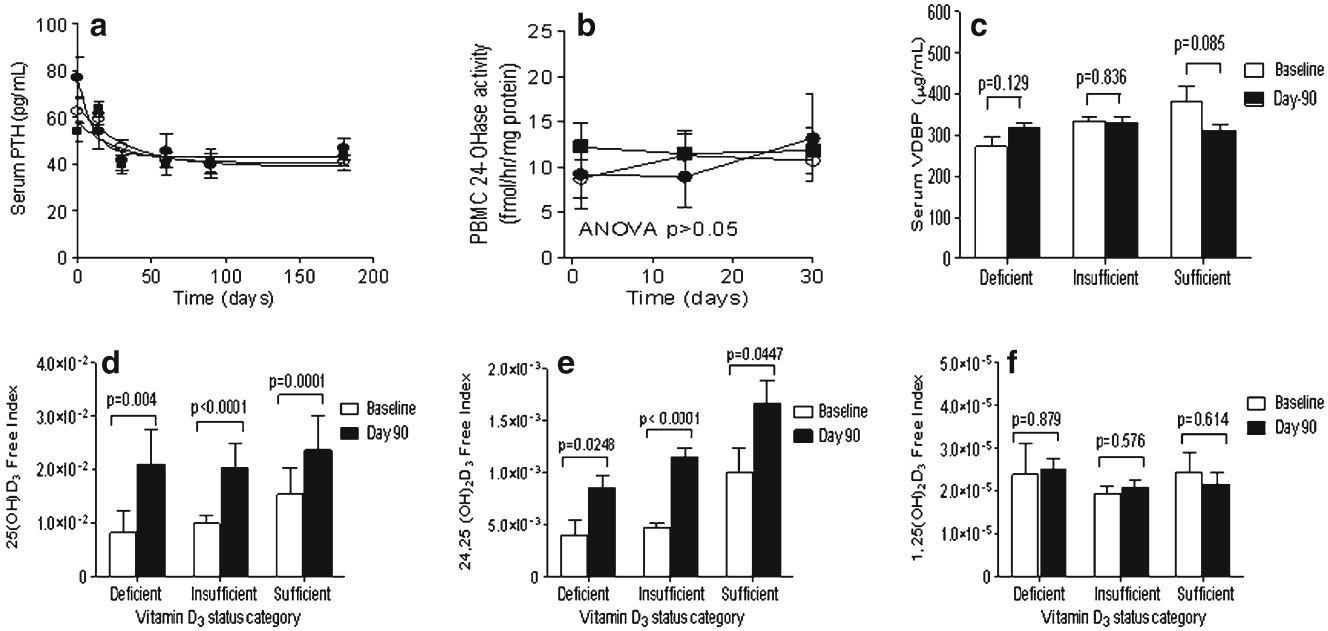

Fig. 2 Time course of the change in serum PTH levels (panel **a**) and PBMC 24-hydroxylase activity (panel **b**) in vitamin D_3 deficiency (*filled circle*, N=9), insufficient (*open circle*, N=24), and sufficient (*filled square*, N=17) CRC patients receiving 2,000 IU of oral cholecalciferol daily; panel **c** shows the baseline and day 90 serum VDBP levels. Net effects of the cholecalciferol supplementation mediated changes in plasma vitamin D_3 metabolite and VDBP levels were evaluated by calculating free index of 25(OH)D_3 (panel **d**), 24,25$(OH)_2D_3$ (panel **e**) and 1,25$(OH)_2D_3$ (panel **f**) at baseline and on day 90 of the D_3 supplementation. Free index=molar ratio of vitamin D_3 metabolite/VDBP

VDBP levels were observed during D_3 supplementation (Fig. 2b and c). Baseline and day 90 serum VDBP levels for all three vitamin D_3 status categories were within the normal range (200–550 μg/mL). D_3 supplementation increased the free indices of serum 25(OH)D_3 and 24,25$(OH)_2D_3$ (Fig. 2d and e); 1,25$(OH)_2D_3$ free index did not change (Fig. 2f).

In summary, the significant consequences of D_3 supplementation were the decrease in serum PTH levels by day 30, and increase in 25(OH)D_3 levels on days 30 and 90; the changes in serum of 24,24$(OH)_2D_3$, 1,25$(OH)_2D_3$ and VDBP levels and PBMC 24-OHase were not significant at any of the time points analyzed (Table 2).

Vitamin D SNPs and Serum Vitamin D_3 Metabolite Levels and Risk of Vitamin D_3 Insufficiency

Associations between the tag SNPs from the 6 genes (*VDR*, *GC*, *CYP2R1*, *CYP27A1*, *CYP27B1*, and *CYP24A1*) with baseline serum levels of 25(OH)D_3, 24,25$(OH)_2D_3$, and 1,25$(OH)_2D_3$ were evaluated in the 313 CRC patients. At $p<0.05$, two *GC* SNPs (rs4588 and rs2282679, in linkage disequilibrium [LD] with each other, $r^2=0.98$), two *CYP24A1* SNPs (rs2762934 and rs2762939) and one *VDR* SNP (rs11168267) were associated with low baseline levels of at least one serum vitamin D_3 metabolite; SNPs associated with high baseline levels of at least one serum vitamin D_3 metabolite were *GC* rs222016, *CYP24A1* rs111675277 and *VDR* rs11574077 (Table 3). After multiple testing, only *GC* rs4588 remained significantly associated with low baseline serum 1,25$(OH)_2D_3$ levels.

The *GC* rs4588 and rs2282679 SNPs were associated with less increase in serum 25(OH)D_3 and 1,25$(OH)_2D_3$ levels after 90 days of D_3 supplementation in 50 patients (Table 3). Six other SNPs, rs10500804 and rs12794714 (*CYP2R1*), rs6022999 (*CYP24A1*) and rs2525044, rs739837, rs7975232 (*VDR*) were also associated with low levels of at least one vitamin D metabolite after supplementation (Table 3). Only two SNPs were associated with higher serum vitamin D_3 metabolite levels after supplementation (Table 3).

The risk of vitamin D insufficiency in association with tagSNPs was evaluated in the 313 CRC patients stratified into 2 groups: vitamin D_3 sufficient (reference, baseline serum 25(OH)$D_3 \geq 32$ng/ml) and vitamin D_3 insufficient (baseline serum 25(OH)$D_3 < 32$ng/mL). *GC* rs4588 and rs2282679 were associated with increased odds of vitamin D_3 insufficiency while *GC* rs222014 and *CYP24A1* rs4809958 and rs6013905 in LD (with $r^2=1$) were associated with reduced odds of insufficiency (Table 4).

Discussion

In this study, 64.9 % of CRC patients had baseline serum 25(OH)D_3 levels less than 32 ng/mL. Similar high percentage of vitamin D_3 deficient and insufficient individuals have been reported in healthy controls and patients with acute myeloid leukemia, colorectal, prostate, and breast cancer in Western New York [24–26]. Therefore, the high prevalence of vitamin D_3 deficiency in these CRC patients simply reflects the vitamin D_3 status of the general population in

Table 2 Changes in vitamin D_3 metabolites and other vitamin D_3 related variables measured during D_3 supplementation in 50 CRC patients categorized by vitamin D_3 status

Time (days)	Changes in serum D_3 variables from baseline	Vitamin D_3 status			*p* value
		Deficient (*N*=9)	Insufficient (*N*=24)	Sufficient (*N*=17)	
30	Δ25(OH)D_3	20.6±5.9	15.5±7.8	11.0±9.6	0.009
	Δ24,25$(OH)_2D_3$	0.8±0.6	0.6±0.6	0.7±1.2	0.80
	Δ1,25$(OH)_2D_3$	7.5±20.5	6.5±15.1	4.4±18.7	0.9
	ΔPTH	−35.5±18.2	−15.4±25.0	−16.3±19.9	0.04
	ΔPBMC CYP24A1 activity	3.5±10.1	2.0±6.0	−0.3±8.4	0.70
90	Δ25(OH)D_3	34.5±11.1	26.3±11.6	13.8±11.3	0.0007
	Δ24,25$(OH)_2D_3$	1.5±1.0	1.7±1.0	1.3±1.7	0.30
	Δ1,25$(OH)_2D_3$	23.7±20.9	9.4±20.5	1.0±23.1	0.10
	ΔPTH	−32.0±14.0	−21.6±29.4	−15.1±23.4	0.20
	ΔVDBP	44.4±71.2	−3.4±61.8	−69.3±166.1	0.09
180	Δ25(OH)D_3	32.5±14.6	27.5±12.0	21.7±15.4	0.20
	Δ24,25$(OH)_2D_3$	2.0±1.3	2.3±1.2	2.1±2.0	0.60
	Δ1,25$(OH)_2D_3$	31.1±19.6	29.3±27.4	12.0±27.0	0.10
	ΔPTH	−30.3±23.4	−24.1±24.4	−13.3±21.7	0.20

Statistics represent mean±SD. Tests for equality for all the variables between baseline 25(OH)D_3 groups at baseline, day 30, 90 and 180 done by Chi-square tests

Table 3 Associations between SNPs in the vitamin D_3 pathway genes with serum $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ levels at baseline and after 90-days of D_3 supplementation

D_3 metabolites	Gene	SNP ID	Function	Baseline (N=313)		After D_3 supplementation (N=50)[a]	
				β	p value[b]	β	p value[b]
$25(OH)D_3$	*GC* (*VDBP*)	rs4588	Exon/Missense	−2.43	0.017	−0.19	0.0009
		rs2282679	Intron	−2.49	0.015	−0.19	0.0008
		rs222016	Intron	2.24	0.035	Not significant	
	CYP2R1	rs10500804	Intron	Not significant		−0.13	0.04
		rs12794714	Exon/Synonymous			−0.13	0.04
		rs7129781	Intron			0.23	0.04
	CYP24A1	rs111675277	Intron	5.15	0.028	Not significant	
	VDR	rs11168267	Intron	−3.59	0.030		
$24,25(OH)_2D_3$	*CYP2R1*	rs7129781	Intron	Not significant		0.53	0.02
	CYP24A1	rs6022999	Intron			−0.32	0.02
	VDR	rs11574077	Intron	0.71	0.023	Not significant	
$1,25(OH)_2D_3$	*GC* (*VDBP*)	rs4588	Exon/Missense	−4.86	0.0008[c]	−0.28	0.01
		rs2282679	Intron	−4.69	0.0012	−0.27	0.02
		rs222016	Intron	3.40	0.025	Not significant	
	CYP24A1	rs2762934	mRNA-untranslated	−4.01	0.010		
		rs2762939	Intron	−3.72	0.007		
	VDR	rs2525044	Intron	Not significant		−0.34	0.007
		rs11574077	Intron			0.59	0.01
		rs739837	mRNA-untranslated			−0.42	0.002
		rs7975232	Intron			−0.43	0.001

Only significant association (p<0.05) between SNPs and either baseline or day-90 plasma vitamin D_3 variables are shown. The only baseline and day-90 overlapping associations were between *GC* (rs4855 and rs2282679) with both $25(OH)D_3$ and $1,25(OH)_2D_3$. SNPs in linkage disequilibrium [LD]: rs4855/rs2282679 (r^2 =0.98), rs10500804/rs12794714 (r^2 =1.0) and rs7975232/rs739837 (r^2 =0.9). *CYP2R1* rs7129781 was associated with high $25(OH)D_3$ and $24,25(OH)_2D_3$ levels after D_3 supplementation while *VDR* rs11574077 was associated with high $1,25(OH)_2D_3$ levels

[a] Most frequent genotype was used as reference and compared with pooled heterozygous and rare genotypes because of small sample size

[b] Raw p values (not corrected for multiple testing)

[c] The only significant association after multiple testing

Western New York. As expected, $25(OH)D_3$ levels were higher in blood samples collected in summer/fall than in samples collected in winter/spring. There was no association between $25(OH)D_3$ levels and race perhaps because of the small sample size of Black Americans in this study.

Table 4 Genotype association with vitamin D_3 status at baseline

Gene	SNPs ID	OR (95 % CI)	p value
GC	rs222014	0.476 (0.27–0.84)	0.009
CYP24A1	rs4809958[a]	0.570 (0.35–0.92)	0.021
CYP24A1	rs6013905[a]	0.580 (0.36–0.93)	0.021
GC	rs4588[b,c]	1.65 (1.07–2.58)	0.026
GC	rs2282679[b]	1.65 (1.07–2.58)	0.026

$25(OH)D_3$ reference group is sufficient (coded as zero), 1=deficient+insufficient; increase in odds of deficient/insufficient for each additional copy of variant allele

[a] SNPs in linkage disequilibrium [LD] with each other

[b] SNPs in linkage disequilibrium [LD] with each other

[c] This SNP is located in an exon, all others are in introns

The increase in $25(OH)D_3$ and decrease in PTH levels which we observed in all vitamin D_3 status categories, is consistent with prior reports and were expected as physiologic response to D_3 supplementation. However, other reports indicate that D_3 supplementation has no effect on serum PTH levels [27, 28]. These contradictory observations may reflect differences in D_3 supplementation doses utilized, calcium homeostasis related to dietary calcium intake and other calciotropic hormones. Our data suggest that serum $24,25(OH)_2D_3$, $1,25(OH)_2D_3$, and VDBP levels are of limited value as measures of response to D_3 supplementation. Changes in serum $25(OH)D_3$ without associated change in serum $1,25(OH)_2D_3$ levels during D_3 supplements has been reported [29]. Moreover, there is no difference in baseline serum $24,25(OH)_2D_3$ levels in these CRC patients (1.4±0.5 ng/mL) and that reported in healthy controls [30, 31]. This finding suggests that $25(OH)D_3$-24-hydroxylation may not play a critical role in the pathogenesis

of systemic vitamin D_3 insufficiency in CRC patients. The use of serum 24,25$(OH)_2D_3$ levels in evaluating the role of 24-hydroxylase in vitamin D_3 status and responses to cholecalciferol supplementation is, however, of limited value because of its further metabolism, by the same enzyme, to 25$(OH)D_3$-23,26-lactone and cholecalcoic acid. Whereas, the decrease in 25$(OH)D_3$ is not a useful measure of 24-hydroxylase because it is also a substrate of non 24-hydroxylase enzymes such as CYP11A1 [32, 33].

We found two major kinetic differences between the changes in total and free serum 25$(OH)D_3$, 24,25$(OH)_2D_3$, and 1,25$(OH)_2D_3$ levels during D_3 supplementation: First, while the increase in total serum 25$(OH)D_3$ and 24,25$(OH)_2D_3$ levels was biphasic, the increase in total serum 1,25$(OH)_2D_3$ levels was linear. Second, free indices of both 25$(OH)D_3$ and 24,25$(OH)_2D_3$ increased whereas 1,25$(OH)_2D_3$ free index did not change. These observations suggest that changes in total and free serum 1,25$(OH)_2D_3$ levels were insufficient to trigger changes in PBMC 24-OHase and serum calcium levels. Free 1,25$(OH)_2D_3$ index is the serum measure highly correlated with hypercalcemia [34]. Although underlying mechanisms of the kinetic differences in 25$(OH)D_3$ and 1,25$(OH)_2D_3$ changes during D_3 supplementation are unknown, we suggest tight reciprocal regulation of 1,25$(OH)_2D_3$ levels by CYP24A1 and CYP27B1 catalyzed metabolism prevents the increase serum 1,25$(OH)_2D_3$ levels. Differences in the VDBP capacity and binding affinity along with variations in adipose tissue storage may play a further role in regulating serum 1,25$(OH)_2D_3$ levels. The percentages of free forms of 25$(OH)D_3$ and 1,25$(OH)_2D_3$ in circulation are 0.04 % and 0.4 %, respectively while VDBP affinity for 25$(OH)D_3$ is 5×10^{-8}–9.5×10^{-11} M compared to ~4×10^{-7} M for 1,25$(OH)_2D_3$ [35, 36].

Our results show that genetic variants of *GC* SNPs rs4588 and rs2282679 were associated with low baseline levels of 25$(OH)D_3$ and 1,25$(OH)_2D_3$, with increased odds of vitamin D_3 insufficiency and with suboptimal response to D_3 supplementation in CRC patients. These findings are consistent with previous reports where these same SNPs have been associated with low serum 25$(OH)D_3$ in both healthy and patients with a variety of diseases including cancer [7–11] and reduced serum 25$(OH)D_3$ changes after vitamin D_3 supplementation in healthy volunteers [12]. Although, genome wide studies have associated *CYP2R1* SNPs rs10500804 and rs12794714, with vitamin D levels and vitamin D insufficiency [7, 8] the present study only showed an association after supplementation.

GC SNP rs222014 and *CYP24A1* SNPs rs4809958 and rs6013905 were associated with reduced risk of vitamin D_3 insufficiency, while *GC* SNPs rs4588 and rs2282679 were associated with increased risk of insufficiency. However, it is unclear why *GC* rs222016 and *CYP24A1* rs111675277, SNPs associated with high 25$(OH)D_3$ levels, were not associated with reduced risk of D_3 insufficiency. Possible explanations may include differences in statistical analysis (continuous vs. categorical OR), the character of the colorectal cancer disease itself and/or small sample size. Thus *GC* rs222014 and *CYP24A1* rs4809958 and rs6013905 SNPs could be potential predictive markers for low risk vitamin D_3 insufficiency, while *GC* rs4588 and rs2282679 may be both predictive of insufficiency and reduced response to D_3 supplementation in CRC patients.

In summary, we found that baseline serum 25$(OH)D_3$ levels and polymorphisms in the vitamin D_3 pathway genes, especially in the *GC* and *CYP24A1* genes affect response to D_3 supplementation in CRC patients and are associated with increased risk of vitamin D_3 insufficiency. Further studies, in large populations, are needed to confirm and determine if individuals with *GC* rs4588 or rs2282679 SNPs require higher doses of D_3 supplementation to achieve vitamin D_3 sufficiency.

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Conflict of interest The authors declare that they have no conflict of interest

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