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High Oral Vitamin D_3 Intake Does Not Protect Against UVR-induced Squamous Cell Carcinoma in Mice

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Abstract. *Background/Aim: The effect of vitamin D on skin carcinogenesis is unclear. Vitamin D derivatives may protect against ultraviolet radiation (UVR)-induced DNA damage, immune suppression, and skin carcinogenesis. However, some epidemiological studies have reported an increased incidence of skin cancer associated with high serum vitamin D levels. We investigated the effect of vitamin D supplementation on serum, skin, and tumor vitamin D levels and on skin cancer development in hairless immunocompetent mice. Materials and Methods: Female C3.Cg-Hr^{hr}/TifBomTac immunocompetent mice (n=125) were randomly separated into five groups. Two groups received a high vitamin D_3 diet (4.5 μg/day/mouse). One group received a medium vitamin D_3 diet (2.3 μg/day/mouse). Two groups received a standard diet (0.045 μg/day/mouse). Three standard erythema doses of UVR were given three times per week to three groups. Results: Animals on a high vitamin D_3 diet had ~150-fold higher serum vitamin D_3 levels (p=0.00016) and 3-fold higher serum 25-hydroxyvitamin D_3 [25(OH)D_3] levels (p=0.00016) than those on a standard diet. For mice on the medium vitamin D_3 diet, serum vitamin D_3 and 25(OH)D_3 levels were 18-fold and 2.3-fold higher than for the standard diet, respectively (p=0.00016). All UVR-exposed mice developed tumors. Vitamin D_3 levels were lower in the tumor than the skin (p<0.0001). High and medium supplementation with vitamin D_3 did not affect tumor development (p>0.05). Conclusion: In mice, vitamin D levels in the serum, skin, and tumors were augmented by supplementation, but this did not affect the development of UVR-induced skin tumors.*

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Vitamin D is a fat-soluble vitamin synthesized in the skin after exposure to ultraviolet radiation B (UVB) or obtained from dietary sources (1). In the skin, 7-dehydrocholesterol is photochemically converted into vitamin D_3 by ultraviolet radiation (UVR) exposure (2-4). In the liver, vitamin D_3 is metabolized to 25-hydroxyvitamin D_3 [25(OH)D_3], the major circulating form in the blood. Levels of 25(OH)D_3 in serum (serum vitamin D) are measured to determine vitamin D status in the body (2).

In vitro and animal studies have shown that vitamin D is involved in cell growth regulation, differentiation, proliferation, and apoptosis (4-7). Several studies suggest that vitamin D may prevent and improve outcomes for many diseases, including cancer (5, 8-12). The essential role of vitamin D in bone health is well known and physicians have encouraged the general population to take vitamin D supplements, especially in parts of the world where exposure to sunlight is low for extended periods (13, 14).

A Danish study involving a cohort of 217,244 people investigated whether there was an association between vitamin D levels and cancer incidence (15). No information on vitamin D supplementation was collected, but the authors emphasized that Danish people frequently use supplements. The study showed an association between higher serum vitamin D levels (30-40 ng/ml) and a higher incidence of skin cancer (both keratinocyte cancer and melanomas), prostate cancer, and hematological cancer. In contrast, higher vitamin D levels were associated with lower lung cancer incidence (15). Furthermore, there was no association between serum vitamin D levels and the incidence of breast, urinary or colon cancers (15).

Table I. *Diet, ultraviolet radiation exposure schedule, and vitamin D concentration in the serum, skin, and tumors.*

Group	n	Diet	Irradiation dose (3 SEDs)	Serum 25(OH)D_3 ng/ml M (Q_1-Q_3)††	Serum Vitamin D_3 ng/ml M (Q_1-Q_3)††	Skin vitamin D_3 ng/g M (Q_1-Q_3)††	Tumor vitamin D_3 ng/g M (Q_1-Q_3)††
1	8	High vitamin D_3	NA	29.9 (28.3-33.3)	66.6 (62.1-76.7)	161.3 (150.6-175.3)	No tumors
		p-Value‡		0.00016	0.00016	0.00016	
2	8	High vitamin D_3	Yes	30.0 (23.2-34.7)	69.3 (56.9-74.7)	184.8 (169.3-196.3)	150.1 (132.8-186.8)
		p-Value‡		0.00016	0.00016	0.00016	0.00016
3	8	Medium vitamin D_3	Yes	22.7 (19.7-25.0)	8.1 (5.0-47.5)	140.4 (116.6-149.5)	60.4 (44.2-84.7)
		p-Value‡		0.00016	0.00016	0.00031	0.00067
4	8	Standard vitamin D_3	Yes	9.5 (8.8-12.4)	0.45 (0.25-0.54)	45.1 (40.1-82.7)	15.5 (12.2-24.9)
5	5	Standard vitamin D_3	NA	12.1 (9.8-21.1)	0.2 (0.15-1.9)	<LOQ	No tumors
		(Control group) *p*-value‡		0.28	0.50		

††M: median and Interquartile range: Q_1=25th percentile and Q_3=75th percentile. ‡The *p*-Value for each group is derived from a comparison with group 4, which had the standard vitamin D_3 diet and same ultraviolet radiation dose. SEDs: Standard erythema doses; NA: not administered; LOQ: limit of quantification [LOQ was 0.1 ng/ml for vitamin D_3 and 25(OH)D_3, and 5 ng vitamin D_3/g skin and tumor].

The association between vitamin D levels and skin cancer was assessed in a prospective study involving 1,191 Australians (16). Individuals with serum vitamin D levels that were greater than 30 ng/ml had an increased risk of developing basal cell carcinomas (BCCs) and melanomas but a decreased risk of developing squamous cell carcinomas (SCCs), compared to those with levels below 30 ng/ml (16). However, no increased risk of skin cancer was associated with vitamin D levels of 20-30 ng/ml (16).

In human studies, it may be difficult to determine whether high vitamin D serum levels are attributable to exposure to sunlight or to food and supplements. The positive association between high serum vitamin D levels and skin cancer could be due to higher exposure to UVR, leading to more DNA damage and skin cancer.

A study on immunocompetent mice showed inhibition of SCC development after topical use of 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2$D] (17). Conversely, a study on hairless mice in which animals were given different vitamin D diets and exposed to UVB showed that vitamin D did not protect against UVB-induced SCCs (18). The same trend was observed in a mouse BCC model in which vitamin D_3 produced in the skin by UVB protected against oncogenic effects by inhibiting the Hedgehog signaling pathway. In contrast, dietary vitamin D_3 provided no protection (19).

Thus, the effects of vitamin D supplementation on skin cancer remain unclear. Because people are exposed to innumerable substances throughout their lives, evaluating the protective or co-carcinogenic effects of vitamin D is difficult. One major advantage of a murine study is the use of a controlled environment. Here, immunocompetent mice were fed a standard, medium, or high vitamin D diet and exposed to UVR to investigate whether vitamin D supplementation altered vitamin D levels in the serum, skin, or tumors, and whether it affected the risk of skin cancer. In addition, the development of pigmentation was measured.

Materials and Methods

Animals. Female 14-24-week-old C3.Cg-*Hr^{hr}*/TifBomTac immunocompetent mice (Taconic, Ry, Denmark) were tattooed with consecutive numbers on the abdomen prior to the start of the experiment (*n*=125). They were randomly separated into five groups of 25 animals each and housed in separate boxes in a 12-h light/12-h dark cycle in a 23-24˚C warm facility. All experiments were approved by the appropriate national (permit number, 2019-15-0201-00131) and institutional ethical committees and followed national guidelines for the care and use of animals in research.

Diets and light sources. Animal diets and UVR were administered as shown in Table I. Food consumption was equivalent for each group (~3 g/day per animal). Groups 1 and 2 had diets enriched with vitamin D_3 (1,500 μg/kg) (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) corresponding to 4.5 μg/day per mouse, which we labelled as a high dose of vitamin D_3 supplementation.

Group 3 had the enriched Vitamin D_3 diet every second week and a standard diet containing 15 μg/kg vitamin D_3 during the other weeks (n˚1324; Altromin Spezialfutter GmbH & Co. KG), which we labelled as a medium dose of vitamin D_3 (2.3 μg/day per mouse). Groups 4 and 5 had standard diets (0.045 μg/day per mouse).

Animals had access to water and food *ad libitum* and were observed daily. Three standard erythema doses of UVR were given three times per week to mice in groups 2, 3, and 4. Light sources and UVR dose measurements were as described by Lerche *et al*. (20).

Tumor development. The first four tumors measuring at least 1 mm were registered and measured weekly until three tumors reached 4 mm or one tumor reached 12 mm in diameter (21). The 'time to the first tumor' was defined as the number of days until the first 1 mm-diameter tumor appeared. Only tumors that later grew to 4 mm in diameter were included in the statistical analyses (21). The times to the second and third tumors were

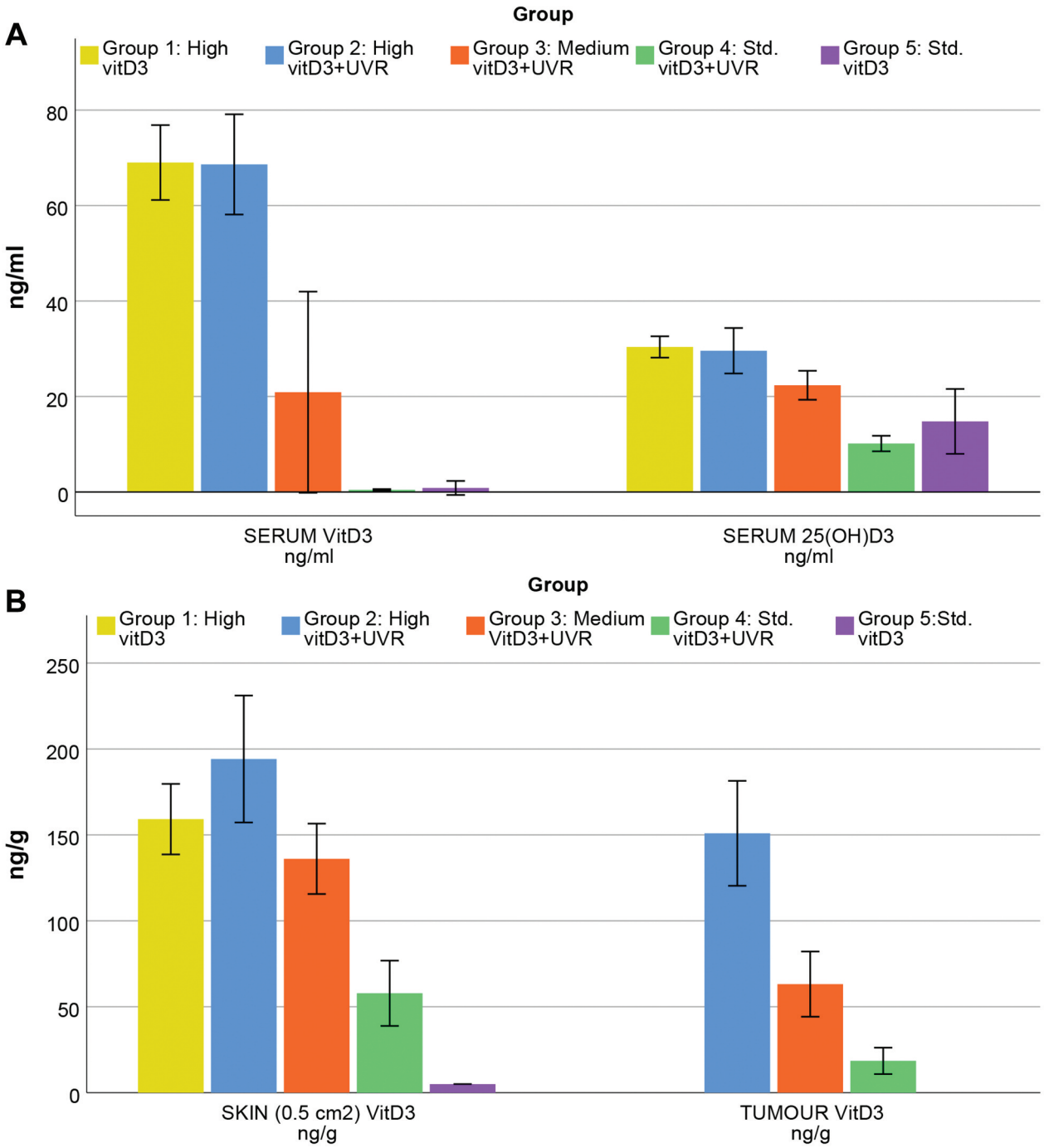

Figure 1. *Concentrations of vitamin D_3 and 25(OH)D_3 in serum, skin, and tumor in the different experimental groups. A) Concentrations of vitamin D_3 and 25(OH)D_3 in serum, quantified by liquid chromatography tandem mass spectrometry. B) Concentrations of vitamin D_3 in the skin and tumors, quantified by liquid chromatography tandem mass spectrometry.*

also recorded in the same way. A detailed inspection for tumors was carried out once a week. Euthanasia was performed after 365 days, or when mice had developed three tumors of 4 mm or one tumor of 12 mm in diameter. The skin was fixed in 4% buffered formaldehyde. Two randomly selected mice from each group were examined histopathologically to confirm that the tumors were SCCs.

Blood and skin sampling. Blood, dorsal, and ventral skin samples (8 mm punch biopsies: *i.e.*, 0.5 cm^2) were collected from all animals.

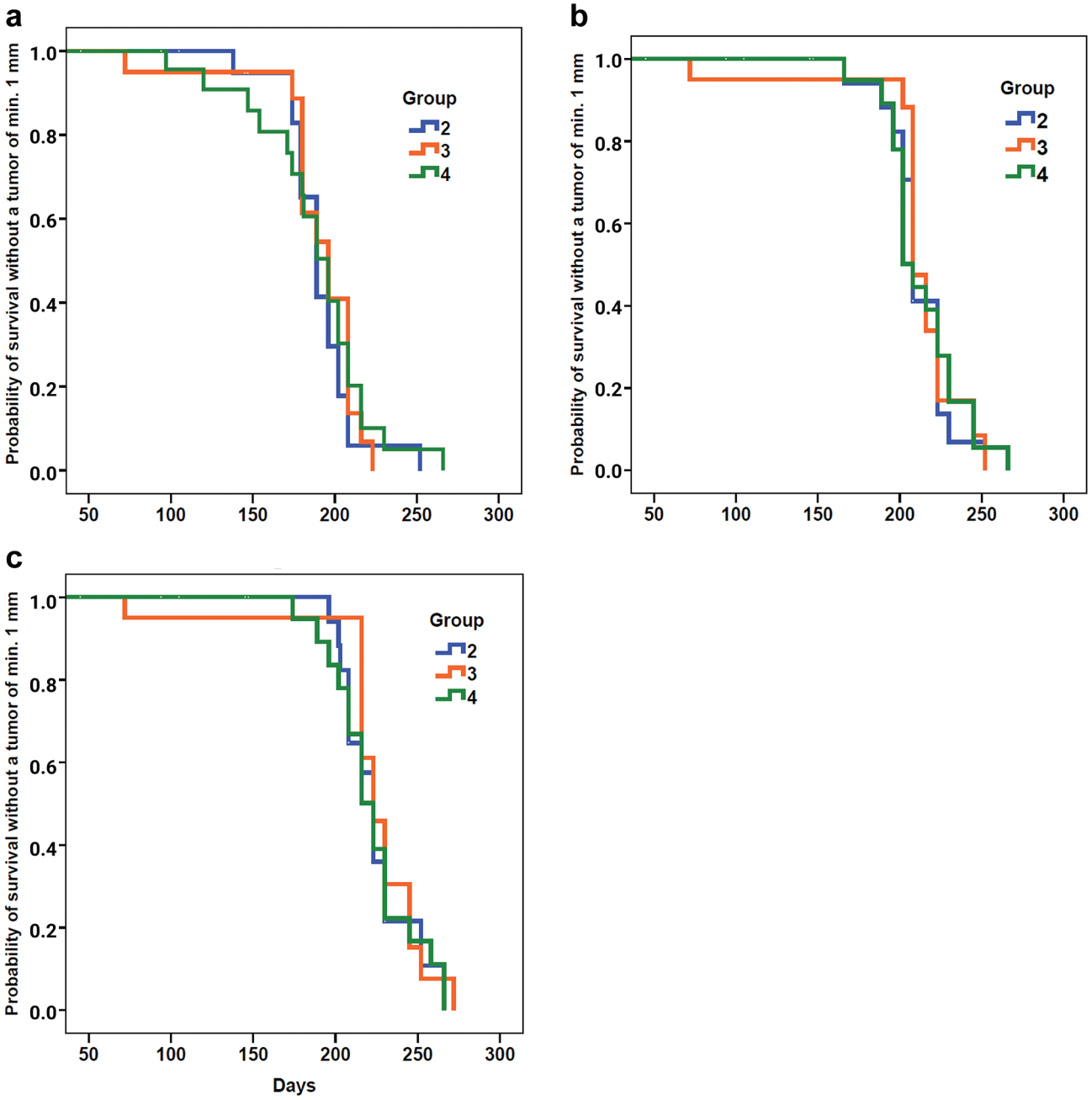

Figure 2. *Kaplan-Meier survival plots. A) First tumor. B) Second tumor. C) Third tumor. There are no significant differences in time to tumor development among the groups (p>0.05).*

Tumor samples were collected and weighed from all mice that had developed tumors. Vitamin D_3 and 25(OH)D_3 were quantified in serum and vitamin D_3 was quantified in the dorsal skin from eight randomly selected mice from groups 1, 2, 3, 4 and from five mice from group 5. Vitamin D_3 was quantified in tumor samples from eight mice from the tumor bearing groups 2, 3, and 4.

Analysis for vitamin D. Serum, skin and tumor samples were analyzed by a method described previously (22), but modified by using 13C-labelled internal standard as described by Loznjak *et al*. (23). In brief, 100 μl serum was treated with acetonitrile to precipitate protein. Vitamin D in the skin (0.040-0.089 g) and tumors (0.065-0.38 g) was extracted by alkaline saponification and liquid–liquid partition. The extract (from serum, skin, or tumors) was cleaned up by solid-phase extraction and derivatized using 4-phenyl-1,2,4-triazoline-3,5-dione. Finally, samples were separated by liquid chromatography coupled with triple quadrupole mass spectrometer for quantification (Agilent 1200 liquid chromatography coupled to

Table II. *Diet, ultraviolet radiation exposure schedule, and median number of days until 50% of the mice had a first, second, and third tumor.*

Group	n	Diet	Irradiation dose (3 SEDs)	Median days to first tumor $(Q_3\text{-}Q_1)^{\dagger}$	Median days to second tumor $(Q_3\text{-}Q_1)^{\dagger}$	Median days to third tumor $(Q_3\text{-}Q_1)^{\dagger}$
1	25	High vitamin D_3	NA	No tumors	No tumors	No tumors
2	25	High vitamin D_3	Yes	189 (202-179)	208 (223-202)	223 (230-208)
		p-Value‡		0.577	0.793	0.939
3	25	Medium vitamin D_3	Yes	196 (208-180)	208 (223-208)	223 (245-216)
		p-Value‡		0.965	0.820	0.507
4	25	Standard vitamin D_3	Yes	196 (208-174)	208 (230-202)	223 (230-208)
5	25	Standard vitamin D_3 (Control group)	NA	No tumors	No tumors	No tumors

†Interquartile range: Q_1=25th percentile and Q_3=75th percentile. ‡The *p*-Value for each group is derived from a comparison with group 4, which had the standard vitamin D_3 diet and same ultraviolet radiation dose. SEDs: Standard erythema doses; NA: not administered.

Agilent 6470 mass spectrometer; Agilent Technologies, Santa Clara, CA, USA). The limit of quantification was 0.1 ng/ml for vitamin D_3 and 25-hydroxy vitamin D_3 in serum and 5 ng vitamin D_3/g for the skin and tumors.

Weight and pigmentation. A single investigator scored skin pigmentation every month until 50% of the mice in a group developed tumors. The actual pigmentation was determined on a 20-point categorical scale in arbitrary units (au) using the Kodak Gray Scale. The pigmentation score was assessed under a bank of six TL08 fluorescent tubes (Philips Healthcare, Best, the Netherlands) in a dark room (24, 25). Weights were measured monthly.

Statistics. Tumor data (time to the first, second and third tumors) were visualized in Kaplan-Meier plots, and groups were compared using the log-rank (Mantel-Cox) test. Pigmentation, weight, vitamin D_3 and 25(OH)D_3 levels comparisons between groups were made using the Mann-Whitney test. The Wilcoxon signed-rank test was used to compare skin and tumor levels of vitamin D_3. All analyses were carried out using SPSS software (ver. 25.0; SPSS Inc., Chicago, IL, USA) and *p*-values less than 0.05 were considered significant.

Results

Vitamin D_3 and 25(OH)D_3 levels in serum. Animals supplemented with high vitamin D_3 diet (groups 1 and 2) had approximately 150-fold higher serum vitamin D_3 levels (p=0.00016) and 3-fold higher serum 25(OH)D_3 levels (p=0.00016) than those that had a standard diet (group 4) (Table I and Figure 1A). There was no significant difference between groups 1 and 2 regarding vitamin D_3 (p=1.00) or 25(OH)D_3 levels (p=1.00) although group 2 was UVR exposed.

In mice supplemented with medium dose vitamin D_3 (group 3), serum vitamin D_3 levels were 18-fold higher and serum 25(OH)D_3 levels were 2.3-fold higher than those in group 4 (p=0.00016) (Table I and Figure 1A). However, the serum increases observed in group 3 were considerably lower than those exhibited by groups 1 and 2 for both vitamin D_3 (p<0.002) and 25(OH)D_3 (p<0.04).

There was no significant difference in serum vitamin D_3 levels (p=0.28) or 25(OH)D_3 levels (p=0.50) between mice from group 4 and group 5 although group 4 had been UVR exposed. Both groups had a standard diet.

Vitamin D_3 levels in skin and tumors. The skin vitamin D_3 levels of mice fed with a high or medium dose vitamin D_3 diet (groups 1, 2 and 3) were 3- to 4-fold higher than those of group 4 mice (p<0.0005; Table I and Figure 1B). Detection of vitamin D_3 in the skin of control group mice (group 5), which did not receive UVR, was below the limit of quantification. Skin vitamin D_3 levels were lower in group 3 than in group 2 mice (p=0.00124) but not significantly different from group 1 mice (p=0.054). For the high vitamin D_3 diet groups, there was more vitamin D_3 in the skin of mice that had received UVR, but the difference was not significant (p=0.065).

Vitamin D_3 levels in the tumors of group 2 and group 3 mice were 9.6- and 3.9-fold higher than those in group 4 mice, respectively, and the differences among all three groups were significant (p<0.00134). Overall, the levels of vitamin D_3 in the tumors were significantly lower than those in the surrounding skin (p<0.0001).

Tumor development. All UVR-exposed mice developed tumors (Figure 2). An SCC diagnosis was confirmed for all evaluated tumors. Animals in the non-irradiated groups (groups 1 and 5) did not develop tumors. High and medium supplementation with vitamin D_3 did not affect the timing of tumor development (p>0.05; Table II and Figure 2).

Pigmentation. All groups exposed to UVR developed identical dorsal pigmentation during the study (Figure 3). Most of the mice in Group 1 developed pigmentation close to the front legs but it was not measured as it was done on the back of the mice (Figure 4). No weight differences were observed among the groups (data not shown).

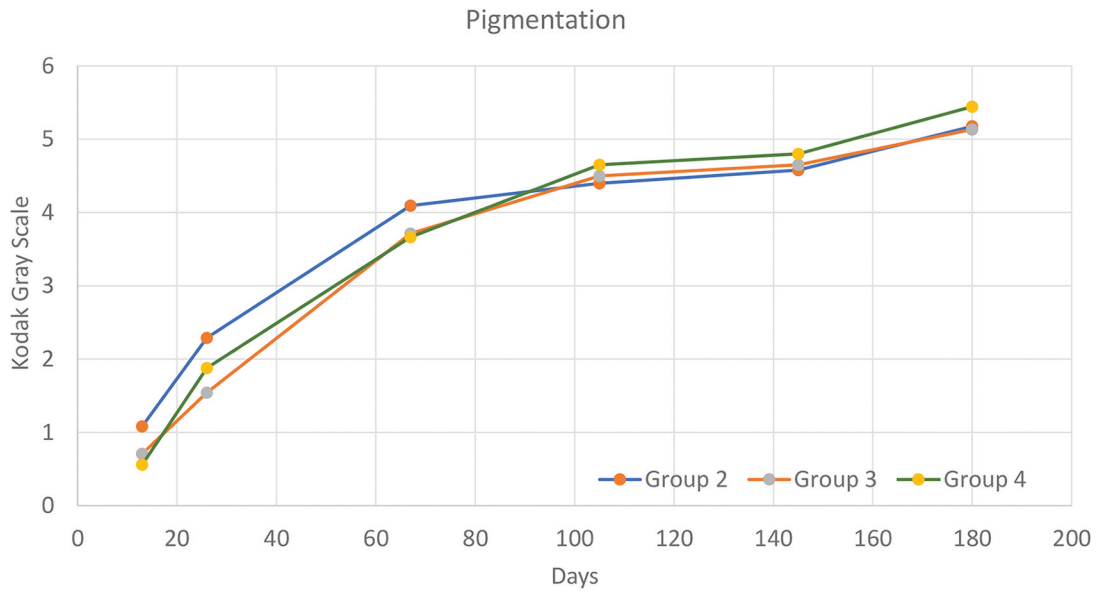

Figure 3. *Pigmentation development over time for Groups 2, 3 and 4. There was no development of pigmentation in the unirradiated Groups 1 and 5.*

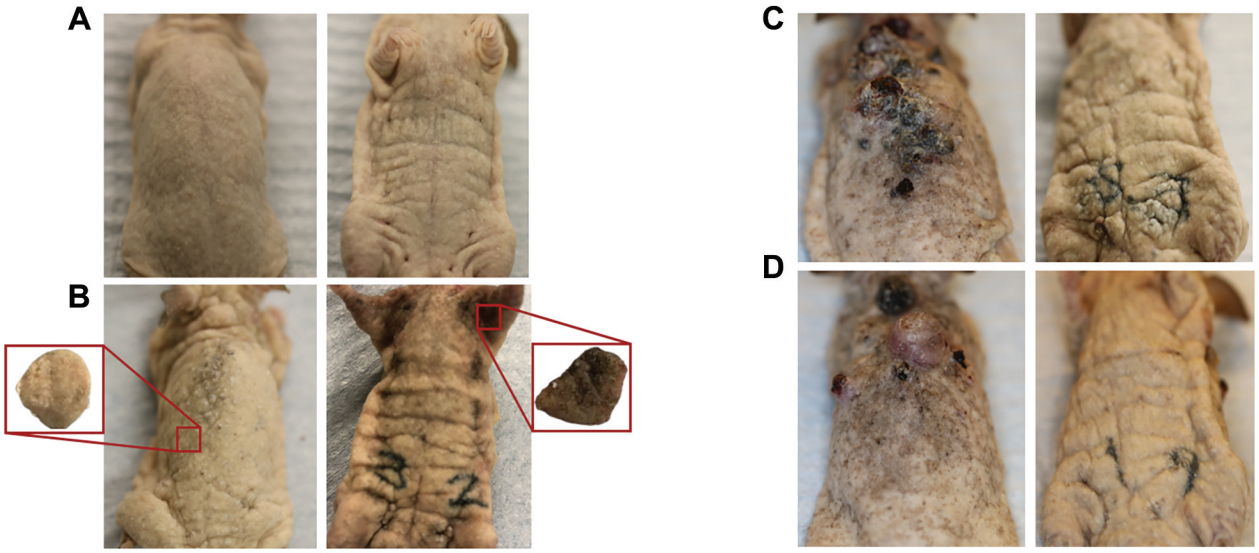

Figure 4. *Photos of representative mice. (A) Control, group 5. (B) High Vitamin D, group 1. (C) High vitamin D+UV, group 2. (D) UVR control, group 4.*

Discussion

Vitamin D supplementation has been associated with preventing and alleviating various cancers, such as colon, breast, and prostate cancer (2, 3). However, studies have produced conflicting results, especially for skin cancer (12, 18). Therefore, we investigated whether vitamin D supplementation could influence vitamin D levels in the serum, skin, and tumors and whether there was a further effect on UVR-induced SCC in a hairless mouse model.

High and medium vitamin D_3 supplementation increased serum 25(OH)D_3 and vitamin D_3 levels compared with those in mice that had a standard diet. The 25(OH)D_3 levels were ~30 ng/ml in the high vitamin D groups, ~23 ng/ml in the medium vitamin D group, and ~10 ng/ml in the standard diet group. The levels of 25(OH)D_3 we observed for the high

vitamin D_3 supplementation diet are similar to those reported in the literature for a normal to high vitamin D_3 supplementation diet (32.8-38 ng/ml), and the low vitamin D_3 supplementation 0.045 μg/day serum levels are similar to those reported for a normal diet, 11-12 *vs*. 10 ng/ml (26, 27).

An increase in vitamin D_3 levels was also observed in the skin and tumors of animals supplemented with high to medium doses of vitamin D_3, compared with the group fed the standard diet. However, the levels of vitamin D_3 in the serum, skin, and tumors showed no correlation with SCC development in our hairless mouse model. Moderate to high vitamin D consumption had no effect on photocarcinogenesis.

Although the concentrations of vitamin D_3 that we used were much higher, our results are consistent with those reported by Hill *et al*. (18), who fed SKH-1 mice with different doses of vitamin D_3 (0.625, 3.75 or 25 μg/kg) and exposed them to UVB. In that study, Vitamin D_3 had no protective effect against SCCs, regardless of the level of supplementation (18). However, Hill *et al*. suggested that increased vitamin D_3 consumption increased serum vitamin D_3 and 25(OH)D_3 and could enhance the development and progression of SCCs due to decreased expression of the epidermal vitamin D receptor (VDR) and increased expression of the oncogene ΔNp63 (18), which is involved in cell proliferation, differentiation, and adhesion, and is over-expressed in keratinocyte cancers (28).

To our surprise, UVR did not increase serum vitamin D_3 or 25(OH)D_3 levels in the mouse model, either in the high vitamin D_3 supplementation groups (group 1 *vs*. 2) or the standard diet groups (group 4 *vs*. group 5). However, for vitamin D_3 levels in the skin, UVR did generate a significant increase in the standard diet groups (group 4 *vs*. group 5) and a trend towards an increase in the high vitamin D_3 supplementation groups (group 2 *vs*. 1).

This study was carried out using female mice only. Results may be different for male mice. Sex bias has been reported for the protective role of topical 1,25$(OH)_2$D against UVR-induced oedema, DNA damage, and immunosuppression in mice. Female mice exhibited more effective DNA damage repair than male mice (29). In addition, the UVR response in the BCC mouse model was affected by the sex of the mice. In female mice, UVR produced vitamin D_3, but did not accelerate BCC carcinogenesis; in male mice, UVR did not produce vitamin D_3 but did accelerate BCC carcinogenesis (1).

Pigmentation development was the same for the three UVR exposed groups independent of vitamin supplementation. However, the high vitamin D_3 group that was not UVR exposed (group 1) developed some pigmentation on the ventral side close to the front legs that has not been observed before in the mouse model (Figure 4).

In conclusion, we showed that in female mice, vitamin D_3 levels in the serum, skin and tumors could be augmented by supplementation but this did not affect the development of UVR-induced SCCs.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

CML: Designed and performed the experiments, analyzed the data, and wrote the manuscript. FEP: analyzed the data and wrote the manuscript. PAP: analyzed the data and wrote the manuscript. EB: contributed to experimental execution and revision of manuscript. JJ: contributed to experimental execution and revision of manuscript. MH: Critical revision of the manuscript and final approval of the version to be published. HCW: Conceptualization, supervision, writing, reviewing, and editing.

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