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Use of $\alpha,25$ -Dihydroxyvitamin D₃ treatment to stimulate immune infiltration into head and neck squamous cell carcinoma

Jarrett E. Walsh^{a,b}, Anna-Maria Clark^{a,b}, Terry A. Day^b, M. Boyd Gillespie^b, and M. Rita I. Young^{a,b,c,*}

^aResearch Services, Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina, USA

^bDepartment of Otolaryngology—Head and Neck Surgery, Charleston, South Carolina, USA

^cDepartment of Medicine, Medical University of South Carolina, Charleston, South Carolina, USA

Abstract

Prior studies have shown that treatment of head and neck squamous cell carcinoma (HNSCC) patients with $1\alpha,25$ -dihydroxyvitamin D₃ [$1,25(\text{OH})_2\text{D}_3$] reduced intratumoral levels of immune inhibitory CD34⁺ progenitor cells while increasing levels of mature progeny dendritic cells. This finding was extended to a pilot study to determine whether $1,25(\text{OH})_2\text{D}_3$ treatment concurrently increases levels of intratumoral CD4⁺ and CD8⁺ T cells, increases intratumoral levels of immune cells expressing the early activation marker CD69, and prolongs time to HNSCC recurrence. The clinical trial comprised 16 patients with newly diagnosed HNSCC being untreated and 16 patients being treated with $1,25(\text{OH})_2\text{D}_3$ during the 3-week interval between cancer diagnosis and surgical treatment. Immunologic effects of treatment were monitored by immunohistochemical analyses of surgically removed HNSCC. Clinical effectiveness of $1,25(\text{OH})_2\text{D}_3$ treatment in this study was measured by the time to HNSCC recurrence. HNSCC tissues of patients who received treatment with $1,25(\text{OH})_2\text{D}_3$ contained increased levels of CD4⁺ cells and, more significantly, CD8⁺ T cells. Also prominent was an increase in cells expressing the lymphoid activation marker CD69. Results of this pilot study suggest that patients treated with $1,25(\text{OH})_2\text{D}_3$ had a lengthier time to tumor recurrence compared with patients who were not treated before surgery.

Keywords

Head and neck cancer; Head and neck squamous cell carcinoma; Recurrence; T-cell; vitamin D

1. Introduction

Squamous cell carcinoma of the head and neck (head and neck squamous cell carcinoma (HNSCC)) is an aggressive malignancy with a 5-year survival in patients that remains at approximately 50%. Thus the possibility of immunotherapeutic approaches for HNSCC patients has gained interest. Feasibility of immunotherapeutic strategies is supported by studies showing infiltration of CD4⁺ and CD8⁺ T cells within HNSCC and a correlation between lymphocyte activity and patient prognosis [1,2]. Immunotherapeutic strategies such as adoptive T-cell transfer, treatment with various immune stimulatory cytokines, or treatment with tumor cell vaccines have suggested therapeutic activity in HNSCC patients [3–7]. Unfortunately, HNSCC patients have profound immune deficiencies that are associated with increased recurrence [1]. CD34⁺ progenitor cells are among the tumor-

*Corresponding author. rita.young@va.gov (M.R.I. Young).

induced suppressor cells that contribute to this immune dysfunction [8–10]. CD34⁺ cells are a part of a spectrum of immature myeloid-lineage cells, the levels of which become prominent in HNSCC patients and which have defects in maturation into dendritic cells able to stimulate T-cell reactivity [11,12]. Our prior *in vitro* studies had shown that 1 α ,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] induced maturation of immune suppressive CD34⁺ progenitor cells into immune stimulatory dendritic cells [13,14]. Expanding this to a pilot clinical trial with HNSCC patients showed that 1,25(OH)₂D₃ diminishes intratumoral levels of tumor-induced immune inhibitory CD34⁺ cells. Concurrent with the decline in intratumoral CD34⁺ cells was an increase in levels of mature dendritic cells.

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Dendritic cells are highly efficient antigen presenting cells that can generate T_h1 cells and cytotoxic T-lymphocytes responses to cancer [15]. Cancers, including HNSCC, are vulnerable to such generated immune effector cell responses [16,17]. However, there has been an accumulation of evidence of defects in the maturation and differentiation of dendritic cells in cancer patients, including those with HNSCC [11,18]. Maturation of dendritic cells is critical in regard to their capacity to stimulate activity of naive T cells, as their ability to form stable adhesions with T cells only develops after maturation [15]. Consistent with this are our past studies showing only low levels of mature dendritic cells within cancers in HNSCC patients and, instead, the presence of dendritic cells expressing markers of immature dendritic cells [19].

Whether decreases in levels of immune inhibitory CD34⁺ progenitor cells and increases in intratumoral mature dendritic cells previously observed after treatment of HNSCC patients with 1,25(OH)₂D₃ [19] also translates into increased levels of immune infiltrating cells and, in particular, T cells within the HNSCC had not been previously determined. The present study used immunohistochemical analyses to determine whether the intratumoral content of cells expressing CD69, which is primarily indicative of early stimulated T cells and monocytes, and intratumoral levels of CD4⁺ and CD8⁺ T cells were increased in tissues from 1,25(OH)₂D₃-treated patients compared with untreated patients. Also determined was whether there were any suggestions of clinical responses to the 1,25(OH)₂D₃ treatment, as measured by the time between surgical treatment and HNSCC recurrence.

2. Subjects and methods

2.1. Study population

Recruitment of patients with a pathologic diagnosis of HNSCC into this 1,25(OH)₂D₃ (Calcitriol) study was institutional review board approved. Pathologic findings were read before patient enrollment by a pathologist who was not affiliated with this study and was therefore blinded to the treatment arms into which the patients would be enrolled. Patients with newly diagnosed HNSCC who were being scheduled for surgery were eligible for enrollment into this randomized trial with preoperative treatment of 1,25(OH)₂D₃. Control patients with HNSCC did not receive 1,25(OH)₂D₃ treatment. Patients were excluded if they had received immunotherapy or radiation treatment in the previous 3 weeks or had concurrent malignancies.

2.2. (OH)₂D₃ treatment and collection of specimens

Patients were treated orally with 4 µg of 1,25(OH)₂D₃ for each of 3 sequential days, followed by 4 days of no treatment. This treatment schedule has previously been shown to be associated with minimal toxicity [19,20]. Nevertheless serum calcium and parathyroid hormone levels were measured weekly to monitor possible toxicity. At the conclusion of three cycles of treatment, patients underwent surgical treatment for their HNSCC. Sixteen patients enrolled in this study completed the 1,25(OH)₂D₃ treatment, and 16 patients enrolled to the control untreated arm underwent surgical excision and were clinically followed up after surgery.

2.3. Immunohistochemistry

Upon surgical collection of HNSCC tissues, the tissues were frozen in optimum cutting temperature (OCT) compound (Miles Laboratories, Elkhart, IN). Tissue blocks were then cryosectioned into 10–nm-thick slices and placed onto slides. Approximately 50 slides were made per frozen block. Slides were stored at –80°C until used for immunohistochemical staining. Cryosectioned HNSCC tissue was used to measure levels of infiltrating immune cells, namely CD4⁺, CD8⁺, and CD69⁺ cells. The procedure for immunostaining for these cells was similar to that previously used [19]. Briefly, the cryosections were fixed onto slides with 100% acetone for 10 minutes and slides were then allowed to dry. The tissue was outlined with a PAP pen. Slides were then washed and incubated in phosphate-buffered saline (PBS) for 10 minutes. Endogenous peroxidase was quenched by incubating the tissues three times for 5 minutes each time in a 0.3% H₂O₂/PBS solution. Nonspecific mouse antibody was added for 20 minutes to bind to Fc epitopes and to reduce background staining. Next, the primary antibodies and corresponding isotype control antibodies were added to slides for 1 hour at room temperature. Primary antibodies that were used to detect infiltrating cells within HNSCC tissues were against CD4 to detect CD4⁺ T cells, CD8 to detect CD8⁺ T cells, and CD69 to detect T cells and monocytes expressing this early activation marker (BD Biosciences, San Jose, CA). Slides were rewashed with buffer for 5 minutes, and then positive staining cells were visualized using the Vecastain ABC immunoperoxidase kit (Vector Labs, Burlingame, CA) with counterstaining using hematoxylin.

2.4. Quantification of data and analysis

The number of positive staining cells in each microscopic field was quantitated by individual cell counts. Four random areas of slides were identified and graded independently by four graders. At least two randomly selected slides were scored for each patient sample. The same four graders were used for each of the primary antibodies. The graders were blinded as to the identity of the tissues. Data for the treated and untreated patients were statistically compared using a two-tailed Student's *t*-test.

2.5. Clinical outcomes

The possibility of clinical effectiveness of treatment with 1,25(OH)₂D₃ was determined by measuring the time to recurrence after surgical treatment and comparing results for patients who received 1,25(OH)₂D₃ treatment or no treatment during the interval between cancer diagnosis and surgery. Results were analyzed using Kaplan–Meier Curves.

3. Results

3.1. Patient population

In all, 16 patients with newly diagnosed HNSCC who were enrolled in this pilot study completed a 3-week course of 1,25(OH)₂D₃ treatment before surgical treatment, and 16

newly diagnosed HNSCC patients were untreated during the interval between diagnosis and surgical treatment (Table 1). There was no evidence of toxicity resulting from 1,25(OH)₂D₃ treatment, based on serum calcium and parathyroid hormone measurements. Patient ages ranged from 45 to 92 years. The mean ages of patient groups were similar after randomization into groups receiving 1,25(OH)₂D₃ treatment preoperatively for 3 weeks (mean 66 years of age) versus those who received no treatment during the same interval (mean 63 years of age). Most of the patients had cancers that originated in the oral cavity. The range of disease stages, based on pathologic evaluation, also didn't differ among patients enrolled in the two arms. For each of the patient groups, seven of 16 patients were female (44%) and nine of 16 were male (56%).

3.2. Levels of CD4⁺ and CD8⁺ T cells in untreated HNSCC patients or those treated with 1,25(OH)₂D₃ before surgery

Prior studies had shown diminished immune functions and altered T-cell content in patients with HNSCC as well as with other cancer types [21,22]. Furthermore reduced immune content and function in HNSCC patients has been correlated with a poor clinical outcomes [1,23]. Therefore this study aimed to quantitatively assess levels of T-cell subpopulations within HNSCC tissue of newly diagnosed HNSCC patients who were either untreated before surgery or who received treatment with the immune modulator 1,25(OH)₂D₃, which we had previously shown to diminish levels of immune inhibitory progenitor cell and stimulate differentiation of dendritic cells within the tumor mass [19].

Immunostaining for CD4⁺ cells showed increases in the numbers of these cells within tissues of patients who received 1,25(OH)₂D₃ treatment (sample micrographs in Fig. 1). Levels of CD4⁺ cells within each microscopic field of tissues of untreated patients averaged 14 ± 6. This level was approximately tripled to 44 ± 12 CD4⁺ cells per field in tissue from patients who had received 3 weeks of 1,25(OH)₂D₃ treatment ($p = 0.038$; Fig. 2).

Although HNSCC tissues from patients who received 1,25(OH)₂D₃ treatment had an increased level of intratumoral CD4⁺ cells, there was a more highly significant increase in intratumoral CD8⁺ cells (sample micrographs in Fig. 3). Tissue from untreated patients had an average of 17 ± 6 CD8⁺ staining cells per field. In contrast, tissue from 1,25(OH)₂D₃-treated patients had an approximate 4.5-fold increase in CD8⁺ cells, with an average of 75 ± 13 cells per microscopic field ($p = 0.0004$; Fig. 4). These findings show that a 3-week course of treatment with 1,25(OH)₂D₃ resulted in an increase in intratumoral infiltration of CD4⁺ cells, and a more highly significant intratumoral infiltration of CD8⁺ T cells.

3.3. Increased numbers of intratumoral infiltrating cells expressing the early activation marker CD69

CD69 is an early activation marker that has been shown to be expressed on lymphocytes as well as natural killer cells and monocytes [24–26]. Thus HNSCC tissue from patients who were either untreated or treated with 1,25(OH)₂D₃ were examined by immunohistochemistry to determine whether treatment with 1,25(OH)₂D₃ increased levels of immune infiltrating cells that expressed this activation marker (sample micrographs in Fig. 5). CD69⁺ cells either were not detectable within HNSCC tissues of untreated patients or, occasionally, a few cells could be detected that stained weakly for CD69 (3 ± 2 positive-staining cells/field). In contrast, the presence of cells expressing the activation marker CD69 was approximately 10-fold greater ($p = 0.0007$; Fig. 6) in tissues from patients who had completed 3 weeks of 1,25(OH)₂D₃ treatment. Levels of CD69⁺ staining cells in HNSCC of patients treated with 1,25(OH)₂D₃ averaged 30 ± 7 cells per microscopic field. Although these studies could not further determine the identity of the cells expressing CD69, they did

show that, after 1,25(OH)₂D₃ treatment, there was a prominent increase in infiltrating intratumoral cells with an activated phenotype.

3.4. Clinical outcomes for HNSCC patients treated with 1,25(OH)₂D₃ before surgical treatment

The clinical profile of the population of patients that were recruited into this study is shown in Table 1. Whether treatment of these patients with 1,25(OH)₂D₃ before surgical cancer treatment might impact on the clinical course of disease was examined. This was accomplished by quantitating possible differences among the patient groups in the time between surgical treatment and recurrence of detectable disease. Although some patients received radiation treatment and/or chemotherapy postoperatively (Table 1), there were no differences in the numbers of control or 1,25(OH)₂D₃-treated patients who received these added treatments. Therefore the time to postsurgical recurrence of detectable disease was analyzed without stratification for these added treatments.

Shown in Figure 7 is the Kaplan–Meier analysis for the time to HNSCC recurrence for the two patient groups. The HNSCC patient group who received preoperative 1,25(OH)₂D₃ treatment had a longer time to recurrence than did the group receiving no treatment before surgery ($p = 0.048$). The median time to recurrence for the control group was 181 days, whereas the median time to recurrence for the patients receiving 1,25(OH)₂D₃ treatment was 620 days. Although this was a small clinical trial, it nevertheless suggested that presurgical treatment with 1,25(OH)₂D₃ stimulated intratumoral infiltration of T cells and early activation marker–expressing cells, which coincided with a reduced rate of recurrence of HNSCC disease.

4. Discussion

T-cell functional competence is critical for stimulating antitumor immune reactivity. Unfortunately, patients with HNSCC are particularly deficient in their immune competence [1]. There are multiple immune inhibitory mechanisms that are mediated by HNSCC, including their induction of immune suppressor cells that block host immune reactivity [27–29]. Among the immune suppressor cells that are induced to appear in elevated numbers in the peripheral blood and within HNSCC tumor tissue are immature progenitor cells that can be identified by their surface expression of CD34 [29,30]. These CD34⁺ immune inhibitory cells appear to be defective in their differentiation, but can be driven to differentiate into several different types of mature cells, including immune stimulatory dendritic cells.

Our prior *in vitro* studies and studies with tumor-bearing animals have shown that, in the presence of the correct cytokine milieu, 1,25(OH)₂D₃ overcomes the defects in differentiation of CD34⁺ cells of tumor-bearers. The required cytokines for differentiation toward the dendritic cell lineage include GM-CSF, which HNSCC produce [9,31]. Consequently, several pilot studies were conducted using vitamin D₃ analogs to modulate maturation of immune inhibitory progenitor cells. The first study showed that treatment of patients with advanced HNSCC disease with 25-hydroxyvitamin D₃ diminished levels of immune inhibitory CD34⁺ cells in the peripheral blood and concurrently increased immune reactivity of peripheral blood T cells. A subsequent study with newly diagnosed HNSCC patients showed that treatment with 1,25(OH)₂D₃ reduced intratumoral levels of CD34⁺ cells and, concurrently, increase levels of mature dendritic cells within the HNSCC tissue.

The above studies using vitamin D₃ analogs to modulate D34⁺ cell differentiation in HNSCC patients prompted further evaluation of whether treatment of newly diagnosed HNSCC patients with 1,25(OH)₂D₃ between the time of cancer diagnosis and surgical treatment would not only increase the intratumoral levels of mature dendritic cells but also

increase infiltrating T-cell levels within the HNSCC tissue. The present study showed that a 3-week course of treatment with 1,25(OH)₂D₃ increased levels of CD4⁺ T cells and, to a somewhat more significant extent, increased intratumoral levels of CD8⁺ cells. Furthermore 1,25(OH)₂D₃ treatment increased levels of CD69⁺ cells, which represent mainly T cells and monocytes expressing this early activation marker.

Increased levels of vitamin D analogues have been shown in multiple studies to be associated with reduced cancer development [32,33]. Whether this is a direct effect on the developing malignant cells or occurs via immune modulatory effects of vitamin D analogs is not known. Although vitamin D analogs have been shown to stimulate immune reactivity in cancer patients, other studies have shown immune inhibitory effects of vitamin D analogs [34,35]. The immune dampening effects of vitamin D analogs has typically been tested either in models in which there is no immune dysfunction or in instances of chronic immune activation, such as in inflammatory bowel disease [36]. Whether the increase in immune cells infiltrating into HNSCC after treatment with 1,25(OH)₂D₃ reflected immune activation within the HNSCC tissue, and whether it was a consequence of our prior observation of diminished levels of immune inhibitory CD34⁺ cells and increased levels of mature dendritic cells, cannot be determined through the present studies, as they were immunohistochemical analyses of HNSCC tissues and were not designed to study function or causality. Of interest would be whether the observed increase in the intratumoral T-cell infiltration after 1,25(OH)₂D₃ treatment was a consequence of increased infiltration or increased intratumoral proliferation. Although it is not feasible to determine this in a clinical trial, it would be expected that the increased T-cell content could be due to enhanced infiltration, as vitamin D has previously been shown to modulate levels of inflammatory chemokines [37].

As a part of the analyses in this pilot study, the clinical effectiveness of 1,25(OH)₂D₃ treatment for newly diagnosed HNSCC patients was assessed by measuring the time to cancer recurrence after surgical treatment. In addition to the demonstration of an increase in infiltration by immune cells showing markers of activation within the HNSCC tumor of 1,25(OH)₂D₃-treated patients, results also suggested a postsurgical increase in the time to recurrence. Patients receiving 1,25(OH)₂D₃ treatment showed a lengthier time to recurrence, with a median time that was almost 3.5-fold that for the control patients who were untreated before surgery. Although this lengthier time to recurrence in 1,25(OH)₂D₃-treated HNSCC patients coincided with its immune enhancing effect, it cannot be definitively determined that these effects are causally related. It is important to note that these results suggesting a clinical response are from a small study that needs to be expanded to a larger cohort of patients before making definitive conclusions about clinical effectiveness. A larger study would also allow analysis of the effect of presurgical treatment with 1,25(OH)₂D₃ on correlations between the extent of the increase in immune infiltration and time to cancer recurrence or to patient responsiveness to salvage treatments following recurrence.

HNSCC patients are known for the multitude of their immune inhibitory cell populations that are induced by the HNSCC, with CD34⁺ cells being among these inhibitory cells. Thus it was surprising that 1,25(OH)₂D₃ treatment aiming to diminish levels of these CD34⁺ suppressor cells to stimulate immune infiltration within the HNSCC was sufficient to suggest a clinical response. Not known is the impact of the extent of vitamin D deficiency on responsiveness to the 1,25(OH)₂D₃ treatment. Future studies with a larger patient cohort could assess whether patients who are more profoundly vitamin D deficient have a greater increase in immune infiltration and time to cancer recurrence than patients who are less deficient in vitamin D. Whether the 1,25(OH)₂D₃ treatment also had an impact on other tumor-induced immune inhibitory populations, such as regulatory T cells (Treg) or tumor-associated macrophages, needs to be determined in subsequent studies. Also of interest for

future studies to assess the immune enhancing effectiveness of 1,25(OH)₂D₃ would be an analysis of the functional activity of the intratumoral immune infiltrate. This would include assessment of whether there is not just an increase in the number of tumor-infiltrating T cells but whether there is stimulation of tumor-specific reactivity by these T cells or by the T cells of the regional lymph nodes. Such analyses of the effect of 1,25(OH)₂D₃ treatment on immune inhibitory cell populations other than CD34⁺ cells and on functional activity of intratumoral T cells are ongoing in a new trial that we have recently initiated with HNSCC patients. Finally, future studies might consider a combination immunotherapeutic approach with 1,25(OH)₂D₃ to stimulate intratumoral immune infiltration, plus a second form of immunotherapy involving a tumor antigen-containing vaccine for stimulating dendritic cells to trigger tumor-specific immune reactivity against any residual HNSCC cells.

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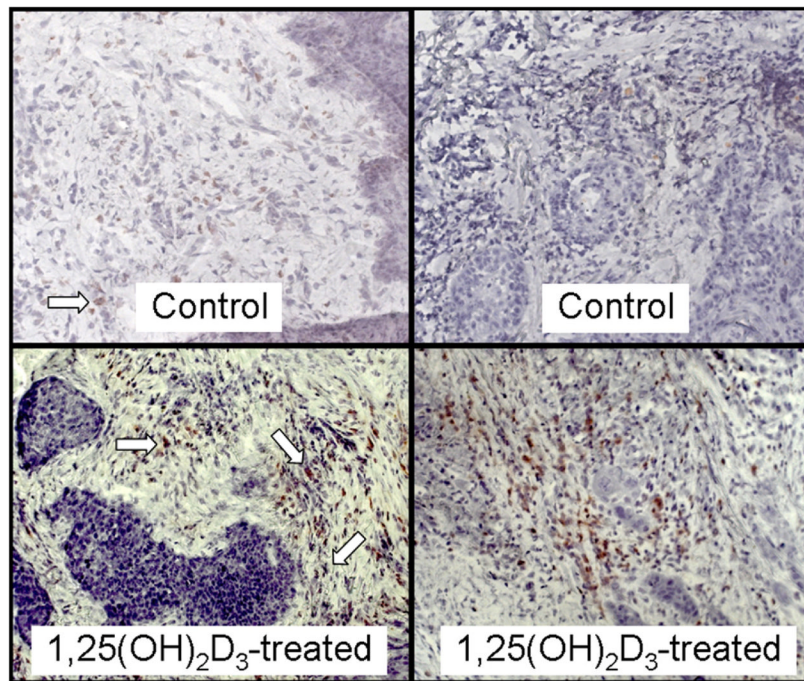


Fig. 1. Representative microscopic images of increased intratumoral levels of CD4⁺ cells in HNSCC tissues from two sample patients treated with 1,25(OH)₂D₃ compared with levels in HNSCC tissue of two sample untreated patients. Arrows indicate examples of positive-stained cells. Images are shown at ×400 magnification.

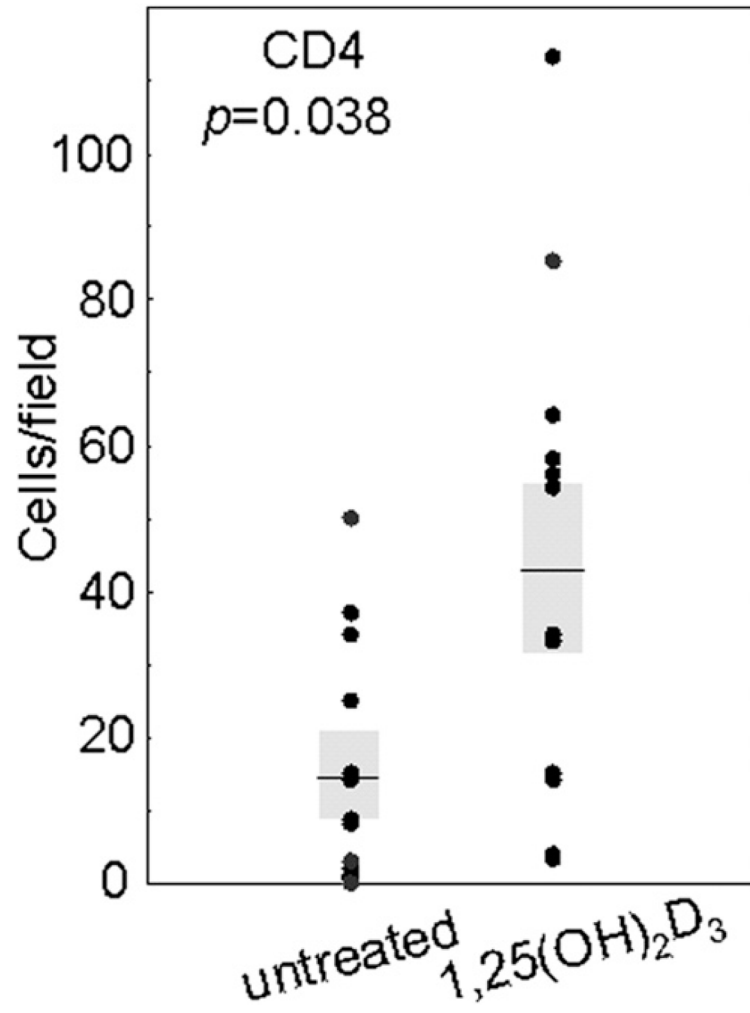


Fig. 2. Increased levels of CD4⁺ cells in HNSCC tissue from patients treated with 1,25(OH)₂D₃. Numbers of immunostained cells per microscopic field were determined in HNSCC tissues from untreated and 1,25(OH)₂D₃-treated patients. Shown are the averages of the numbers of immunostained cells for at least two separate slides for each patient, each counted independently by four blinded scorers. Shaded areas represent SEM.

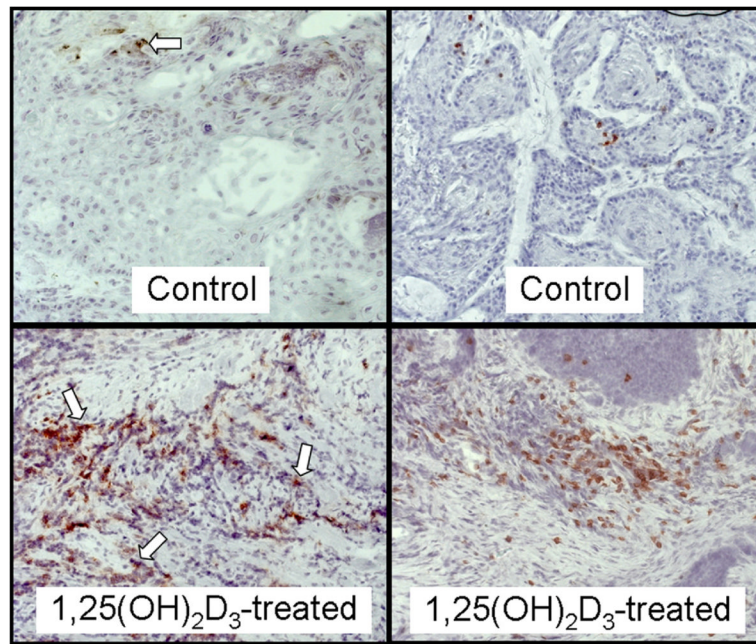


Fig. 3. Representative microscopic images of increased intratumoral levels of CD8⁺ cells in HNSCC tissues from two sample patients treated with 1,25(OH)₂D₃ compared with levels in HNSCC tissue of two sample untreated patients. Arrows indicate examples of positive-stained cells. Images are shown at ×400 magnification.

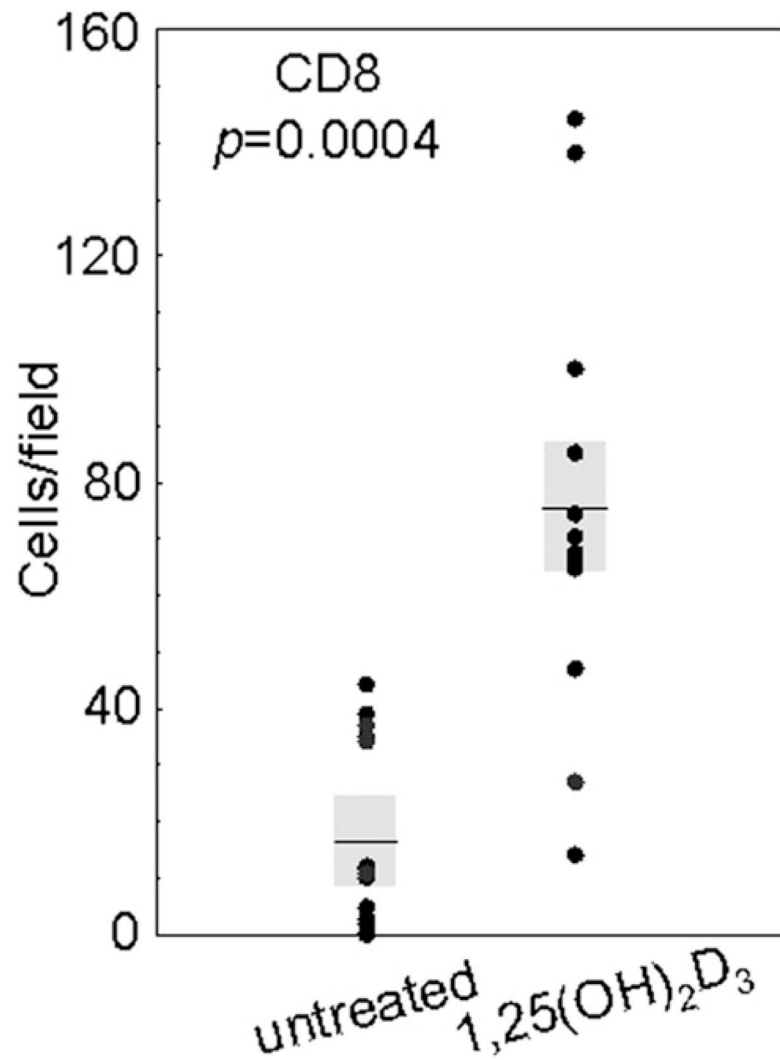


Fig. 4. Increased levels of CD8⁺ cells in HNSCC tissue from patients treated with 1,25(OH)₂D₃. Numbers of immunostained cells per microscopic field were enumerated in HNSCC tissues from untreated and 1,25(OH)₂D₃-treated patients. Shown are the averages of the numbers of immunostained cells for at least 2 separate slides for each patient, each counted independently by four blinded scorers. Shaded areas represent SEM.

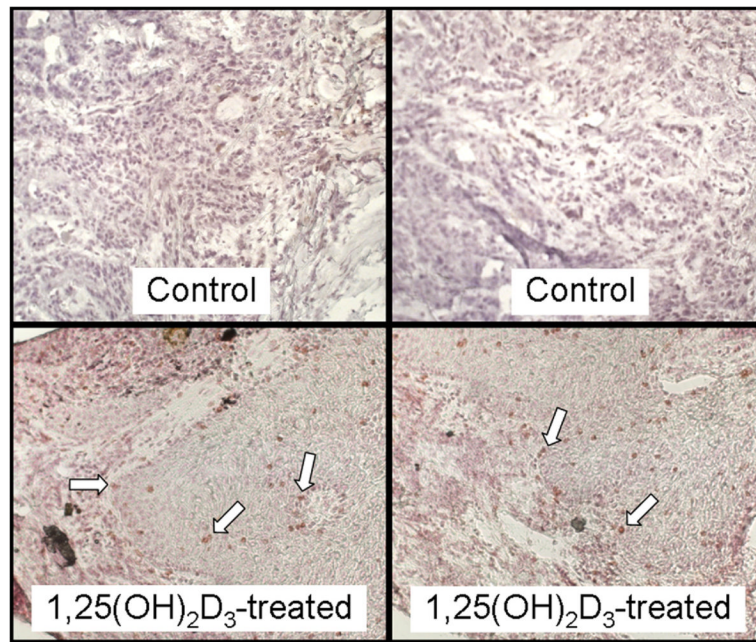


Fig. 5. Representative microscopic images of increased intratumoral levels of cells expressing the early activation marker CD69 in HNSCC tissues from two sample patients treated with 1,25(OH)₂D₃ compared with levels in HNSCC tissue of two sample untreated patients. Arrows indicate examples of positive-stained cells. Images are shown at $\times 400$ magnification.

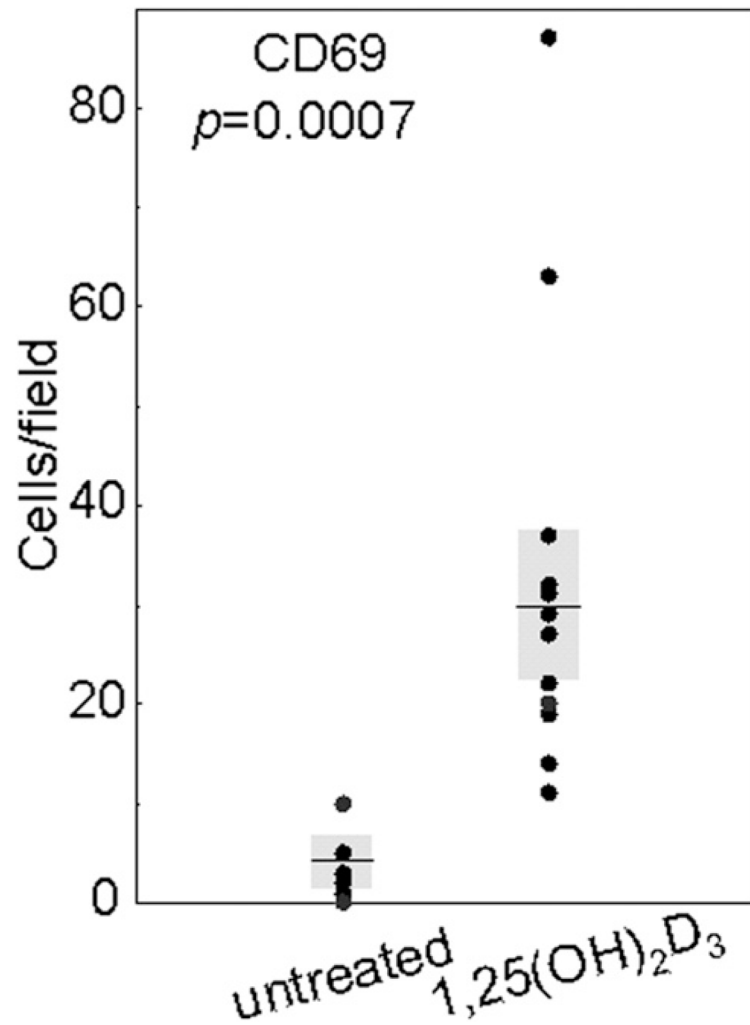


Fig. 6. Increased levels of cells expressing the early activation marker CD69 in HNSCC tissue from patients treated with 1,25(OH)₂D₃. Numbers of immunostained cells per microscopic field were determined in HNSCC tissues from untreated and 1,25(OH)₂D₃-treated patients. Shown are averages of numbers of immunostained cells for at least two separate slides for each patient, each counted independently by four blinded scorers. Shaded areas represent SEM.

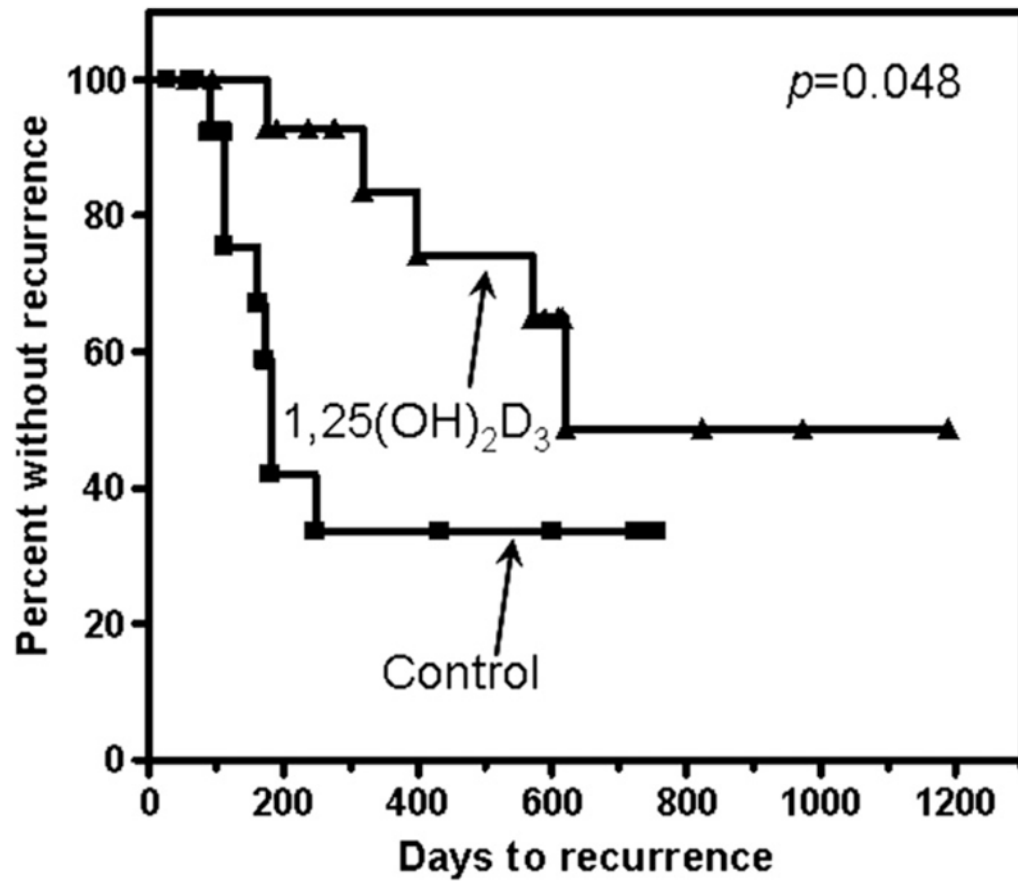


Fig. 7. Time to HNSCC recurrence in patient treated with 1,25(OH)₂D₃. Clinical effectiveness of 1,25(OH)₂D₃ treatment was analyzed by the number of days between surgical treatment and HNSCC recurrence. Data shown are from Kaplan–Meier analyses of days to recurrence.

Table 1

Patient population

Age	Gender	Primary site	Stage	Margins	Postsurgical treatment
Unreated					
74	M	Oral cavity	T3N2bM0	Negative	RT
70	M	Oral cavity	T4N2cM0	Negative	Chemo/RT
49	M	Oral cavity	T4aN2bM0	Negative	Chemo/RT
59	M	Sinonasal	T2N0M0	Positive	RT
80	M	Floor of mouth	T4aN0M0	Positive	RT
54	M	Oral cavity	T3N1M0	Negative	Chemo/RT
64	F	Tongue	T3N0M0	Negative	None
64	M	Tongue	T2N0M0	Positive	None
92	F	Palate	T4aN2bM0	Positive	RT
51	M	Floor of mouth	T4N2bM0	Positive	RT
48	F	Floor of mouth	T4aN2bM0	Positive	Chemo/RT
58	F	Floor of mouth	T4aN2cM0	Positive	Chemo
50	M	Floor of mouth	T4aN2cM0	Positive	RT
48	F	Tongue	T2N2cM0	Negative	None
67	F	Tongue	T2N2cM0	Negative	Chemo/RT
72	F	Oral cavity	T2N2bM0	Negative	None
1,25(OH) ₂ D ₃ treated					
72	F	Larynx	T3N2cM0	Positive	Chemo/RT
76	M	Floor of mouth	T2N0M0	Positive	RT
45	M	Floor of mouth	T2N2cM0	Positive	Chemo/RT
72	M	Anterior mandible	T4aN1M0	Negative	RT
74	M	Tongue/floor of mouth	T4aN2cM0	Positive	RT
58	M	Oropharynx	T4N2bM0	Negative	Chemo/RT
63	M	Oral cavity	T4aN3M0	Positive	Chemo/RT
47	M	Lip	T2N0M0	Negative	None
47	M	Tongue	T4N0M0	Positive	RT
59	F	Floor of mouth	T3N1M0	Positive	RT
76	F	Hard palate	T2N2bM0	Negative	Chemo/RT

Age	Gender	Primary site	Stage	Margins	Postsurgical treatment
76	F	Oropharynx	T2N2bM0	Negative	Chemo/RT
75	F	Oropharynx	T2N0M0	Negative	None
73	F	Tongue	T3N2bM0	Negative	Chemo/RT
66	F	Oral cavity	T2N0M0	Negative	None
73	M	Floor of mouth	T4N2bM0	Positive	None