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Immunological modulation by 1 α ,25-dihydroxyvitamin D₃ in patients with squamous cell carcinoma of the head and neck

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Abstract

Prior studies showing that treatment of head and neck squamous cell carcinoma (HNSCC) patients with 1 α ,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] stimulated intratumoral immune infiltration were extended to an analysis of cytokine profiles in the periphery and in oral tissues. Most prominent was the disparity between cytokine levels in plasma and in either pathologically normal oral tissue or HNSCC tissue from patients that were untreated or treated with 1,25(OH)₂D₃. Levels of IL-6 and IL-10, but not IL-2, IFN- γ or TNF- α , tended to be increased in the plasma of HNSCC patients and 1,25(OH)₂D₃ further increased plasma levels of all of these cytokines. While these cytokines tended to be increased in HNSCC tissue, 1,25(OH)₂D₃ resulted in variable cytokine responses that showed a general tendency toward further increased levels. Levels of IL-8 and VEGF were increased in plasma and tissue of untreated HNSCC patients, and were further increased in plasma, but not in tissues, of patients treated with 1,25(OH)₂D₃. Levels of IL-1 α and IL-1 β were similar in plasma of controls and HNSCC patients, but were increased in HNSCC tissues. In contrast to that seen in plasma where 1,25(OH)₂D₃ increased levels of IL-1 α and IL-1 β , this was not seen in tissue following 1,25(OH)₂D₃ treatment. These results show a discordant relationship between systemic and intratumoral cytokine profiles and suggest a tendency of 1,25(OH)₂D₃ to increase a multitude of cytokines within tumor tissue.

Keywords

cytokines; head and neck cancer; HNSCC; vitamin D

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Conflict of interest

The authors declare that there are no conflicts of interest.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common type of malignancy worldwide, and represents over 6% of the global cancer burden [1]. HNSCC accounts for nearly 650,000 new cases of cancer worldwide, and over 35,000 deaths each year. Historically, HNSCC has been a challenging disease to manage, with locally advanced disease often requiring a multidisciplinary approach of surgery, chemotherapy and radiation. Despite significant advances in treatment approaches, the current 5-year survival rate has improved very little over the last 30 years, remaining stagnant at approximately 50% [1,2]. This poor improvement in prognosis is a reflection of the unique treatment challenges presented by HNSCC, which include advanced stages at diagnosis, high recurrence rate after surgical removal and second primary tumor development [3]. Not only are current treatment options often non-curative, but they are associated with significant morbidity, including substantial physical deformity and functional deficits. Together, these challenges underscore the importance of developing novel anti-neoplastic treatment strategies which may improve survival and quality of life among HNSCC patients.

An alternative treatment strategy is immunotherapy. While HNSCC, like all cancers, is theoretically susceptible to a number of host immune reactivities, there is failure to mount protective immunity to the HNSCC. This failure of normal immunosurveillance occurs through a myriad of survival mechanisms on the part of the actively growing tumor including tissue sequestration and localization, failed chemoattraction of immune cells, alteration of antigenicity, and direct immunosuppression by activation of inappropriate, ineffective, and uncoordinated immune responses.

T-helper cells play a critical role in the early steps of normal immune responses and are often modulated by tumor-sparing mechanisms. A normal immune response involves antigen presentation to T-helper cells that results in differentiation toward either a Th1 response and, ultimately, cytotoxic and anti-neoplastic activity or a Th2 response with a primarily humoral-mediated immunity [4]. A Th1 response is associated with IFN- γ , TNF- α , IL-2, IL-12, and IL-18, and a Th2 response is characterized by IL-4, IL-6, and IL-10. These responses are typically not concurrent and each will result in relative downregulation of the opposing pathway [5,6].

HNSCC is a particularly aggressive cancer with regards to its immunosuppressive activities [7] and is implicated in the production of numerous tumor-derived cytokines including IL-4, IL-6, IL-8, IL-10, GM-CSF, TGF- β 1 and VEGF among other inflammatory mediators and growth factors [8,9]. There is evidence to suggest skewing of the immune response with a Th2 bias and a cascade of downstream events. For example, IL-12 and IL-18 have well-established anti-tumor activities via the Th1 response but are antagonized by HNSCC induction of the Th2 cytokine IL-10 [10]. IL-10 in turn results in the migration of immunosuppressive T-regulatory cells into the intra-tumoral environment with an overall dampening of anti-tumoral immunity by altering dendritic cells, macrophages, natural killer cells, and CD8⁺ and CD4⁺ T-cells [11]. In addition, lower levels of IFN- γ , a cytokine involved in anti-tumor immunosurveillance and anti-neoplastic cytotoxicity, are seen in tumor samples as a result of this increase in IL-10 [9,11].

A prominent immune suppressive mechanism induced by HNSCC is the increased appearance of immune inhibitory CD34⁺ progenitor cells [12,13]. While these are immature cells with immune inhibitory activity, they can be induced to differentiate *in vitro* into antigen-presenting dendritic cells by supplementing cultures with 1 α ,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] [14,15]. In a small clinical trial, treatment of HNSCC patients with 1,25(OH)₂D₃ was shown to reverse this immunosuppressive mechanism and to

approximately double the time to cancer recurrence following surgical treatment [16]. Studies using a hamster buccal pouch tumor model similarly showed clinical effectiveness of 1,25(OH)₂D₃ treatment [17]. However, it is important to note that the exact role of 1,25(OH)₂D₃ has not been fully elucidated and that it incurs a myriad of various and seemingly paradoxical effects depending on the particular scenario. For example, in pulmonary tuberculosis, 1,25(OH)₂D₃ suppresses the inflammatory effects of the Th1 response (IFN- γ and TNF- α) but simultaneously promotes the activation of macrophage bactericidal activity [18-20]. In animal models, it has been shown to protect from experimentally induced autoimmunity, and to prevent dendritic, Tc1, and Th1 cell differentiation [18,21]. In contrast to the autoimmune and infectious setting, 1,25(OH)₂D₃ has been shown to activate the immune system in cancer patients in part by promoting the differentiation of CD34⁺ immature cells into antigen presenting cells capable of eliciting an immune response [12]. In mouse models, 1,25(OH)₂D₃ therapy resulted in a reduction of the extent of metastatic disease and, when combined with adoptive immunity, reduced metastasis and improved locoregional control [22]. These improvements in anti-tumoral activity in association with 1,25(OH)₂D₃ are intuitively in contrast to the Th2 promoting effects of 1,25(OH)₂D₃ in other settings. However, the full effects of 1,25(OH)₂D₃ have not been fully characterized in cancer patients and are not entirely clear with relation to systemic versus local downstream immune effects (i.e., cytokine production as a marker of Th1/Th2 balance). Evaluation of the mechanisms of 1,25(OH)₂D₃ immune modulation associated with HNSCC may lead to effective immunotherapies that are not compromised by the intrinsic immunosuppression associated with these aggressive neoplasms.

2. Materials and methods

2.1. Recruitment of patients with HNSCC

Recruitment of patients into this study was approved by the Institutional Review Board of record. Newly diagnosed patients with stage II-IV HNSCC who were being scheduled for surgery were eligible for enrollment into this randomized trial with preoperative treatment of 1,25(OH)₂D₃. Cancer control patients with HNSCC did not receive 25(OH)₂D₃ treatment. Exclusion criteria included prior immunotherapy or radiation treatment in the previous 3 weeks or concurrent malignancies.

2.2. 1,25(OH)₂D₃ treatment and collection of specimens

Patients were treated for 3 cycles with 4 μ g of enteric 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 have minimal toxicity [23]. Nevertheless, serum calcium levels were measured weekly to monitor toxicity. At the conclusion of 3 cycles of this treatment, patients underwent resective surgery. At the time of surgical treatment, peripheral blood samples and HNSCC tissues were collected from patients who were either treated or not treated with 1,25(OH)₂D₃. Surgically excised HNSCC tissues were cryopreserved at -80° C until used for cytokine analyses. HNSCC tissues from untreated patients were also obtained from the Medical University of South Carolina Tissue Biorepository. Normal, non-carcinogenic oral tissue was procured from the adjacent areas bordering oral cancer tissue. These tissues were deemed pathologically normal, and contained no microscopic evidence of invasive carcinogenic disease.

2.3. Cytokine bead array

All reagents used for the cytokine bead array were from BD Biosciences unless otherwise specified. Levels of cytokines in both plasma and tissue lysates were determined using human cytometric bead array Th1/Th2/Th17 cytokine kits. The frozen plasma samples were first allowed to return to room temperature and then mixed with a predetermined volume of

capture beads for each of the relevant cytokines (IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, TNF- α , IFN- γ , VEGF,). This solution was then combined with phycoerythrin (PE) detection reagent and allowed to incubate for a standard time (incubation depends on the particular set of cytokines being evaluated). Next, the capture beads were centrifuged at 200g for 5 min and resuspended in 300 μ L of buffered solution in preparation for quantification by flow cytometry. A FACS Canto (Becton Dickinson) flow cytometer was used for quantification of the cytokine profiles and data were analyzed with the use of FCAP Array Software (manufactured by Soft Flow Hungary Ltd. for BD Biosciences).

To prepare tumor homogenates, tissue samples were thawed and then dissociated in serum-free media using the Stomacher 80 (Seward Laboratory Systems, Inc, Bohemia, NY) for 2 minutes. The resulting material was then mechanically homogenized with the use of an ultrasonic processor for 2 minutes. The remaining lysate was then analyzed per the capture bead assay exactly as outlined above. In order to compare cytokine quantities from different amounts of homogenized tissue, protein concentration was determined with a BCA protein assay kit (Becton Dickinson). The cytokine levels measured were normalized to pg of cytokine per 100 micrograms of total protein.

2.4. Statistical analysis

Data were reported using the mean as a measure of central tendency $\pm$ standard error of the mean. To compare one variable condition between groups, the Wilcoxon signed-rank test was used. Significance was reported in the 95% confidence interval.

3. Results

3.1. Patient Distribution

Plasma samples were collected, processed, and analyzed from 23 patients without cancer, 35 HNSCC patients who were not treated with 1,25(OH) $_2$ D $_3$, and 18 HNSCC patients who were treated with 1,25(OH) $_2$ D $_3$. Tissue specimens were collected, processed and analyzed from 10 pathologically normal adjacent tissue specimens of HNSCC patients and 30 HNSCC patients who did not receive 1,25(OH) $_2$ D $_3$ treatment. A more limited number (seven) of HNSCC tissue specimens were also collected from HNSCC patients who were treated with 1,25(OH) $_2$ D $_3$. Within each treatment group, tissue and plasma samples are not necessarily matched. Thus, there are enrolled patients that contributed only plasma, only tissue, or both plasma and tissue to the final specimen analysis. However, for nine of the HNSCC patients, plasma was available prior to and following 1,25(OH) $_2$ D $_3$ treatment. Thus, paired analyses were also conducted for these plasma cytokine levels.

3.2. Increase in peripheral plasma IFN- γ and TNF- α levels in HNSCC patients treated with 1,25(OH) $_2$ D $_3$ prior to surgery

The Th1 immune response is mediated by a collective cytokine profile which includes IL-2, IFN- γ , and TNF- α . When comparing levels of these cytokines in plasma of healthy subjects versus plasma of HNSCC patients that did not receive 1,25(OH) $_2$ D $_3$, there is little evidence of a Th1-promoted response. Indeed, comparisons of IL-2, IFN- γ , and TNF- α levels revealed no significant difference between the two groups of subjects (Fig. 1, top panel). However, when compared to the untreated HNSCC patients, the 1,25(OH) $_2$ D $_3$ treatment arm exhibited significant increases in IFN- γ , TNF- α , and IL-2, suggesting Th1 skewing as a result of treatment with 1,25(OH) $_2$ D $_3$. Paired plasma cytokine levels were also analyzed for 9 patients by comparing pre-treatment cytokine levels of HNSCC patients to levels following completion of 1,25(OH) $_2$ D $_3$ treatment. Similar to what was seen for group analyses, levels of IL-2 and IFN- γ were increased following treatment with 1,25(OH) $_2$ D $_3$ (Fig. 1, bottom panel).

3.3. Increase in peripheral plasma Th2 cytokine profile of HNSCC patients treated with 1,25(OH)₂D₃ prior to surgery

The Th2 immune response is mediated by a collective cytokine profile that includes IL-4, IL-6, and IL-10. As compared to normal plasma, plasma from untreated HNSCC patients has significantly increased levels in IL-6 and a small, but significant increase in IL-10 (Fig. 2, upper panel). However, no significant differences were seen in IL-4 concentrations between the two groups. Compared to untreated HNSCC patients, HNSCC patients treated with 1,25(OH)₂D₃ had significant increases in IL-6 and IL-10. Similar results were seen when conducting paired analyses of the same patients' plasma cytokine levels prior to and following 1,25(OH)₂D₃ treatment (Fig. 2, bottom panel). IL-4 tended to also be increased, but the increase was not statistically significant. These Th2 cytokine results together with the Th1 cytokine results described above, suggest that treatment with 1,25(OH)₂D₃ stimulates both cytokine arms.

3.4. Comparisons in Th1 and Th2 cytokine levels within tumor versus in plasma

Several differences emerge when tissue cytokine levels were measured and compared to plasma levels. Compared to pathologically normal tissue, levels of several Th1 cytokines, including IFN- γ and TNF- α , were increased in HNSCC of untreated patients (Fig. 3). Levels of IL-2 also tended to be increased, but this increase was not statistically significant. In contrast, plasma levels of these cytokines were not increased. Comparisons of the same two groups also revealed there was a small, but significantly increased level in the Th2 cytokine IL-10 (Fig. 4) in the HNSCC tissue. IL-4 was not detectable in any of the tissue specimens. IL-6 levels trended toward an increase in the HNSCC patients, but this increase was not statistically significant. The Th2 tissue and plasma profiles for normal and HNSCC patients were more closely aligned than what was seen for Th1 cytokines.

Comparisons of cytokines in HNSCC tissue from untreated and 1,25(OH)₂D₃ treated HNSCC patients also revealed interesting results, even though the number of HNSCC tissue specimens from patients that were treated with 1,25(OH)₂D₃ was limited. Compared to levels in HNSCC tissues of untreated patients, both IL-2 and IFN- γ levels were increased in HNSCC tissues from 1,25(OH)₂D₃-treated patients (Fig. 3). However, TNF- α levels were reduced. With the exception of TNF- α , which increased in plasma of 1,25(OH)₂D₃-treated HNSCC patients, the increase in IL-2 and IFN- γ in tissue reflected what was seen in plasma (Fig. 1). In the HNSCC tissue of 1,25(OH)₂D₃-treated patients, levels of the Th2 cytokine IL-6 and, to a lesser extent, IL-10 were increased compared to levels in tissue from untreated HNSCC patients, while IL-4 was not detectable in tissues from any of the patient groups (Fig. 4). These increases in Th2 cytokines were comparable to what was seen in plasma of HNSCC patients (Fig. 2).

3.5. Treatment with 1,25(OH)₂D₃ promotes decreases in pro-angiogenic cytokines in the HNSCC tissue, but not in plasma

VEGF and IL-8 are two cytokines whose angiogenic properties are particularly well described. In fact, their levels have been shown to correlate with clinical disease progression. For instance, VEGF has been demonstrated to be a marker for tumor metastasis, and thus has been used as a prognostic factor in HNSCC patients [24,25]. When plasma was analyzed from healthy subjects and untreated HNSCC patients, elevations were shown in the HNSCC patients for both VEGF and IL-8 (Fig. 5). Interestingly, when the plasma of untreated and 1,25(OH)₂D₃ treated HNSCC patients were compared, those treated with 1,25(OH)₂D₃ had significant elevations in both proangiogenic cytokines VEGF and IL-8 (Fig. 5, top panel). Paired analyses of the same patients' plasma samples prior to and after 1,25(OH)₂D₃ treatment similarly showed a significant increase in VEGF following treatment (Fig. 5, bottom panel).

The HNSCC tissue analyses yielded several surprising findings that failed to correlate with data from peripheral plasma analysis. When compared to pathologically normal tissue, HNSCC tissue from untreated patients had significant elevations in IL-8 and VEGF (Fig. 6), as was seen in plasma of these patients. However, when tissue from 1,25-(OH)₂D₃ treated and untreated HNSCC patients were analyzed, the results did not correlate with peripheral plasma values. In fact, HNSCC patients treated with 1,25-(OH)₂D₃ had decreasing trends of IL-8 levels and unchanged levels of VEGF (Fig. 6). While, these latter trends lacked significance, they were markedly different than the increases seen in plasma of 1,25-(OH)₂D₃-treated HNSCC patients (Fig. 5).

3.6. Tumor-promoting cytokines in HNSCC tissue versus plasma of untreated patients or patients treated with 1,25(OH)₂D₃

Both IL-1 α and IL-1 β have been associated with the promotion of tumor activity and survival. IL-1 α promotes expression of genes involved in cell survival, proliferation, and angiogenesis [26]. IL-1 β has been associated with the promotion of cancer cell adhesion to vascular endothelium [27]. Examinations of plasma from healthy and untreated HNSCC patients revealed no significant changes in either IL-1 α or IL-1 β (Fig. 7, top panel). However, treatment with 1,25(OH)₂D₃ resulted in significant increases in plasma levels of both IL-1 α and IL-1 β . A significant increase in plasma levels of IL-1 α and a tendency toward an increase in IL-1 β was also seen when comparing each patients' pre-treatment and post-treatment cytokine levels (Fig. 7, bottom panel).

When lysates of pathologically normal tissue or HNSCC from untreated patients were compared, significant increases were seen in both IL-1 α and IL-1 β in the HNSCC tissues (Fig. 8). However, HNSCC tissue from patients that were treated with 1,25(OH)₂D₃ did not have the increase in either IL-1 α or IL-1 β that was seen in plasma. Instead, there was a slight tendency toward a decline in levels of these cytokines. Although these studies were conducted with a limited number of tissue specimens, the results suggest a dissociation in multiple cytokine categories between levels in plasma versus in tissue.

4. Discussion

In order to thrive within the immunocompetent host, HNSCC evades the host immune system via several mechanisms. One key method of tumor escape is direct modulation of the host immune profile. In the past, our lab has shown that patients with advanced HNSCC have a diminished Th1/Th2 balance [28]. Numerous efforts have attempted to alter this skewed pro-tumorigenic cytokine phenotype, thus encouraging the host immune system to recognize and mount a defense against the tumor. Our lab has been investigating the immunomodulatory effects of 1,25(OH)₂D₃ in patients with HNSCC. These studies have shown that, in patients with advanced HNSCC, oral administration of 1,25(OH)₂D₃ diminishes levels of immune inhibitory CD34⁺ progenitor cells and increases levels of mature dendritic cells within tumor tissue [12]. While these findings are promising, the downstream immune effects of 1,25(OH)₂D₃ treatment remain poorly defined. This is particularly true with respect to effects on systemic versus intratumoral cytokine levels.

Our efforts to further elucidate the effects of HNSCC presence and the effects of treatment of HNSCC patients with 1,25(OH)₂D₃ on systemic and local intratumoral immune profiles revealed several novel findings. Perhaps most surprising was the discordant relationship between the systemic and intratumoral cytokine profiles. For example, plasma Th1 cytokine levels in HNSCC patients were comparable to levels of healthy controls while, surprisingly, tissue Th1 cytokine levels were increased in HNSCC tissue compared to pathologically normal tissue. With respect to Th1 and Th2 cytokines, treatment with 1,25(OH)₂D₃ tended to result in an overall stimulation in levels of both of these cytokine groups in plasma as well

as tissue. Treatment with $1,25(\text{OH})_2\text{D}_3$ also stimulated plasma levels of the angiogenic and pro-tumorigenic cytokines that were measured, but this stimulation was not evident in HNSCC tissue. The implications of these findings suggest that, because of the variability in the relationship between plasma and tissue cytokine levels, a patient's peripheral cytokine profile may not be an accurate reflection or interpretation of the intra-tumoral immunologic profile. On the other hand, the tumor would be a more highly necrotic microenvironment, which can also impact on cytokines and $1,25(\text{OH})_2\text{D}_3$ treatment responses.

The most interesting findings within the study is the counterintuitive correlation in the stimulation by $1,25(\text{OH})_2\text{D}_3$ of both Th1 and Th2 cytokine levels, with our prior studies showing increases in levels of mature dendritic cells and T-cells expressing the early activation marker, CD69, within HNSCC tissue of patients treated with $1,25(\text{OH})_2\text{D}_3$ as compared to untreated HNSCC patients [16]. This concept is also extended to the positive clinical responses that we showed in that same study where $1,25(\text{OH})_2\text{D}_3$ -treated HNSCC patients had an increased time between surgical treatment and cancer recurrence by approximately 2-fold. Of interest is that most of the HNSCC specimens that were used for the present study were the same as those used in the prior study showing that $1,25(\text{OH})_2\text{D}_3$ treatment stimulates immune infiltration and prolongs the time to HNSCC recurrence.

While treatment with $1,25(\text{OH})_2\text{D}_3$ tended to stimulate both Th1 and Th2 cytokines in the periphery and in HNSCC, $1,25(\text{OH})_2\text{D}_3$ treatment stimulated the angiogenic and pro-tumorigenic cytokine levels in plasma, but not in HNSCC tissue. Thus, there is a discordant relationship not only between cytokine levels in plasma and HNSCC tissue, but also among the different functional cytokine categories.

The study is not without limitation. The main limitation is the limited sample size within the $1,25(\text{OH})_2\text{D}_3$ treatment arm. This low sample size limits statistical significance of certain relationships but, instead, showed non-statistically significant trends which can still offer an understanding of the downstream immunologic effects of $1,25(\text{OH})_2\text{D}_3$. It is also important to note that within the treatment groups, tissue and plasma samples are not matched. This finding accounts for the noticeable discrepancy between the tissue and plasma sample sizes within each treatment arm, most noticeably within the $1,25(\text{OH})_2\text{D}_3$ treated group. Also, of note is that normal plasma was obtained from healthy controls, while normal tissue was pathologically normal tissue obtained from HNSCC patients. This does not, however diminish the strength of the cytokine analyses for untreated and $1,25(\text{OH})_2\text{D}_3$ -treated HNSCC patients.

As a whole, the above findings indicate a cytokine-stimulated environment within the HNSCC and a further stimulation by $1,25(\text{OH})_2\text{D}_3$ of some, but definitely not all, of the cytokine categories. Of interest for future studies would be to also study the functional consequence and reactivity of host cells to the cytokine milieu in HNSCC and the impact of $1,25(\text{OH})_2\text{D}_3$ treatment to this responsiveness.

5. Conclusions

The present study has shown the ability of $1,25(\text{OH})_2\text{D}_3$ to alter the cytokine balances associated with HNSCC. The Th2 skewing associated with HNSCC allows the tumor to escape immune recognition by the host. Administration of $1,25(\text{OH})_2\text{D}_3$ appears increase levels of both Th1 and Th2 cytokines within the tumor tissue. However, treatment with $1,25(\text{OH})_2\text{D}_3$ did not stimulate levels of proangiogenic factors VEGF and IL-8 as well as procarcinogenic cytokines such as IL-1 α and IL-1 β within HNSCC tissue. The relationships demonstrated within this study provide a sound foundation for further investigations into the immunomodulatory effect of $1,25(\text{OH})_2\text{D}_3$ in patients with HNSCC.

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Highlights

Head and neck cancer tissue contains a cytokine-stimulated environment.

Vitamin D treatment of HNSCC patients increases Th1 and Th2 cytokines.

Vitamin D does not stimulate VEGF, IL-8, IL-1 α or IL-1 β in HNSCC tissue.

Systemic cytokine levels do not parallel oral tissue cytokine levels.

Fig. 1. Plasma Th1 cytokine levels

Levels of the Th1 cytokines IL-2, IFN- γ and TNF- α were measured in the plasma of healthy control subjects and in HNSCC patients who were either untreated or treated with 1,25(OH) $_2$ D $_3$. Cytokine concentrations are reported as pg/ml. Shown in the upper panel are group analyses, while the lower panel shows paired analyses for 9 patients where pre-treatment and post-treatment plasma samples were available for each patient.

Fig. 2. Plasma Th2 cytokine levels

Levels of the Th2 cytokines IL-4, IL-6 and IL-10 were measured in the plasma of healthy control subjects and in HNSCC patients who were either untreated or treated with $1,25(\text{OH})_2\text{D}_3$. Cytokine concentrations are reported as pg/ml. Shown in the upper panel are group analyses, while the lower panel shows paired analyses for 9 patients where pre-treatment and post-treatment plasma samples were available for each patient.

Fig. 3. Tissue Th1 cytokine levels

Levels of the Th1 cytokines IL-2, IFN- γ and TNF- α were measured in pathologically normal tissue of HNSCC patients and in HNSCC tissues of patients who were either untreated or treated with 1,25(OH) $_2$ D $_3$. Cytokine concentrations are reported as pg/100 μ g tissue protein.

Fig. 4. Tissue Th2 cytokine levels

Levels of the Th2 cytokines IL-4, IL-6 and IL-10 were measured in pathologically normal tissue of HNSCC patients and in HNSCC tissues of patients who were either untreated or treated with 1,25(OH)₂D₃. Cytokine concentrations are reported as pg/100 µg tissue protein.

Fig. 5. Plasma angiogenic cytokine levels

Levels of the angiogenic cytokines IL-8 and VEGF were measured in the plasma of healthy control subjects and in HNSCC patients who were either untreated or treated with 1,25(OH)₂D₃. Cytokine concentrations are reported as pg/ml. Shown in the upper panel are group analyses, while the lower panel shows paired analyses for 9 patients where pre-treatment and post-treatment plasma samples were available for each patient.

Fig. 6. Tissue angiogenic cytokine levels

Levels of the angiogenic cytokines IL-8 and VEGF were measured in pathologically normal tissue of HNSCC patients and in HNSCC tissues of patients who were either untreated or treated with 1,25(OH)₂D₃. Cytokine concentrations are reported as pg/100 μg tissue protein.

Fig. 7. Plasma pro-tumorigenic cytokine levels

Levels of the pro-tumorigenic cytokines IL-1 α and IL-1 β were measured in the plasma of healthy control subjects and in HNSCC patients who were either untreated or treated with 1,25(OH) $_2$ D $_3$. Cytokine concentrations are reported as pg/ml. Shown in the upper panel are group analyses, while the lower panel shows paired analyses for 9 patients where pre-treatment and post-treatment plasma samples were available for each patient.

Fig. 8. Tissue pro-tumorigenic cytokine levels

Levels of the pro-tumorigenic cytokines IL-1α and IL-1β were measured in pathologically normal tissue of HNSCC patients and in HNSCC tissues of patients who were either untreated or treated with 1,25(OH)₂D₃. Cytokine concentrations are reported as pg/100 μg tissue protein.