

Aus der Universitätsklinik für Zahn-, Mund- und Kieferheilkunde Tübingen  
Abteilung: Klinik und Poliklinik für Mund-, Kiefer- und Gesichtschirurgie

# **Expression of 1,25-Dihydroxyvitamin D3 receptor in oral squamous cell carcinoma**

Inaugural-Dissertation  
zur Erlangung des Doktorgrades  
der Zahnheilkunde

der Medizinischen Fakultät  
der Eberhard Karls Universität  
zu Tübingen

vorgelegt von  
Shokri, Beshr

2018

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Tag der Disputation: 13.06.2018

Meiner Mutter, meiner Frau und meiner Familie  
In Liebe und Dankbarkeit gewidmet

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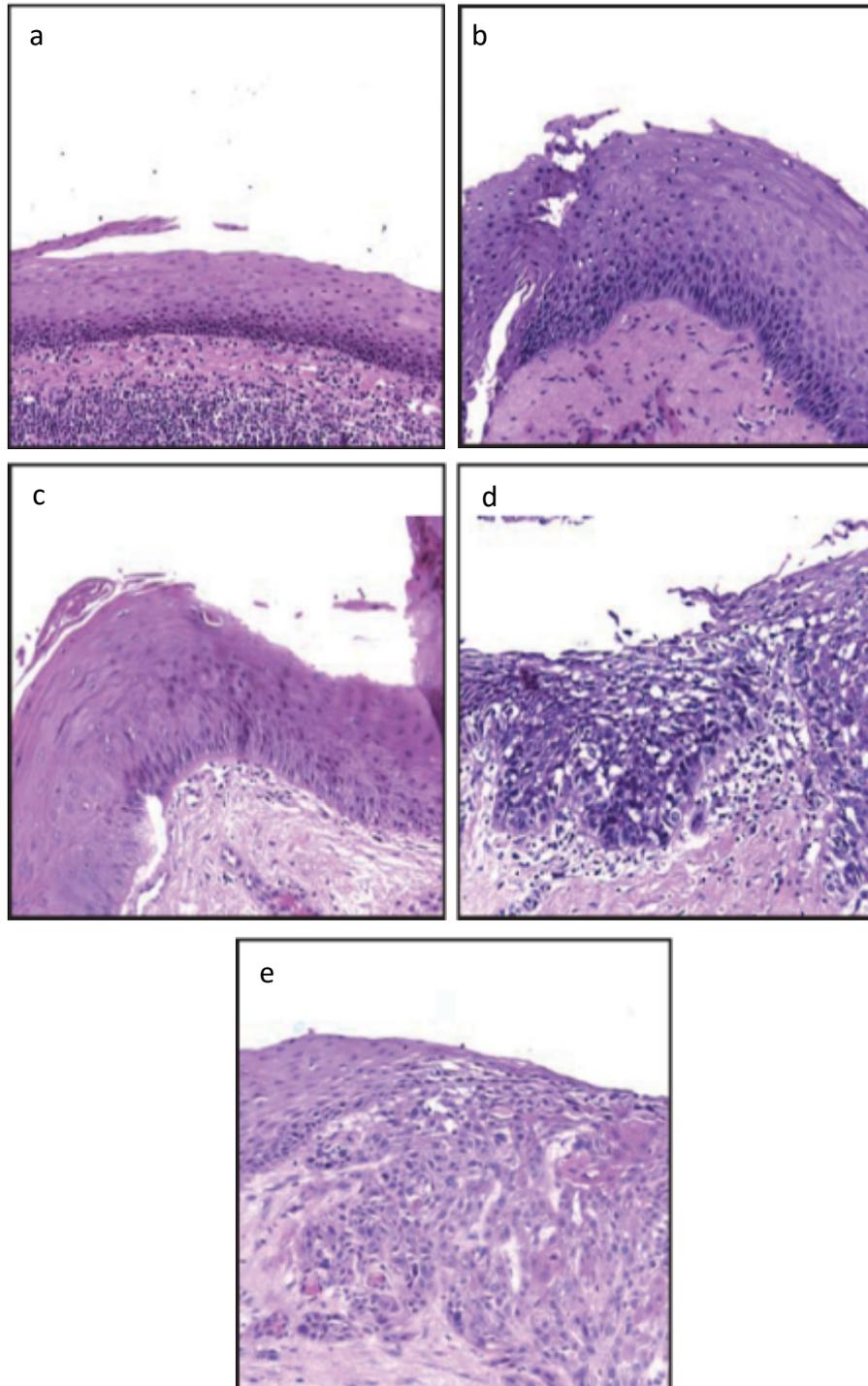
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## A. Introduction

### A.1. Oral squamous cell carcinoma (OSCC)

#### A.1.1. Definition and epidemiology

Oral cancer is a malignant disease that affects the structure of the mouth and its tissues. It can arise from the mouth tissue and may be a result of metastatic invasion from a distant body location or a junction with a tumor of an adjacent tissue [5]. Squamous cell carcinoma (SSC) is found in 90% of various oral cancer types worldwide [6]. SCC, which is also considered as one important type of head and neck malignancies, is the most common malignant transformation that can also arise from the “paranasal sinuses, nasal cavity, pharynx and larynx”. Microscopically, OSCC results after a series of tissue morphological premalignant changes starting from tissue hyperplasia then mild to severe epithelial dysplasia (also known as carcinoma in situ) then malignant carcinoma (Fig. 1) [4]. The most common habitual etiological risk factors that lead to OSCC are tobacco and alcohol consumption, which when combined have synergic action [7]. Another major causative risk factor that has recently been intensively studied, is the persistent infection with human papillomavirus which is particularly important in oropharyngeal squamous cell carcinoma including tonsillar and base of the tongue cancer [8-10]. As for most cancers, squamous cell carcinoma is the result of genetic alterations that lead to malignant behavior, in which cellular DNA undergoes increased exposure to extrinsic damaging factors and a decrease in the function of intrinsic DNA protecting factors. Although many patients are unaware of its presence, oral cancer is found to be the eighth most frequent cause of death among other human body cancer diseases. [11].



**Figure 1: Histopathological stages of malignant transformation in epithelial squamous cell carcinoma (HNSCC tissue samples).** Stages of malignant transformation in which **normal epithelial tissue (slide a)** undergoes hyperplastic cellular activity to from **epithelial hyperplasia (slide b)**. Persistent etiological factors then **lead to mild dysplasia (slide c)** then to the last precancerous lesion; **carcinoma insitu (slide d)** and finally reaching the phase of **invasive squamous cell carcinoma (slide e)**. Clinically, Hyperplastic changes need at least regular professional control while dysplastic to invasive neoplastic changes demand surgical intervention or other cancer therapy. Modified from [4].

In general, mouth cancers including the lip, salivary gland, mouth, gum and tongue cancers have a 2.6% worldwide incidence rate with about 212.000 new cases every year. The occurrence in males (141.200 cases yearly) is more than females (70.300 new cases yearly) with a gender-ratio (M:F) of 2 to 1. The highest incidence rate in reference to the geographic classification is in Melanesia , “a sub-region of Oceania, northeast of Australia” and South Central Asia. In Western, North and South Europe, nearly 32.200 cases are diagnosed every year, where the prevalence of key risk factors like tobacco and alcohol consumption in these areas is at increasing levels [12]. Statistical studies concluded that the sixth leading cancer among the most common malignancies worldwide is the head and neck cancer. In the meantime, 25% to 40% of the oral squamous cell carcinomas are affecting the tongue tissue [13]. In the European Union including Germany, oral and pharyngeal tumor is the seventh tumor affecting men and account for 3.5% of tumors and sixteenth occurring tumor among women and accounts for 1.5% of occurring tumors. In Germany alone, ten thousand new cases of oral and pharyngeal tumors are diagnosed yearly [14, 15]. In 2004, 40.100 death cases in Europe resulting from oral and pharyngeal cancer were registered [14]. The overall five-year relative survival rate of oral cancer patients in Germany is 54.6%. OSCC survival rate is in general statistically associated with the age of the patient, tumor grade and stage for both sexes. In Germany for example, the five-year survival rate is around 54.6%. Although the incidence of OSCC is increasing in women, it is still less than in male patients, in which the incidence in women is 8% lower than in men among all age groups under 75 years [16].

### A.1.2. Detection of OSCC

In general, oral cavity cancer can be detected early using various diagnostic methods. Among these are conventional methods such as taking a tissue biopsy and modern non-invasive methods such as “oral brush biopsy, saliva-based oral diagnosis and optical biopsies”. Optical biopsy and saliva-based oral diagnosis techniques are found to be promising and effective measures in diagnosing oral squamous cell carcinoma [17]. Other cancer diagnostic modalities have, to some extent, proven their efficiency in detecting malignant changes. Although, it is

always required to take a tissue biopsy and use newly developed referral criteria for definitive confirmation of any malignant change [18].

Early detection of the signs and symptoms of SCC of the head and neck can largely prompt the diagnosis of the disease. Unfortunately, there is no known better proven screening method yet other than the usual clinical diagnostic means like regular clinical examination and inspection in areas of high disease prevalence [19]. Furthermore, delays in disease detection can play a role in prognosis determination of oral cancer. In addition, the patients' age, the rate and nature of disposition to etiological factors are important to the tumor's behavior influential factors [20]. The high prevalence of OSCC in developing countries in comparison to developed countries could be related to the latency in disease detection, which in turn limits the proper treatment choices [21].

### A.1.3. Management of OSCC

Choosing the appropriate treatment and decision making for the management of OSCC, as well as for other head and neck squamous cell carcinomas, is usually complicated and requires the systematic collaboration of the dentist with head and neck surgeons, medical oncologists, radiotherapy specialists as well as plastic surgeons. For treatment guidance, important factors like the primary malignancy site and stage as well as the possibility of a surgical tumor resection should be considered. Other patient-related factors like swallowing, airways and the patients' desire for preserving the affected organ and the presence of other morbid diseases must be considered [22].

In combination to surgical treatment and adjuvant radiotherapy, which are the best known effective treatment methods, drugs with cytostatic effects such as cisplatin, 5-fluorocil (5-FU) and paclitaxel as well as for palliative treatment proved to be very efficient in treating oral cancer [23] , [24] & [25]. The most important factor that guides the treatment planning is the accurate staging, which is usually carried out by an oral and maxillofacial surgeon and is usually based on the radiographic examination with computer tomography (CT) or magnetic resonance

imaging (MRT) [16] or mostly both. Distant metastasis of the OSCC can take place in the lungs, which is the most common place of metastasis that makes the chest imaging a very important routine initial assessment tool. Other areas and organs like mediastinal lymph nodes, liver and skeletal bone also could be affected [22]. To detect the presence of a distant malignant invasion, the combination of radiological techniques like CT imaging and the positron emission tomography (PET) scan after premedication with radioactive tracer agents like [18F] Fluorodeoxyglucose (18F-FDG) is usually a routine procedure, which is known as PET/CT [26]. This diagnostic procedure has proven to be efficient in detecting body distant malignant invasions as well as lymph node metastasis or both together [27].

## A.2. Cancer stem cell compartments (CSCs), CD44 a CSCs extracellular marker in oral and head and neck squamous cell carcinoma (HNSCC) and their role in tumor

### A.2.1. Cancer stem cells (CSCs) in OSCC

Stem cells in general are type of cells that have the capability of self-renewal and the differentiation to generate mature cells of a particular tissue [28]. Also known as physiological stem cells, stem cells have two types: embryonic and adult stem cells. Embryonic stem cells arise from the inner mass of the mammalian blastocyst and can differentiate into cells of the known three germ layers that can develop to all tissue types. On the other hand, adult stem cells have more limited differentiation ability which is more restricted to cell types of the tissue where they are located and can assist in tissue homeostasis, regeneration of new cells as well as their major role in the pathogenesis of various diseases including cancer, which then can be called cancer stem cells (CSCs). CSCs can be found within cancer cells and have the ability of self-renewal to serve as precursors for tumor cells sustaining tumor growth [29]. CSCs were first detected in myeloid leukemia. Studies on mice and humans have increased the evidences of their importance in the development of many solid tumors including oral cancer. Due to biological variations and so often experimental technical reasons, the presence of CSCs in solid tumors is variable and may have no similar properties from primary CSCs.

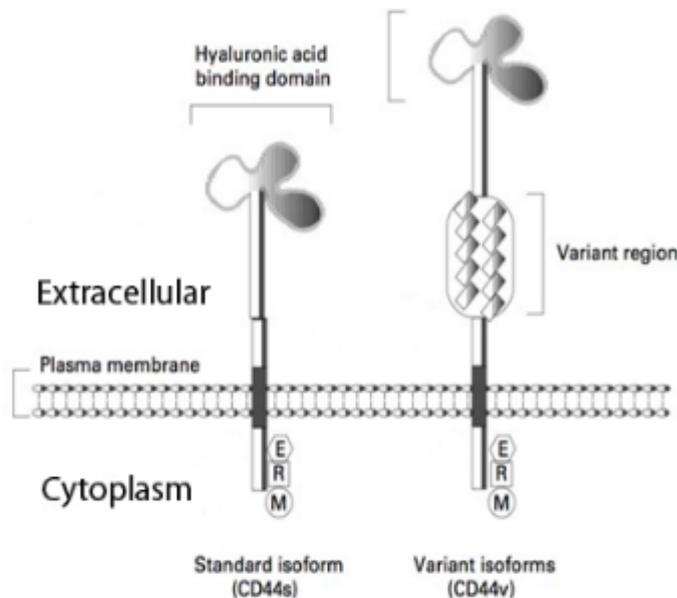
More importantly, CSCs have shown more resistance to chemo- as well as radiotherapy [30]. CSCs are also known to share similar normal stem cell capabilities like the state of inactivity, resistance to drugs and toxins due to the involvement of ATP-binding cassette transporters (ABC transporters) expression ability, active DNA repair capacity and resistance to programmed cell death (apoptosis). These factors can lead to increased chances of CSCs survival against chemotherapy and the evolution of more tumor cell populations [31].

In recent studies, it is becoming more evident that most, if not all, malignancies are developing from the so called cancer stem cell compartment [1, 2, 32]. Also, from clinical observation of tumor recurrence and relapse after treatment with chemotherapy, theories arose initially to doubt the existence of residual stem cells after therapy correlating to their responsibility for tumor regeneration. Secondly, they encouraged the development of new treatment modalities that can target the critical CSCs cancer regenerating and self-renewal properties resulting in more treatment efficiency in head and neck cancer as well as in other cancers [1, 2]. Although not all tumor cells have the ability to metastasize, epithelial CSCs can metastasize firstly through a process which utilize the so-called epithelial-mesenchymal transition (emT), an instrumental event in the metastatic process and secondly with the help of the disturbed cellular homeostasis [1, 2].

### A.2.2. CD44 and cancer stem cells CSCs in OSCC

In order to understand the involvement of CSCs and to study the mechanism of carcinogenesis in head and neck squamous cell carcinoma, CD44 extracellular receptor must be studied. CD44 glycoprotein is an extracellular matrix receptor, which main site of action is the cell surface and is one of the most recognized and studied CSCs related marker [29, 33, 34]. In addition to its role in lymphocytic function, cellular adhesion, migration and homing, CD44 protein overexpression in CSCs can play major roles in neoplastic diseases like in head and neck cancers, breast cancer, prostate cancer and ovarian cancer [3, 35, 36]. On the contrary, high expression level of CD44 in renal cancer and non-Hodgkin's lymphoma is not always a relevant indicator [37].

The expression of a CD44 gene can result in multiple isoforms like the standard form (CD44s), variant form (CD44v) and other forms depending on the site of expression on the CD44 gene. When one of these isoforms bind to the so-called hyaluronic acid (HA) at the cellular membrane, the formed profile is called HA-binding profile and can improve tumor growth (Fig. 2). However not all CD44 receptors tend to bind to HA and accomplish this new structural formation.



**Figure 2: CD44s and CD44v cellular markers.** The structure of the variant isoform (CD44v) and the standard isoform (CD44s). Hyaluronic acid binds itself to the "N-terminal of the extracellular domain" resulting in the active form of CD44. CD44 is important marker in cancers like Oral squamous cell carcinoma, head and neck cancer, breast cancer etc.. CD44v isoform is believed to have neoplastic activities Modified from [3]

Studies on animal tissue have shown a high concentration of CD44 in tumor cell lines and more aggressive tumors forms [3, 30, 38]. In addition, CD44 is recognized to be actively involved in the tumor metastatic process, probably because of its rule in the lymphocytic migration process, especially the CD44v form that has metastatic properties [3, 30, 38].

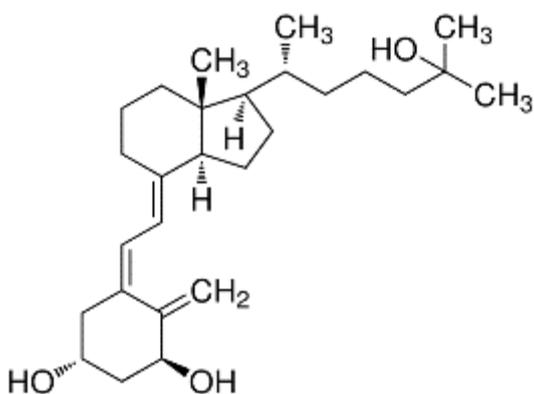
### A.3. Vitamin D, its active metabolite 1,25-dihydroxyvitamin D<sub>3</sub>, Vitamin D receptors and their role in malignancy

#### A.3.1. Vitamin D and its active metabolite 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>)

Vitamin D (Calcitriol) is represented by a group of fat-soluble secosteroids most importantly vitamin D<sub>3</sub> (Cholecalciferol) and vitamin D<sub>2</sub> (ergocalciferol). The most important physiological application of these compounds is to enhance the intestinal absorption of various elements like calcium, iron, magnesium,

phosphate and zinc. Many studies have shown the importance of this vitamin in preventing some bone diseases such as rickets, as well as its need for proper muscular function and its role in maintaining proper bone mass, in which inadequate vitamin D levels may lead to elderly osteoporosis. Furthermore, other studies suggested the importance of vitamin D in the prevention of type 1 diabetes mellitus (with controversial evidence), hypertension as well as various common cancers. Vitamin D synthesis is mainly cutaneous, where sunlight-exposure mainly to ultraviolet-beam (wavelengths of 290-315 nm) irradiation is the primary source. Mostly, dietary sources of vitamin D are less relevant [39].

1,25-dihydroxycholecalciferol (synonyms are 1,25-dihydroxyvitamin D<sub>3</sub> and 1 $\alpha$ -25-dihydroxyvitamin D<sub>3</sub> also abbreviated in 1,25-(OH)<sub>2</sub>D<sub>3</sub>) is an active hormonal metabolite of vitamin D. Its structural form consists of three hydroxyl groups [40] (Fig. 3). 1,25-(OH)<sub>2</sub>D<sub>3</sub> is produced in the kidney, keratinocytes, macrophages and other tissues like breast, prostate and colon cells. 1,25-(OH)<sub>2</sub>D<sub>3</sub> synthesis is resulted from the hydroxylation of 25-hydroxycholecalciferol. One of the main functions of 1,25-(OH)<sub>2</sub>D<sub>3</sub> is to increase the absorption of calcium in the intestine by mediating the biochemical interactions for this process [41]. Aside from controlling calcium absorption and homeostasis by interacting with its specific receptor in the target tissue cytoplasm, 1,25-(OH)<sub>2</sub>D<sub>3</sub> is widely recognized to be the hormonal form of vitamin D responsible for all its functions [42-45].



**Figure 3: Organic structure of 1,25-dihydroxycholecalciferol** Other scientific names include 1,25-dihydroxyvitamin D<sub>3</sub> (Abbreviated 1,25-(OH)<sub>2</sub>D<sub>3</sub>) which is commonly known as Calcitriol.

### A.3.2. Antineoplastic activities of vitamin D, its active metabolites like 1,25-(OH)<sub>2</sub>D<sub>3</sub> and vitamin D analogues

In the past decades, many researchers studied the role of vitamin D and its metabolites, mainly 1,25-(OH)<sub>2</sub>D<sub>3</sub>, and its implications on cancer prevention, development, pathogenesis and treatment. The essential vitamin D role in cell growth maintenance and regulation, the inhibition of proliferation and differentiation induction is well established in many researches [39, 46-50]. Other studies provided evidence that 1,25-(OH)<sub>2</sub>D<sub>3</sub> can prevent cancer through the role of cell growth maintenance [46, 49, 51, 52]. This mode of action is explained as 1,25-(OH)<sub>2</sub>D<sub>3</sub> reduces angiogenesis, increases cellular apoptosis of cancer cells, as well as reduces cellular proliferation, and metastasis [46, 49-51, 53-60]. In addition, vitamin D analogues like paricalcitol is known to have anticancer action against myeloid leukemia, myeloma, and colon cancer cells [61]. For the first time, in a study on human melanoma cells Colston et al. established that 1,25-(OH)<sub>2</sub>D<sub>3</sub> successfully inhibited the growth of tumor cells and that the “time of malignant melanoma cells” was doubled after incubation with the hormone which confirms that melanoma cells are responsive to the hormone in vitro [62]. Later, the report of Miyaura et al. concluded that another role of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in leukemia cancer cells HL60 is to be added, where vitamin D is involved in cell growth and differentiation of this type of cancer cells. The presented effect was the induction of the differentiation of human promyelocytic leukemia into mature myeloid cells (myelocytes, metamyelocytes and mature granulocytes). For this function, 1,25-(OH)<sub>2</sub>D<sub>3</sub> was the most potent vitamin D metabolite [63]. In the same year, Abe et al. confirmed the presence of vitamin D<sub>3</sub> action on bone marrow cells and provided evidence for the differentiation of myeloid leukemia cells (M1 cells) into macrophages [64]. Another study described the mechanism of proliferation inhibition of leukemic NCI-H929 cells by Vitamin D<sub>3</sub> analog [65]. Other than their role in leukemia, 1,25-(OH)<sub>2</sub>D<sub>3</sub>, also through its antiproliferative activity, found to be involved in inhibiting other cancers [51].

Breast cancer risk and lower levels of vitamin D are proportionally correlated in white women [66]. In a study on women in a region of high solar radiation, the reduction in breast cancer risk in 25% is noted and is associated with high vitamin D exposure [67]. In addition, it is found that a novel Gemini vitamin D analogue (BXL0124) was able to repress CD44 expression in vitro and in xenograft tumors inhibiting breast cancer cells [68].

In brain cancer, 1,25-(OH)<sub>2</sub>D<sub>3</sub> proved to have cytotoxic effects on rat as well as human glioma tumor cell lines and successfully induced cellular death in the study of Naveilhan et al [69]. In prostate cancer, the antineoplastic antiproliferative responses of 1,25-(OH)<sub>2</sub>D<sub>3</sub> through multiple mechanisms in human prostate cancer cell lines were also reported [70-72]. It is also concluded that prolonged insufficient vitamin D intake might increase the risk of prostate cancer. Therefore, inducing the synthesis of 1,25-(OH)<sub>2</sub>D in the prostate may decrease the risk of developing prostate cancer [70, 73].

In colorectal cancer, according to epidemiological studies, 1,25-(OH)<sub>2</sub>D<sub>3</sub> deficiency is associated with an more risk of colon cancer [74]. Palmer et al. Found 1,25-(OH)<sub>2</sub>D and other non-hypercalcemic Vitamin D analogues to be potential anticancer agents as they induced the differentiation of human colon carcinoma cells [75].

In head and neck SCC, a study on a hamster buccal pouch with SCC found that the administration of systemic vitamin D<sub>3</sub> aided in delayed carcinogenesis [76]. It was also shown that treating HNSCC with 1,25-(OH)<sub>2</sub>D<sub>3</sub> promoted the maturation of dendritic cells and reduced the anti-immune cellular activity of CD34+ cells [77], which are known to negatively affect the cancer prognosis and increase its recurrence [78, 79]. Moreover, EB1089, a vitamin D<sub>3</sub> analogue, can completely inhibit the growth of human laryngeal squamous cell carcinoma cells [80]. With less hypercalcemic activity, vitamin D analogues are found to induce G0/G1 cell cycle arrest when they or an active 1,25-(OH)<sub>2</sub>D<sub>3</sub> targeted the cell cycle protein p21 responsible for this action [81]. Other studies on the

antineoplastic activity of Vitamin D and its derivatives like EB 1089 on HNSCC supported the claims about their preventive and therapeutic value [82, 83]. Also, the in vitro growth-inhibitory action in various OSCC after the administration of 1,25-(OH)<sub>2</sub>D<sub>3</sub> have been recently proven [84].

### A.3.3. Vitamin D receptor and VDR expression in tumor cells of various cancers

Vitamin D receptor (VDR) is one of the nuclear receptor members of transcription factors family which is also known as NR111 (nuclear receptor subfamily 1, group I, member 1) [85]. The activation of VDR leads to the formation of “heterodimer with the retinoid-X receptor” that bind to the hormone response elements on DNA leading to the production of specific gene products through the known DNA expression process. VDR is also involved in “microRNA-directed posttranscriptional mechanisms”. [86].

In the earlier mentioned study of Kumagai et al., VDR is found to mediate the action of anticancer Vitamin D and its analogues in myeloid leukemia, myeloma and colon cancer cells [61]. In addition, in a study on human melanoma cells, Colston et al., observed 1,25-(OH)<sub>2</sub>D<sub>3</sub> receptors that were similar to normal well characterized receptors in 1,25-(OH)<sub>2</sub>D<sub>3</sub> target organs. [62]. Later, Tanaka et al demonstrated the growth inhibition in mouse and human leukemic cells where VDR was expressed and led to the differentiation stimulation of leukemia cells (M1 cells) into mature macrophages among cells which are exposed 1,25-(OH)<sub>2</sub>D<sub>3</sub> [87].

In breast cancer, Eisman et al. Made the first evidence of the presence of low-capacity binding protein specific for 1,25-(OH)<sub>2</sub>D<sub>3</sub>, which was found in cloned human breast cells. His study discussed its relevance to developing bony complications and the implication of these findings on tumor behavior [88]. Later, other studies confirmed the antiproliferative properties of 1,25-(OH)<sub>2</sub>D<sub>3</sub> causing lower tumor cells activity and reduction of cell growth in breast cancer cells in relation to the high presence of VDR in cancer cell lines [89-93].

In head and neck squamous cell carcinoma, the  $1,25\text{-(OH)}_2\text{D}_3$  receptor mediated inhibition of neoplastic cellular growth is already demonstrated. In addition, it was found that this action of the hormone is dose-dependent and leads to down-regulation of DNA synthesis and a reduction in cell numbers [94].

## **B. Aim of the study**

### **B.1. Rational of the study**

The association between putative CSC compartment and VDR expression in CD44+ breast cancer has already been demonstrated [68]. However, this is the first study to assess this link in putative CSC compartment of OSCC. The treatment of OSCC with the aid of nontoxic endocrine cancer therapy has not yet been intensively discussed, which also mandates further investigations of VD, its analogues and their therapeutic value.

### **B.2. Specific aims of this study**

1. To analyze the expression of VDR in OSCC as well as in normal mucosal tissue.
2. To identify the relation between VDR expression and tumor relapse.
3. To evaluate whether oral tissue may be a new potential target for biologically active 1,25-(OH)<sub>2</sub>D<sub>3</sub> or its analogues, and to discuss their potency.
4. To evaluate whether biologically active 1,25-(OH)<sub>2</sub>D<sub>3</sub> or its analogues are helpful in guiding supportive treatment of patient with OSCC in accordance with the “carcinogenesis model based on putative CSC hypothesis” [30, 68].

## C. Materials and methods

### C.1. Patients and Tumor Specimen

We did a retrospective review of 191 patients' records after a primary radical R0 tumor resection in the Department for Oral and Maxillofacial Surgery, University Hospital Tuebingen, over a period of ten years as well as 10 healthy individuals with normal oral mucosa tissue [1, 2]. Samples of patients with non-resectable disease, inadequate follow-up data as well as patients with preoperative antineoplastic radio- or chemotherapy were not included in the study [1, 2]. The specimens used in this study were retrospectively retrieved from the pathology archives, in which histopathological diagnostic conformation of squamous cell carcinoma was carried out by the Department of Pathology, University of Tuebingen. After routine histopathological work-up, specimens were formalin-fixed and paraffin embedded tissues [1, 2]. Experienced pathologists have selected the paraffin-embedded tumor blocks and done the routine Haematoxylin-Eosin (H.E) stained sections.

The study was approved by the local ethics committee [1, 2]. For every specimen and prior to surgical resection, an informed consent from the patients was obtained. We depended on the local tumor registry to get the follow-up data of our patients [1, 2]. The records of last follow-up were reviewed from the last recall in the outpatient department or the date of loco-regional recurrence or cancer specific death, respectively [1, 2]. Tumor and patient characteristics are summarized in Table 1. Intensive histopathological assessment was carried out blind to the prior histopathology report [1, 2]. Microscopic slides were prepared after cutting a 2µm thickness tissue-section from (FFPE) samples with a microtome and then were fixed on microscopic slides using warm water. [1, 2]. Tumor staging was carried out according to the Union for International Cancer Control (UICC) and the American Joint Committee of Cancer (AJCC) regulations of 2010.

**Table 1:** Clinicopathological characteristics and prognostic factors of 191 patients with OSCC of low and high VDR expressers. Cutoff value for VDR expression was determined by Receiver Operating Characteristics (ROC) analysis [1, 2].

Characteristics	Number of Patients			p-value
	Total n=191	VDR expression Low ( $\leq 39\%$ ) n=136 (71%)	VDR expression High ( $> 39\%$ ) n=55 (29%)	
Age (y)				0.9286
<60 $\pm$ 11.8	93 (48.7%)	66 (71%)	27 (29%)	
$\geq 60\pm 11.8$	98 (51.3%)	70 (71%)	28 (29%)	
Gender				0.6394
Male	145 (75.9%)	105 (72%)	40 (28%)	
Female	46 (24.1%)	31 (67%)	15 (33%)	
Site distribution of OSCC				0.8589
Lips	11 (5.8%)	9 (82%)	2 (18%)	
Tongue	42 (22.0%)	30 (71%)	12 (29%)	
Floor of the mouth	84 (44.0%)	59 (70%)	25 (30%)	
Palate	17 (8.9%)	10 (59%)	7 (41%)	
Buccal mucosa	10 (5.2%)	6 (60%)	4 (40%)	
Alveolar ridge	27 (14.1%)	22 (81%)	5 (19%)	
Histological Grading				0.6066*
G1	50 (26.2%)	36 (72%)	14 (28%)	
G2	125 (65.4%)	90 (72%)	35 (28%)	
G3	15 (7.9%)	10 (75%)	5 (25%)	
G4	1 (0.5%)	0 (0%)	1 (100%)	
Depth of invasion				0.1834**
pT1	75 (39.3%)	50 (67%)	25 (33%)	
pT2	52 (27.2%)	36 (69%)	16 (31%)	
pT3	18 (9.4%)	16 (89%)	2 (11%)	
pT4	46 (24.1%)	34 (74%)	12 (26%)	
Cervical lymph node metastasis				0.9442
pN0	133 (69.6%)	94 (71%)	39 (29%)	
pN1-3	58 (30.4%)	42 (72%)	16 (28%)	
UICC stage				0.3386***
UICC I	57 (29.8%)	38 (67%)	19 (33%)	
UICC II	42 (22.0%)	29 (69%)	13 (31%)	
UICC III	34 (17.8%)	26 (76%)	8 (24%)	
UICC IV	58 (30.4%)	43 (74%)	15 (26%)	
Locoregional recurrence				0.0002
No	140 (73.3%)	89 (64%)	51 (36%)	
Yes	51 (26.7%)	47 (92%)	4 (8%)	

**Abbreviations:** y, years; G, grading; UICC, Union for International Cancer Control; \*G1/2 vs. G3/4; \*\*pT1/2 vs. pT3/4; \*\*\*UICC I/II vs. UICC III/IV.

Tumor grading was performed depending on the World Health Organization (WHO) criteria [95]. The information about tumor characteristics and UICC stage, pT-categories, pN-categories, pM-categories, lymph nodes infiltration as well as residual tumor status were then gathered [1, 2]. In addition, tumor size and site distribution along with other patients' characteristics like gender, age, personal history and habitual history were considered [1, 2]. All this data was used to construct a Microsoft Excel © database [1, 2]. After tissue fixation, tissues surgical resection margins status was determined in the final histopathological evaluation, in which close margins were deemed positive in all analyses [1, 2]. However, margins that were greater than or equal to 10 mm from resection margin were considered as negative [1, 2].

## C.2. Staining procedure and quantitative immunohistochemistry

Unconjugated specific VDR, CD44, Ki-67, and isotype control antibodies were purchased for immunohistochemical analysis from Santa Cruz Biotechnology (CA, USA, mouse anti-human VDR mAb, D-6 sc-13133, dilution: 1:50), BD Pharmingen (Heidelberg, Germany, mouse anti-human CD44 mAb, 550392, dilution: 1:50), and Novocastra (Newcastle, UK, mouse anti-human Ki-67 mAb, NCL-Ki67-MM1, dilution: 1:50) [1, 2]. Moreover, we purchased VDR clone from LifeSpan Biosciences (Eching, Germany, rabbit anti-human VDR pAb, LS-B2976, dilution: 1:100), in order to confirm the VDR expression stained with anti-VDR mAb from Santa Cruz in a selection of 30 cancer samples and BICR cancer cell lines and we used this clone for double labeling experiments [1, 2].

Then, series of steps followed to carry out the Pretreatment through slide fixation, deparaffinization, rehydration, heat-induced epitope retrieval of FFPE slides blocking to accomplish the immunohistochemical single staining [1, 2]. Briefly, 2 µm thickness tissue sections were cut on a microtome from paraffin blocks and mounted on adhesive microscope slides [1, 2]. Serial sections were deparaffinized using xylene and ethanol and rehydrated in water [1, 2]. Using citrate buffer pH 6.0, Heat induced epitope retrieval (HIER) was performed [96],

[1, 2]. Alkaline phosphatase (AP) conjugated AffiniPure donkey-anti-mouse IgG, at 1:200 dilution (Jackson ImmunoResearch Laboratories Inc., Suffolk, England) was used as “a secondary antibody for immunohistochemical double staining” of CD44 [1, 2]. A horseradish peroxidase (HRP) conjugated AffiniPure donkey-anti-rabbit IgG (Jackson ImmunoResearch) was used as a secondary antibody for VDR (LifeSpan Biosciences, rabbit anti-human VDR pAb, LS-B2976) in dilution at 1:200. Fluorescein isothiocyanate (FITC) conjugated AffiniPure donkey-anti-rabbit IgG in dilution at 1:200 (Jackson ImmunoResearch) was used as a secondary antibody for immunofluorescence double staining of VDR [1, 2]. A Cy3-conjugated AffiniPure donkey-anti-mouse IgG (Jackson Immuno-Research) in dilution at 1:200 was used as a secondary antibody for Ki-67 [1, 2]. Cell counting was carried out for the immunohistochemistry quantification [1, 2]. Results were described in percentages where a number of positive tumor cells within 100 given tumor cells were counted [1, 2]. Results with less than 10% positive cells were considered negative [1, 2]. ImageJ software (<http://rsbweb.nih.gov/ij/>) coupled with immunomembrane plug-in (<http://imtmicroscope.uta.fi/immunomembrane>) was used to have computer-assisted semi-quantitative analysis in order and to assess the quantification of VDR immunoreactivity in microscopically acquired JPEG images of OSCC samples [1, 2]. Staining completeness (0–10 points) and intensity (0–10 points) were added for a combined score (0–20 points) [1, 2]. To assess precision (reproducibility/ repeatability) of computer-assisted semi-quantitative analysis, 5 images per VDR positive sample slide showing representative tumor areas were acquired using 20x objectives [1, 2]. Canon camera (Krefeld, Germany) was used to analyze produced pictures. Microsoft Office Picture Manager © was used to import the photographed images [1, 2].

### C.3. Cell Culture

VDR expression was analyzed in cells ( $1 \times 10^4$ ) from the OSCC cell lines BCR3 and BICR56 (American Type Culture Collection, ATCC) in cytopins as positive control of VDR expression by cancer cells [1, 2]. Preparation and staining of cytopins was performed [1, 2]. We analyzed VDR expression in BCR3 and BIC56 cell lines from patients with OSCC and healthy individuals on protein and

mRNA level in cytopins as positive control for the case of VDR expression [1, 2]. Cytopins were fixed in acetone then dried for 10 minutes [1, 2]. Rehydration, blocking and the staining procedure steps followed as for immunohistochemistry of FFPE sections [96]. We cultured BICR3 and BICR56 cells in DMEM F-12 medium (Invitrogen, Belgium) containing 10% FCS (Sigma-Aldrich, Germany), 1% fungicide, and penicillin/streptomycin (Biochrom, Germany) at 37°C and 5% CO<sub>2</sub> [1, 2].

#### C.4. Immunohistochemical and immunofluorescent double labeling experiments

For VDR-CD44 expression in FFPE tissue and OSCC cell lines (BICR3, BICR56), the sequential immunohistochemical (IHC) double staining (co-expression) was analyzed [1, 2]. For VDR- Ki-67 expression in FFPE tissue, the sequential immunofluorescence double labeling (co-expression) was analyzed [1, 2].

“Slides were incubated with the primary CD44 antibody (mouse mAb) or control antibody overnight at 4°C in a humidified chamber and with secondary AP-conjugated antibody for 30 minutes at room temperature in a humidified chamber followed by 20 minutes of incubation with Fast Red (Biogenex, San Ramon, USA) to perform the IHC double staining. Subsequently, the slides were then incubated with the second primary VDR antibody (rabbit pAb) or control antibody overnight at 4°C in a humidified chamber and with a secondary HRP-conjugated antibody for 30 minutes at room temperature in a humidified chamber” [1, 2]. Then slides were incubated for 5 minutes with 3,3'-Diaminobenzidine (DAB, Biogenex). Counterstaining with Haemalaun and mounting with Glycergel (Dako, Hamburg, Germany) was then performed [1, 2].

Immunofluorescence double labeling was performed after incubating the slides with a primary VDR antibody (rabbit pAb) or control antibody overnight at 4°C using a humidified chamber and then with a secondary FITC-conjugated antibody for 30 minutes at room temperature also in a humidified chamber [1, 2]. Subsequently, the slides were then incubated with the second primary Ki-67 antibody (mouse mAb) or control antibody overnight at 4°C using a humidified

chamber and with secondary Cy3-conjugated antibody for 30 minutes at room temperature in using a humidified chamber [1, 2]. Counterstaining of the slides with DAPI (4',6-Diamidino-2-phenylindoldihydrochloride, Sigma-Aldrich, Taufkirchen, Germany) then covering with Polyvinyl-alcohol mounting medium (DABCO, Sigma-Aldrich) was performed [1, 2]. Microsoft Office Picture Manager © was again used to import the photographed images [1, 2].

### C.5. Real-time quantitative reverse transcription-PCR analysis (RT-PCR)

To measure gene expression of VDR by RT-PCR, cellular RNA was extracted and cDNA synthesis was performed from OSCC cell line (BICR3, BICR56) and FFPE tissue [1, 2, 97]. RNA measurement was carried out by measuring the absorbance at 260 nm. The purity and contamination absence of total RNA was confirmed with a ratio of 260:280 nm of the total RNA [1, 2]. Normal matched human mucosal cDNA was purchased from BioChain (Hayward, CA, USA) as control [1, 2]. Relative quantification and cDNA quality (integrity) was controlled using the reference genes Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin [1, 2]. Commercial primer kits (VDR, GAPDH, beta-actin) were purchased from Search LC (Heidelberg, Germany) [1, 2]. mRNA expression quantitation was determined by establishing RT-PCR with the LightCycler System (Roche Applied Science, Mannheim, Germany), [1, 2]. The RT-PCR for GAPDH and VDR was carried out by using a volume of 20  $\mu$ L which contains 2  $\mu$ L cDNA, 2  $\mu$ L primer mix (commercial primer kits from Search LC, Heidelberg, Germany), 2  $\mu$ L DNA Master Sybr Green I mix (Roche Applied Sciences, Mannheim, Germany) and 14  $\mu$ L DEPC treated water [1, 2]. Target DNA was amplified during 35-38 cycles of 95°C for 10 seconds, 68°C for 10 seconds and 72°C for 16 seconds with temperature transition rate of 20°C/second for each cycle and secondary temperature of 58°C with a step size of 0.5°C [1, 2]. To identify specific reaction products, Melt-curve analysis was used [1, 2]. The relative quantification value, fold difference, is expressed as  $2^{-\Delta\Delta Ct}$  [1, 2]. More specifically, in order to separate unspecific primer dimers and

specific PCR products, melting curve analysis at 95 °C 0 seconds, 58 °C 10 seconds and 95 °C 0 seconds was achieved [1, 2]. With the aid of specific commercial primer kits (Search LC, Heidelberg, Germany), the transcript levels of VDR and housekeeping GAPDH for each sample were determined [1, 2]. Results were then calculated in the form of ratio of target vs. housekeeping gene transcription [1, 2, 98].

### C.6. Western blot analysis and densitometric quantification

Western blot analysis was used to confirm VDR antibody specificity [1, 2]. Protein extraction from OSCC cell lines BICR3 and BICR56 was performed [1, 2, 99]. As a control, normal human mucosal protein purchased from BioChain (Hayward, CA, USA) was used [1, 2]. For this analysis, cells were harvested on ice. Cells were then centrifuged and then dissolved in 60 µl lysis buffer [1, 2]. Cells were then incubated overnight at temperature of -70°C [1, 2]. The removal of cell debris was done by centrifugation [1, 2]. To measure the protein concentrations of the supernatants, colorimetric assay (RD DC Protein Assay, Bio Rad, Germany) was used according the manufacturer's manual [1, 2, 99]. After that, a total of 100µg of denatured cell lysate from the cellular extracts were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes after the addition of reducing Laemmli sample buffer [1, 2, 100]. Using monoclonal mouse anti-human VDR (Santa Cruz Biotechnology, mouse anti-human VDR mAb, D-6 sc-13133, dilution: 1:100) and monoclonal mouse anti-human GAPDH (Abcam, Cambridge, UK, ab8245, dilution: 1:5000) specific primary antibodies incubation overnight at 4°C, the membranes were then analyzed [1, 2]. HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, USA) was used to detect the binding of the primary antibodies [1, 2]. Specific bands showing VDR and GAPDH proteins were visualized by the “enhanced chemiluminescence method” (GE Healthcare, Freiburg, Germany) [1, 2]. To quantify VDR intensity of western blot bands, we used an “automated densitometric quantification digitizing system” (UN-SCAN-IT Gel © software, version 6.1, Silk Scientific Inc., Utah, USA) [1, 2]. JPEG in 8-bit gray scale format at 600 dpi were produced by scanning the developed films followed by the measurement of the pixel intensities within a

band[1, 2]. Quantification of western blot bands with the segmental analysis method was done with the UN-Scan-it © software [1, 2]. The constant pixel measurement was assured by Clone drawing mode which was used to perform pixel summation of individual segments [1, 2]. Pixel intensities correction was performed in the background [1, 2]. Digitized gel data (pixel total) were imported into an Excel database (Excel ©, Microsoft ®) [1, 2].

### C.7. Statistical analysis

For the statistical analysis, MedCalc © Software, Version 12.3.0 (Mariakerke, Belgium) was used [1, 2]. To calculate Disease-specific survival (DSS), duration from the time of tumor resection until obvious loco-regional recurrence or tumor conditional death respectively has been considered [1, 2]. To assess differences in the DSS for patients who received successful (R0) curative surgical resection for OSCC, patients were classified into VDR high expressers (VDR high) and VDR low expressers (VDR low) [1, 2, 101]. ROC analysis was performed in order to determine the best cutoff range for OSCC recurrence group screening VDR expression [1, 2]. Quality measurement of VDR expression, area under the curve (AUC) analyzed [1, 2]. The value of the “highest diagnostic average of sensitivity and specificity” (highest diagnostic accuracy) was determined as the cutoff point and was calculated at 39% VDR expression in correspondence with the highest Youden index [1, 2]. To test for a normal distribution of the data, the D’Agostino-Pearson test was performed [1, 2]. To estimate the DSS times, the Kaplan-Meier method was used then it was compared using the log-rank test [1, 2, 102, 103]. Using the Cox Proportional Hazards Model, multivariate analyses were performed [1, 2]. The resulted significant parameters on univariate analysis were included [1, 2]. 95% Confidence Interval (CI) was provided for hazard ratios (HR) of variables that may influence survival status in univariate and multivariate analysis [1, 2]. To assess the two categorical variables relation, Chi-Square test ( $\chi^2$ ) and Fisher's exact test were used [1, 2]. The accuracy was considered as “the degree of closeness of measurements of a quantity to that quantity's actual value” [1, 2, 100, 104]. Non-parametric Kendall's tau ( $\tau$ ) correlation coefficient measurement was performed in order to assess the accuracy between the two

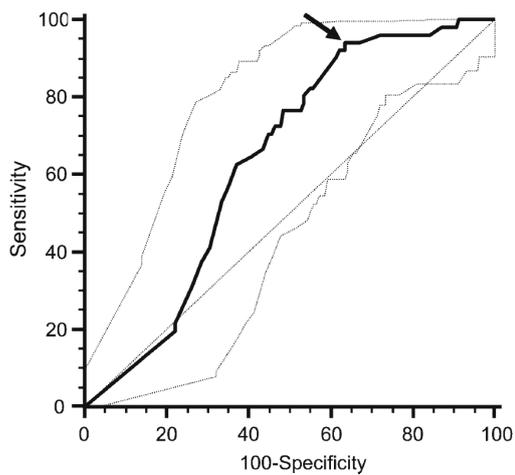
quantification methods of immunohistochemical analysis taking in consideration “the manually counted percentages of positive tumor cells within 100 counted tumor cells vs. combined score of computer-assisted semi-quantitative analysis” [1, 2, 100, 104]. The “non-parametric Spearman Rho rank correlation coefficient” was used to perform Correlation analysis of the VDR count (%) with Ki-67 count (%) [1, 2]. Pearson's correlation (Rr) index was used to measure the co-localization parameter of immunofluorescent double labeling [1, 2, 100, 104]. Significant P-value considered being  $p < 0.05$  in which all P-values were 2-sided [1, 2].

## D. Results

In order to assess the accuracy between our two quantification methods of immunohistochemical analysis, a preliminary study was carried out in which a significant correlation between the first and second assessment was found showing VDR expression results of:  $\tau = 0.979$ ,  $p < 0.0001$ , 95% CI 0.972 to 0.984 [1, 2].

### D.1. There is no association between VDR expression and clinicopathological characteristics of OSCC

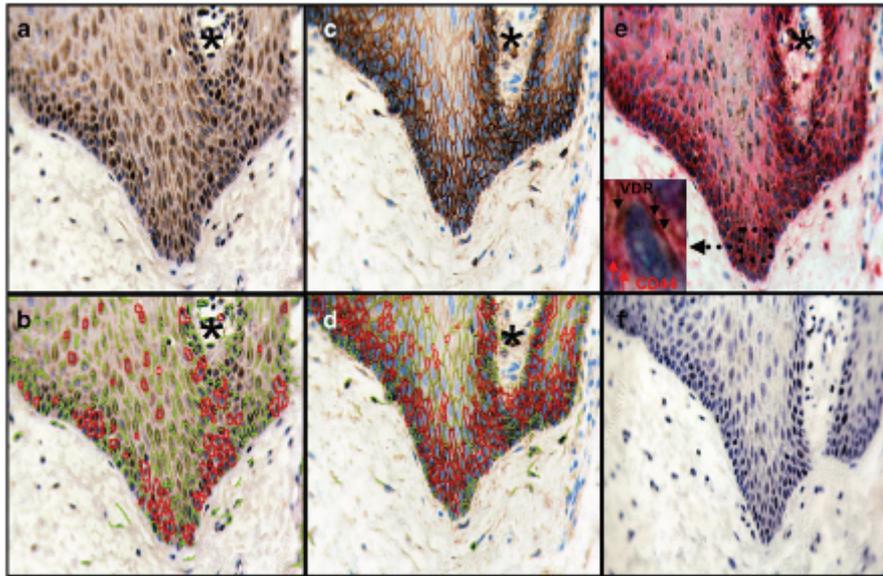
In normal oral mucosa samples (n=9/10), VDR was relatively not highly detected in the basal layer cells in healthy oral squamous epithelium [1, 2]. In stromal cells, VDR expression was very weak but considerably associated with present cancer cells [1, 2]. Clinicopathological characteristics and prognostic factors of 191 patients with OSCC measured by ROC analysis are summarized in table 1. More importantly, VDR expression within the tumor cells was found in 50% of the patients with OSCC (n = 96/191). The mean VDR expression rate in positive tumor samples was 47%, 95 CI for the mean 42.6 to 50.7%, lowest value was 12% and highest value was 87% [1, 2]. However, no associations between VDR expression and clinicopathological characteristics were found [1, 2]. Using area AUC analysis, 39% VDR expression cutoff point of VDR expression led to the optimal sensitivity and specificity for the diagnosis of patients with recurrence (AUC: 0.638,  $p = 0.0006$ , 95% CI 0.566 to 0.706) (Fig. 4) [1, 2]. In 29% of the patients (n = 55/191), high VDR expression was analyzed [1, 2]. VDR with CD44 expression were primarily found in the epithelial basal layer cells, which were shown in representative images of Immunohistochemistry of OSCC serial sections (Fig. 5a,c) [1, 2]. By staining the OSCC cell lines (BICR3 and BICR56) in cytopins as control measure, positive results of cancer cells VDR expression pattern was confirmed (Fig. 6a-c) [1, 2].



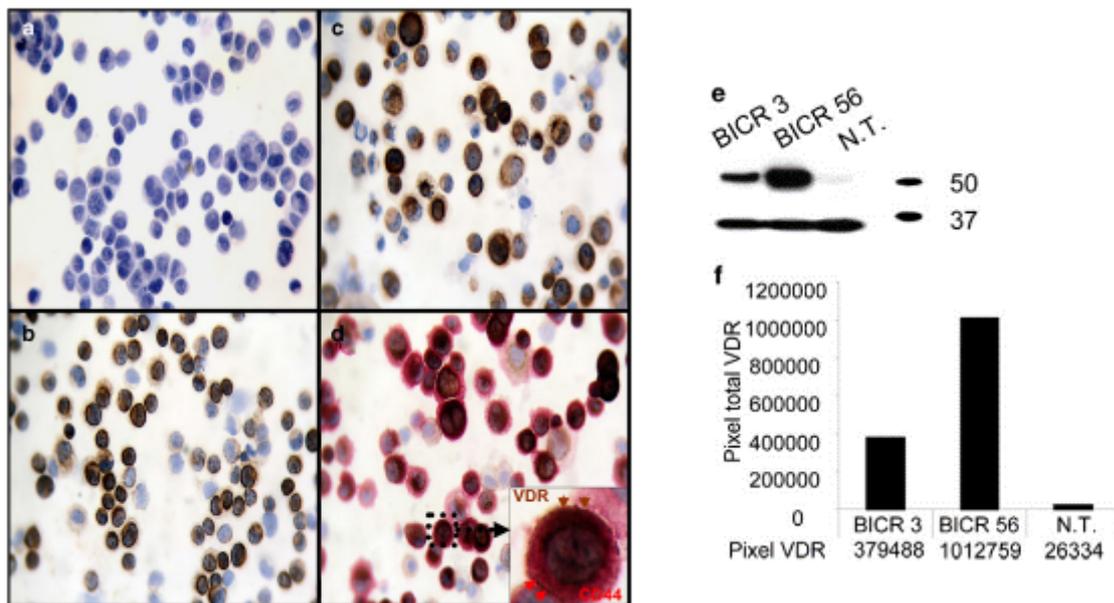
**Figure 4: Receiver operating characteristics (ROC) curve for VDR expression in OSCC patients [1, 2].** For the measurement of the cutoff point, sensitivity (the true positive rates) are plotted in function of false positive rates (100-specificity): the arrow represents the cutoff value with highest diagnostic accuracy of VDR expression analyzed at 39% for the diagnosis of recurrent group. (Sensitivity= 94.12 %, 95 % CI 83.8–98.8 %; specificity= 36.43 %, 95 % CI 28.5–45.0 %). Dotted lines show 95 % CI [1, 2, 104].

## D.2. The confirmed correlation between CD44+ cancer cells and VDR expression in OSCCC cell lines

OSCC serial tissue sections and cell lines were stained using immunohistochemical double-staining experiments to investigate, whether CD44+ CSCs are associated with VDR expression or not [1, 2]. Dominant populations of CD44+/VDR+ tumor cells in the epithelial basal layer of OSCC tissues were found (Fig. 5e), similar to what was suggested by OSCC immunohistochemical stained serial sections with single staining procedure. (Fig. 5a, c) [1, 2]. Another evaluation of BICR3 and BICR56 in OSCC cell lines and immunohistochemical double staining confirmed co-expression of CD44+/VDR+ (Fig. 6d) [1, 2].



**Figure 5: Immunohistochemical single staining (VDR, CD44) and double staining (VDR with CD44) of serial sections in OSCC (magnification  $\times 200$ -fold) [1, 2].** Note the formation of epithelial cells within epithelial layer (Asterisks). The area of interest is shown in the small box. **Slides a, b** represents images of VDR expression and CD44 in **slides c, d** VDR with CD44 single and immunohistochemical double staining in **slide e**. **Slide f** shows IgG control with no staining. In **slides b, d**: the staining parts of "computer-assisted semi-quantitative analysis" on VDR+ and CD44+ OSCC cells is shown, where red labels **b, d** represent heavy or complete staining and green labels represent weak or weaker staining. In **slides a, e**, brown chromogen color (3,3'-diaminobenzidine) indicates positive VDR and in **slide c** indicates CD44 staining. In **slide e**, red chromogen color (Fast red) of double stained tumor cells shows CD44+ cells and the blue color indicates the nuclear counterstaining by hematoxylin. The strong presence of VDR and CD44 expression in the epithelial basal layer cells is noted [1, 2].



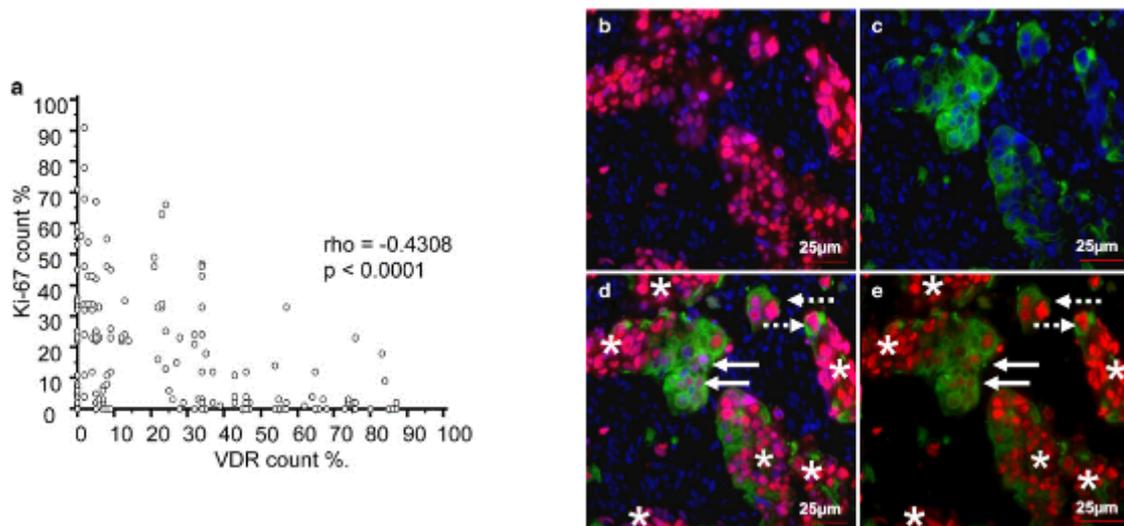
**Figure 6: VDR expression within BICR3 and BICR56 OSCC cell lines**[1, 2]. Slides a-d represent the immunocytochemical single and double staining of VDR expression. Slides e, f represents Western blot analysis of VDR expression. Slide a shows images of IgG control. Slide b shows images of VDR expression in BICR3 cell lines. Slide c shows images of VDR expression in BICR56 cell lines. Slide d shows images of immunocytochemical double staining of VDR in brown areas (3,3'-diaminobenzidine) show with brown arrows. CD44 immunocytochemical double staining in red areas (fast red) showed with red arrows. Original magnification is  $\times 400$ -fold. In slide e, the specificity of immunohistochemical staining and increased VDR expression in comparison to normal tissue was confirmed by Western blot of VDR in BICR3 and BICR56 OSCC cell lines. The graph f represents a densitometric quantification of Western blot protein bands in pixel total VDR expression in BICR3 and BICR56 and normal tissue. As predicted, the detected molecular weight of VDR is in accordance with the predicted molecular weight of 50 kDa. GAPDH (loading control), which is detected as a band of approximately 35 kDa [1, 2].

### D.3. VDR specificity is confirmed by western blot analysis

The performed western blot expression in OSCC cell lines (BICR3 and BICR56) confirmed the specific results of the VDR immunohistochemical staining [1, 2]. That revealed increased VDR in OSCC cell lines compared to normal tissue [1, 2]. The detected molecular weight of VDR (50 kDa) matched the predicted molecular weight [1, 2]. The molecular weight of GAPDH, which we considered as control, was detected on approximately 35 kDa (Fig. 6e) [1, 2]. Compared to normal tissue, densitometric analysis allowed for positive quantification of VDR western blot protein bands (pixel total) in both BICR3 and BICR56 OSCC cell lines [1, 2].

#### D.4. VDR+ tumor cells and proliferating (Ki-67+) OSCC cells are inversely correlated

All OSCC tissue samples (n = 191) were analyzed for Ki-67 immunoreactivity in tissue serial sections in order to evaluate whether VDR+ tumor cells associated have anti-proliferating properties or not. We found that Ki-67 expression in OSCC had a weak but significant inverse correlation ( $r = -0.4308$ ,  $p < 0.0001$ ) with the expression of VDR. This analysis resulted from “quantified immunohistochemical single staining results of VDR with proliferating tumor cells (Ki-67+)” (Fig. 7a) [1, 2]. The results of the immunohistochemical single staining procedure in FFPE slides were confirmed by the representative double-immunofluorescent staining of VDR with Ki-67 [1, 2].



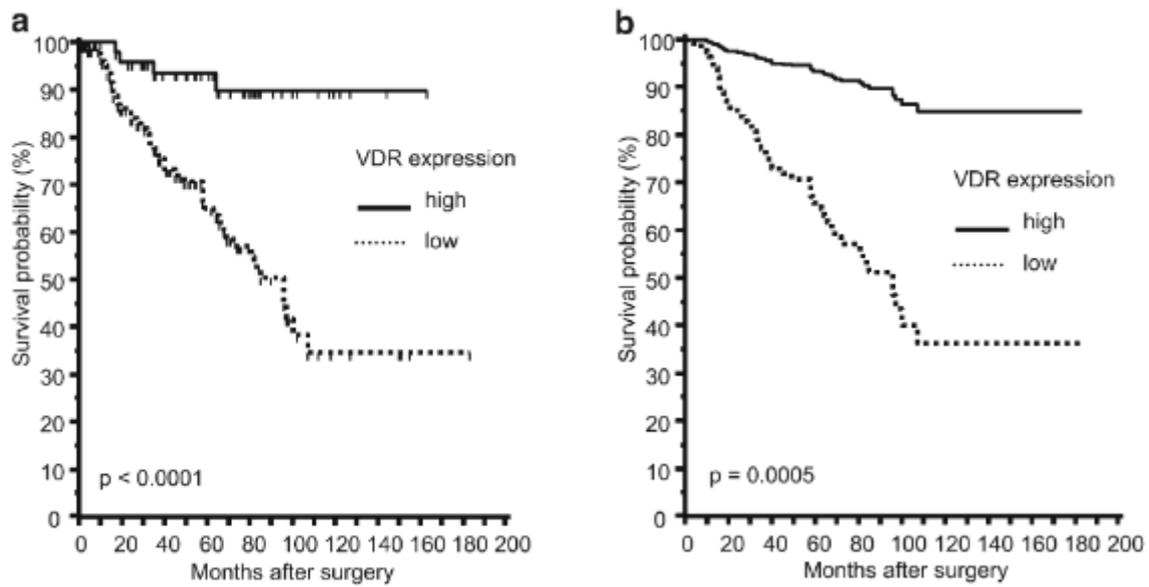
**Figure 7: Correlation and co-expression analysis of VDR with Ki-67. Calibration bar represents 25  $\mu\text{m}$** [1, 2]. Immunofluorescent double staining of VDR with Ki-67 confirms the immunohistochemical single staining results in OSCC FFPE tissues. **Slide a** shows the correlation analysis of VDR with Ki-67 from quantified immunohistochemical single staining results of VDR (measured in %) in OSCC FFPE tissues with proliferating cells (Ki-67 measured in %), Ki-67 expression in OSCC had a weak but significant inverse correlation with the expression of VDR ( $r = -0.4308$ ,  $p < 0.0001$ ). **Slide b** represents the Ki-67 expression (red, Cy), slide (c) represents VDR expression (green, FITC). **Slides d, e** shows the co-expression in OSCC FFPE tissues showing the majority of proliferating (Ki-67+) cancer cells without VDR expression (asterisks, merge). To adjust a better contrast, co-expression of VDR/Ki-67 is demonstrated with (left, **slide d**) and without (right, **slide e**) nuclear counterstaining (blue, DAPI). White dotted arrows point to areas of increased co-expression indicating that VDR expressing cancer cells are seldom strongly associated with proliferating cells but mainly detected without or with weak Ki-67 expression (white arrows). Co-localization parameter (merge, **slides d, e**) of Ki-67/VDR (red and green) measured by Pearson's correlation ( $R_r$ ) index is analyzed at 0.08. FITC, fluoresceinisothiocyanate, Cy3, Cyanine 3 red, and DAPI 4',6-diamidino-2 phenylindoldihydrochloride [1, 2, 100].

## D.5. VDR gene expression analysis

Gene expression of VDR in OSCC cell lines BICR3 and BICR56 was assessed to confirm the results of the immunohistochemical staining [1, 2]. In comparison to normal tissue, VDR gene expression in OSCC tissues was increased [1, 2]. A 3.34-fold and a 11.47-fold higher expression was shown by BICR3 and BICR56 cell lines respectively, in comparison to normal tissue [1, 2]. A 3.9-fold higher expression was shown by the representative analysis of VDR gene expression in OSCC tissue [1, 2].

## D.6. OSCC-VDR expression prognostic value

Patients were divided into two groups (disease recurrence and absence of disease recurrence) in order to analyze the survival rates after successful (R0) surgical resection of OSCC [1, 2]. In univariate analysis of all (n=191) OSCCs, cervical lymph node metastasis (pN1-3, HR = 2.0010, 95% CI = 1.0411 to 3.8458,  $p = 0.0138$ ) as well as depth of invasion (pT3/4, HR = 1.4689, 95% CI = 0.7706 to 2.7999,  $p = 0.1903$ ) and grading (G3/4, HR = 0.8641, 95% CI = 0.2893 to 2.5813,  $p = 0.8052$ ) were shown as unfavorable factors [1, 2, 100]. Patients were also divided into two groups according to the intensity of VDR expression (VDR low and VDR high expression) in order to analyze differences in tumor related survival dependent on VDR expression in OSCC [1, 2]. Worse survival was found in the group of low VDR expression in OSCC (VDR low: n=136,  $p < 0.0001$ , HR = 6.3220, 95% CI = 3.5557 to 11.2404) in compared to the group of patients with high VDR expression (Fig. 8a). Cox Proportional Hazards Model multivariate analysis showed low VDR expression and positive lymph node metastases as independent prognostic factors in all OSCCs samples (VDR low: Exp (b) 6.1791; 95% CI of Exp (b) 2.2352 to 17.0823;  $p = 0.0005$ ; (Fig. 8b). LN positive, pN1-3: Exp (b) 1.8998; 95% CI of Exp (b) 1.0764 to 3.3531;  $p = 0.0276$ ) [1, 2].



**Figure 8: Survival curves of OSCC patients measured by VDR expression [1, 2].** **Curve a:** Kaplan–Meier curve shows survival for disease-specific survival (DSS) stratified by high VDR expression (black line) and low VDR expression (dotted line). **Curve b:** Cox regression curve shows survival for disease-specific survival (DSS) stratified by high VDR expression (black line) and low VDR expression (dotted line). Low VDR expression ( $n=136/191$ ) in OSCC is found to be associated with poorer survival. DSS 1-, 3-, and 5-year survival rates (VDR high vs. VDR low) in univariate Kaplan-Meier survival curve (a) are 100 % vs. 97 %, 94 %, vs. 78 %, and 94 % vs. 65 %, respectively. The times of the censored data are indicated by short vertical lines. DSS 1-, 3-, and 5-year survival rates (VDR high vs. VDR low) in multivariate Cox regression (b) survival curve are 100 % vs. 99 %, 96 %, vs. 82 %, and 94 % vs. 65 %, respectively [1, 2].

## E. Discussion

Vitamin D activities are known to be mediated by its active metabolite 1,25-(OH)<sub>2</sub>D<sub>3</sub> [42-45]. 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediate major cellular activities that can influence carcinogenesis in most body cancers. Among these activities inhibition of proliferation, induction of differentiation, as well as reduction of angiogenesis, and induction cellular apoptosis are most important for cancer therapy. [46, 49-51, 53-60]. It is also suggested that Vitamin D can directly and indirectly play a role in overcoming the HNSCC immune suppressive activity, which is profound characteristic in this disease [105]. Moreover, the treatment with 1,25-(OH)<sub>2</sub>D<sub>3</sub> leads to stimulation of the infiltration of CD44+ and, in higher amount, CD8+ T cells, which in turn leads to lengthening the time to HNSCC tumor recurrence [106]. 1,25-(OH)<sub>2</sub>D<sub>3</sub> is also, in a complex way, able to alter the balance of cytokines within HNSCC tumor cells [107].

The involvement of VDR in cancers other than OSCC like breast cancer, myeloid leukemia, myeloma, colorectal cancer and head and neck cancer is in various studies well established (Bower, 1991; Colston, 1981; Colston, 1992; Eisman, 1979; Frampton, 1983; Kornfehl, 1996; Kumagai, 2003; Lisse, 2013; Moore, 2006; Tanaka, 1982; Vink-van Wijngaarden, 1996). In parallel to that, a recent in vivo and in vitro study, VDR expression has been found to be inversely correlated with CD44+ CSCs in breast cancer, meaning that VDR has performed a repressing activity on CD44+ CSCs [68]. However, no other studies on the role of VD and its receptor in OSCC have been performed yet, which is the main reason for conducting our study.

As far as we know, this is the first report demonstrating VDR expression in association with OSCC prognosis. In this study, we analyzed VDR expression in 191 OSCC samples within two cancer cell lines. In addition, we examined 10 samples of normal oral mucosa. Specialized pathologists carefully selected all samples included in this study. Our study confirmed that VDR expression is present in both cancer and non-cancer tissue samples [1, 2]. VDR expression

was mainly restricted to the basal layer of normal oral mucosa as well as in OSCC tissue [1, 2]. Marked overexpression in human OSCC has been detected. Western blot and RT-PCR analysis confirmed VDR specificity [1, 2]. This study also revealed similar data to a report by Reichrath et al., in which predominant VDR expression within the basal cell layer of normal and SCC cells of cervix were noted. VDR up-regulation at protein level in cervical carcinomas cells in comparison to normal tissue was also observed in that study [1, 2, 108]. ROC analysis helped in identifying a cutoff value for subgroups in immunohistochemical analysis for tumor recurrence. Our finding supports the hypothesis that increased VDR expression may be associated with increased-tumor specific survival amongst patients with high VDR expression compared to patients with a low VDR subgroup [1, 2]. However, no difference was detected concerning other the clinicopathological characteristics [1, 2]. Although CD44 populations “may indeed harbor a subpopulation encompassing stem cells”, this alone does not appear to be an adequate marker for stem cells of normal oral mucosa [109].

On the other hand, CD44 putative CSC marker in normal head and neck epithelium was investigated in various studies with differing results. For instance, one study suggested that CD44 might be expressed in normal oral epithelial stem cells, where isolated CD44 normal oral keratinocytes found to exhibit G2-block associated with apoptosis resistance. It was also suggested that targeting these G2 checkpoints proteins might deactivate these checkpoints making cells no more resistant to cellular apoptosis [110]. Another report by Prince et al. concluded that tumor subpopulation with increased tumorigenicity was isolated using CD44 expression in head and neck cancer [33]. Therefore, the use of CD44 as a stem cell marker in normal tissue as well as in OSCC cancer stem cells may be rational due to its established relevance in breast cancer [33].

VDR could “provide a novel molecular link between the physiologic mucosal stem cell niche and the cell-of-origin for OSCC” for the reason that OSCC can arise from physiologic mucosal stem cells [100, 111] that include CD44+ cell

populations [112]. Due to the lack of further *in vitro* and *in vivo* studies to show tumor development by colony and spheroid properties formation, our results describe VDR expression by CD44+ CSC. However, our observations may help to make more future explanations of tumor cells with stem cell properties. For tumor relapse and recurrence, hypothesis about a possible involvement of putative residual cancer stem cell compartments has been rising. Therefore, in order to understand this, our study emphasized on the “co-expression of putative CD44+ CSC with VDR expression in OSCC tissue and cancer cell lines”. Our analysis of immunohistochemical double staining experiment data (CD44+/VDR+), clearly showed a heavy co-expression of VDR+ with CD44+ cancer cells [1, 2]. In line with this finding, the earlier mentioned study of So et al. discussed a novel Gemini vitamin D analogue (BXL0124) that was able to repress CD44 expression *in vitro* and *in vivo* leading to inhibition of breast CSCs [68]. From a therapeutic point of view, the demonstrated increase of VDR content in OSCC lead us to hypothesize that putative CD44+ CSC in OSCC may be sensitive to endogenous or exogenous therapeutic 1,25-(OH)<sub>2</sub>D<sub>3</sub>. However, in this study, the functional roles of VDR in OSCCs are not to be concluded from our results [1, 2]. Although we know that high VDR expression is associated with increased overall survival rates in other cancers like in esophageal cancer [113], it is not yet known if VDR in OSCCs exhibit a mutational inactivation function leading to more expression [1, 2]. Other hypothesis is that tumor cells defective from VDR may tend to signal a feedback loop, which results in increased VDR expression and consequently more presence of VDR in OSCCs [114].

The enhancement of VDR expression at the mRNA and protein levels *in vitro* by various cell types have been already demonstrated [115, 116]. However, Weise et al. found no change in VDRs mRNA levels after the administration of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, which leads to the conclusion that VDR protein level elevation is due to increased receptor protein lifetime and not directly induced by increased gene transcription [116]. Because of the ability of keratinocytes to hydroxylate vitamin D at the C-1 and C-25 positions [117], it can be concluded that keratinocytes are able to synthesize the biologically active 1,25-(OH)<sub>2</sub>D<sub>3</sub>, the vitamin D metabolite

responsible for almost all its activities [45]. However, OSCCs capability of 1,25-(OH)<sub>2</sub>D<sub>3</sub> synthesis is not yet known and needs to be further investigated. On the other hand, one can speculate that increased expression and volume of VDR on the mRNA and protein level may be the result of increased production of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in these tumor cells. Moreover, it is also possible that VDR expression up-regulation in neighboring tumor cells may be due to cytokines and inflammatory peptides released from tumor-invading leucocytes as well as tumor cells [108].

The higher the number of receptors, the higher the responsiveness of steroidal hormone [115]. Based on the suggestion that the biological effects of calcitriol and its analogs like cellular proliferation and differentiation in target cells are mediated by VDR [118], increased VDR expression and content in tumor cells (mainly CD44+ CSCs) may represent a point of weakness in tumor cells which could open the gate for another module of adjuvant antineoplastic treatment of OSCC [1, 2]. As indicated by our double labeling experiment (Ki-67/VDR+), the demonstrated increased VDR expression was inversely correlated with proliferating cancer cells. This finding may be possible due to the fact that VD poses anti-proliferating properties [1, 2, 94]. However, VDR expression could be determined by other different mechanisms, an interesting suggestion could be that VDR expression may be a function of the state of differentiation [1, 2]. The highest maximal ligand binding of VDR is in the early phase of cell growth. While studying human keratinocytes, Pillai et al. found that the number of VDR receptors have fallen as soon as cells differentiated, which decreased the influence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on these cells. Thus, higher influence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> was more noted at the early stage of cellular differentiation [119].

Although pre-treatment 1,25-(OH)<sub>2</sub>D<sub>3</sub> dietary intake of HNSCC patient and vitamin D serum level may have no influence on disease outcomes [120], the findings of this study along with previous findings lead us to speculate that the therapeutic use of 1,25-(OH)<sub>2</sub>D<sub>3</sub> or its non-hypercalcemic analogues as adjuvant chemopreventive therapy could be even more successful than targeting the vast

majority of cancer cells. This therapy could mainly be aimed to target adjuvant residual tumor cells [1, 2]. Some studies suggested that 1,25-(OH)<sub>2</sub>D<sub>3</sub> could reduce tumor growth of HNSCC. For instance, on a study on hamsters with HNSCC, tumor cells of the buccal pouch responded positively with delayed tumor carcinogenesis [76]. Aside from this, levels of immunosuppressive CD34+ cells in HNSCC were reduced in response to treatment with Vitamin D, which also lead to increased maturation of dendritic cells [77]. These findings were found to have the potential to increase the prognosis of HNSCC [79]. The addition of 1,25-(OH)<sub>2</sub>D<sub>3</sub> to HNSCC cells in vitro [94], as well as adding less hypercalcemic vitamin D analogues [81] resulted in a blockage in the transition of cells from G1 to S phase, inducing G0/G1 cell cycle arrest accumulating both differentiated and undifferentiated tumor cells in this phase. Vitamin D analogue was also able to “inhibit cellular proliferation of human laryngeal squamous carcinoma cells through the cyclin-dependent kinase inhibitor p57 or p21” [79, 81]. The role of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in suppressing cellular proliferation, apoptosis induction and cell cycle arrest as well as down-regulating several angiogenesis factors took place under the influence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> action on oral squamous cell carcinoma cells of the oral floor [83]. A dose related consequence of the administration of Vitamin D3 analogue EB1089 had presented itself in the inhibition of HNSCC cellular growth [82], which could be also related to 1,25-(OH)<sub>2</sub>D<sub>3</sub> ability to upregulate the expression of cell cycle inhibitory proteins p21 and p18 [121].

As we find these results to be encouraging, it should be pointed out that more effort to define the role of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and its analogues in the chemoprevention of OSCC is to be done. In this matter, a very related research done after this study supported our findings and provided more evidence about the increased VDR expression in OSCC basal cell layer and moreover suggested that decreased serum 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations enhances oral epithelial proliferation [122]. Also recently, the adjuvant combination therapy with 1,25-(OH)<sub>2</sub>D<sub>3</sub> and Erlotinib, a chemotherapeutic agent, showed more treatment efficacy for OSCC [123]. Further studies on other non-hypercalcemic VD

analogues will also be valued and may offer therapeutic regimes of fewer side effects, preventing the unfavorable responses to increased body calcium levels.

In conclusion, this study provides the first evidence that decreased VDR expression in OSCC might be associated with tumor relapse [1, 2][1, 2]. VDR expression is found in putative CD44+ CSC compartments, which may represent an entrance point for other adjuvant treatment modality for OSCC. Chemopreventive therapy using 1,25-(OH)<sub>2</sub>D<sub>3</sub> or its analogues to target adjuvant residual tumor cells could be successful, which in turn may help in tuning the tumor supportive treatments in the clinic [1, 2]. However, further in vivo and in vitro investigations are required to support this hypothesis.

## F. Summary

Oral squamous cell carcinoma (OSCC) is the sixth common cancer worldwide. Despite the amount of research and the development of diagnosis and treatment methods, there is no significant improvement in the patient-survival rates. The treatment outcome prognostic and predictive biomarkers in OSCC still lack the clinical routine compared with other tumors in other regions of the human body, where such biomarkers have been more recognized as causative factors.

In recent studies, it is becoming more evident that most, perhaps all, malignancies are developing from the so-called cancer stem cell (CSC) compartment. Also from clinical observation, theories suggested the existence of residual stem cells after therapy that may be responsible for tumor regeneration, which demand developing new treatment modalities that can target the critical CSCs regenerating and self-renewal properties. In this manner, CD44, which is a cell-surface extracellular matrix receptor, is the most recognized and studied CSCs related marker.

Vitamin D (VD) and its metabolites, mainly 1,25-(OH)<sub>2</sub>D<sub>3</sub> are known to regulate cell growth maintenance, cellular proliferation, and differentiation induction. Accordingly, Vitamin D receptor (VDR) expression is the mediator of anticancer VD activity and its analogues in some cancers. In head and neck squamous cell carcinoma and OSCC, VDR mediates the inhibition of neoplastic activities and cellular growth.

This is the first study to assess the link between putative CSC compartment and VDR expression in CD44<sup>+</sup> tumor cells. This study was designed to analyze the expression of VDR in OSCC and normal mucosal tissue and to identify the relation between VDR expression and tumor relapse.

A retrospective analysis demonstrated VDR expression in OSCC and normal tissue samples by immunohistochemistry. Immunohistochemical and immunofluorescent double labeling experiments for VDR<sup>+</sup>, with CD44<sup>+</sup> and Ki-

67+ were carried out. VDR expression was analyzed in cells from the OSCC BICR3 and BICR56 cell lines in cytopins as positive control of VDR expression by cancer cells. VDR gene expression was measured by RT-PCR. Western blot analysis confirmed VDR antibody specificity.

This data confirmed that VDR expression is present in both cancer and non-cancer tissue samples. There was no association between VDR expression and clinicopathological characteristics although univariate analysis and multivariate analysis showed worse survival rates in OSCC patients with low VDR expression. Immunohistochemical double labeling experiments in OSCC tissue serial sections and OSCC cell lines confirmed that VDR expression is associated with CD44+ tumor cells. Our analysis of immunohistochemical double staining experiment data (CD44+/VDR+) showed increased co-expression of VDR+ with CD44+ cancer cells. Western blot expression in OSCC cell lines confirmed increased VDR expression in OSCC cell lines compared to normal tissue. In both tissue samples, VDR expression was mainly restricted to the basal cell layer. Correlation analysis from quantified immunohistochemical single staining revealed that Ki-67 expression in OSCC is inversely correlated to VDR expression.

Our finding supports the hypothesis that increased VDR expression may be associated with increased tumor-specific survival amongst patients with high VDR expression compared to patients with low VDR. From our results, we speculate that the use of 1,25-(OH)<sub>2</sub>D<sub>3</sub> or its non-hypercalcemic analogues as adjuvant chemopreventive therapy could be useful.

In conclusion, this study provides the first evidence that decreased VDR expression in OSCC might be associated with tumor relapse. However, VDR expression found in a putative CD44+ CSC compartment may represent an entrance gate for other adjuvant treatment modalities in OSCC. Chemopreventive therapy using 1,25-(OH)<sub>2</sub>D<sub>3</sub> or its analogues to target adjuvant residual tumor cells could be successful, which in turn may help enhancing tumor

supportive treatments in the clinic. However, further in vivo and in vitro investigations are required to support this hypothesis.

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## H. Zusammenfassung

Mundhöhlenkarzinome gehören zu der sechsthäufigsten Tumorentität weltweit. Trotz Fortschritten in Diagnose und therapeutischen Maßnahmen konnte die Überlebensrate von Patienten mit Mundhöhlenkarzinom in den letzten Jahrzehnten nicht signifikant gesteigert werden.

Während für andere Tumorentitäten bereits standardisierte prognostische Biomarker identifiziert wurden, fehlen diese bis heute in der klinischen Routine für das Mundhöhlenkarzinom.

Das Ziel dieser Studie war es – anhand eines „Hallmark of Cancer“ (Krebsmerkmal) – einen gewebebasierten Biomarker zu analysieren, wodurch möglicherweise langfristig eine schonendere und bessere Therapie für Patienten mit Mundhöhlenkarzinom angeboten werden kann.

Der Nachweis einer reduzierten Expression des Vitamin-D-Rezeptors (VDR) in Tumorzellen kann als Hinweis auf Apoptoseresistenz verstanden werden. Vitamin D ( $1,25(\text{OH})_2\text{D}_3$ , 1,25-Dihydroxyvitamin  $\text{D}_3$ , Calcitriol) wirkt vielfältig auf den Zellmetabolismus und ist maßgeblich an der Aufrechterhaltung und Regulation von normalem Zellwachstum, Reifung von Lymphozyten, Hemmung der Proliferation, Karzinogenese und Metastasierung beteiligt. Calcitriol, der aktive Metabolit von Vitamin D und Agonist des VDR besitzt antiproliferative, proapoptotische und Differenzierung-induzierende Attribute. Außerdem induziert Vitamin D Zell-Zyklus-Arrest und ist an der Regulation von Zellwachstum involviert, was Calcitriol und synthetische Vitamin-D-Analoga für eine Therapie gegen Tumore interessant macht. Aus diesem Grund ist die Untersuchung von VDR bei Tumoren ein wichtiger Schritt auf dem Weg zu neuen Behandlungsmethoden.

Eine Expressionsanalyse des VDR wurde beim Mundhöhlenkarzinom bislang noch nicht durchgeführt. Diese Studie befasste sich daher insbesondere erstmalig mit der Analyse des VDR auf die Prognose des Mundhöhlenkarzinoms.

Für diese Analyse wurden von 191 Patienten mit Mundhöhlenkarzinom immunhistochemische Färbungen von VDR und Ki-67 (Proliferationsmarker) durchgeführt und die Expressionsanalyse mit klinisch-pathologischen Verlaufsparemtern assoziiert. 136 Tumorproben (n = 136/191) zeigten eine erniedrigte VDR Expression, die mit deutlich reduziertem tumorfreien Überleben in Verbindung gebracht wurde. Eine erhöhte VDR Expression war mit einer besseren Prognose assoziiert. In Tumorzelllinien des Mundhöhlenkarzinoms konnte durch die Genexpressionsanalyse (mittels RT-PCR) eine erhöhte VDR Expression analysiert werden. Der VDR Antikörper konnte durch Western Blot Analyse in Proteinlysaten von Tumorzellkulturen spezifische VDR positive Banden aufzeigen. Eine immunhistochemische Doppelmarkierung demonstrierte u.a. in der basalen Epithelzellschicht eine Co-expression VDR positiver Tumorzellen mit CD44 positiven Tumorzellen. Eine Immunfluoreszenz-Doppelmarkierung zeigte die Mehrzahl proliferierender (Ki-67) Tumorzellen ohne oder mit geringer VDR Expression und bestätigte die inverse Korrelationsanalyse von Ki-67 mit VDR aus den Ergebnissen der immunhistochemischen Einfachmarkierungen.

Auf Basis der erzielten Ergebnisse aus dieser Studie kann schlussgefolgert werden, dass zwar die Expression des VDRs keinen Einfluss auf klinisch-pathologische Verlaufsparemter (Tumorgröße, Tumorstadium, Metastasierung, Grading) zu haben scheint, jedoch mit tumor-spezifischem Überleben assoziiert ist.

Die in dieser Arbeit erzielten Ergebnisse können zu einem besseren Verständnis der prognostischen Relevanz des VDRs beim Mundhöhlenkarzinom beitragen. Diese basiswissenschaftlichen Ergebnisse tragen dazu bei, in Zukunft notwendige Therapieentscheidungen zu ergänzen. Insbesondere weiterführende klinische Arbeiten zur funktionellen Rolle von Calcitriol auf die Lebensqualität und das Langzeitüberleben von Patienten mit Mundhöhlenkarzinom sind unerlässlich und Gegenstand aktueller Therapiekonzepte bei anderen Tumorentitäten (z.B. beim malignen Melanom oder Mammakarzinom).

## **I. Author's declaration**

I hereby declare that this thesis is my own work, which is performed under the supervision of Professor Dr. Dr. Siegmar Reinert and Professor Dr. Dr. Martin Grimm. I participated in the planning of this work, carried out immunohistochemistry/immunofluorescence, RT-PCR, and Western Blot studies, statistics, data analysis, and interpretation. Furthermore, I am a co-author in the earlier published article on this study: "Is 1,25-dihydroxyvitamin D3 receptor expression a potential Achilles' heel of CD44+ oral squamous cell carcinoma cells?" and this work represent the thesis expanded format of that publication which is presented to obtain the doctor degree in dentistry (Dr. med. dent).

## **J. Acknowledgement**

I would like to thank Professor Dr. Dr. S. Reinert for the motivation and for giving me the opportunity to make this research on this topic and the provision of the workplace. He always supported me and advised me, and thus enabled the success of the present work.

My special thanks go to Professor Dr. Dr. Martin Grimm, who helped me very much in the planning and execution of this work. Without the valuable discussions with him, his scientific advice, innovative ideas and his motivation this work wouldn't be possible.

I also thank the lovely medical-technical assistant Mrs. Adelheid Munz, whose technical assistant in the laboratory was always very helpful. She also was a competent companion through the time of the laboratory work.

Finally, I would like to thank my great mother Dr. Sahar Shukri for her limitless support, my beloved wife Hend Bashor for her patience and every friend who supported me during the duration of my dissertation especially my special friends Ahmad Algarny, Hamsa Abo Touk and Dr. Hazem Altaki.

## K. Abbreviations

1,25-(OH) <sub>2</sub> D <sub>3</sub>	1,25-dihydroxyvitamin D <sub>3</sub>
18F-FDG	[18F] Fluorodeoxyglucose
5-FU	5-fluorocil
ABC transporters	ATP-binding cassette transporters
AJCC	American Joint Committee on Cancer
AP	Alkaline phosphatase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	Area under the curve
CD44s	CD44 standard isoform
CD44v	CD44 variant isoform
cDNA	Complementary DNA
CSC	Cancer stem cells
CT	Computer tomography
DAB	Diaminobenzidine
DAPI	4,6-Diamidino-2-phenylindoldihydrochloride
DNA	Deoxyribonucleic acid
dpi	Dots per inch
DSS	Disease-specific survival
emT	Epithelial-mesenchymal transition
F	Female
FFPE	Formalin-fixed paraffin-embedded
FFPE	Formalin-fixed paraffin-embedded
Fig.	Figure
FITC	Fluoresceinisothiocyanate
G	Grading
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HA	Hyaluronic acid
HNSCC	Head and neck squamous cell carcinoma
HR	Hazard ratios
HRP	Horseradish peroxidise
IgG	Immunoglobulin G
IHC	Immunohistochemical
JPEG	Joint Photographic Experts Group (file extension)

M	Male
mAb	Mouse antibody
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
OSCC	Oral squamous cell carcinoma
p	P-value
pAb	Primary antibody
PET	Position emission tomography
pM	Stage of distant metastasis (according to UICC)
pN	Stage of nearby lymph node infiltration (according to UICC)
pT	Size of tumor (according to UICC)
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristics
Rr	Pearson's correlation index
RT-PCR	Reverse transcription polymerase chain reaction
UICC	Union for International Cancer Control
UK	United Kingdom
USA	United States of America
VD	Vitamin D
VDR	Vitamin D receptor
WHO	World Health Organization
Y	Years
$\chi^2$	Chi-square
T	Tau

## L. Figures

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DEMONSTRATED WITH (LEFT, SLIDE D) AND WITHOUT (RIGHT, SLIDE E) NUCLEAR COUNTERSTAINING (BLUE, DAPI). WHITE DOTTED ARROWS POINT TO AREAS OF INCREASED COEXPRESSION INDICATING THAT VDR EXPRESSING CANCER CELLS ARE SELDOM STRONGLY ASSOCIATED WITH PROLIFERATING CELLS BUT MAINLY DETECTED WITHOUT OR WITH WEAK KI-67 EXPRESSION (WHITE ARROWS). CO-LOCALIZATION PARAMETER (MERGE, SLIDES D, E) OF KI-67/VDR (RED AND GREEN) MEASURED BY PEARSON'S CORRELATION (Rr) INDEX IS ANALYZED AT 0.08. FITC, FLUORESCENISOTHIOCYANATE, CY3, CYANINE 3 RED, AND DAPI 4',6-DIAMIDINO-2 PHENYLINDOLDIHYDROCHLORIDE [1, 2, 100]..... 27

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## M. Tables

**Table 1:** Clinicopathological characteristics and prognostic factors of 191 patients with OSCC of low and high VDR expressers. Cutoff value for VDR expression was determined by Receiver Operating Characteristics (ROC) analysis [1, 2].

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