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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Vitamin D receptor gene expression in HPV-negative oral carcinomas – limited prognostic value within a complex molecular network

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SUMMARY

Introduction/Objective The vitamin D receptor (VDR) regulates cell proliferation, differentiation, apoptosis, and immune responses and has been proposed as a potential cancer biomarker. This study investigated VDR gene expression in oral squamous cell carcinoma (OSCC), an aggressive malignancy with unfavorable outcomes, particularly in HPV-negative tumors.

Methods VDR mRNA expression was analyzed by reverse transcription quantitative PCR in tumor samples from 109 HPV-negative OSCC patients, and relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Associations with clinicopathological variables were assessed using χ^2 and Fisher's exact tests, while survival was evaluated by Kaplan–Meier and Cox regression analyses.

Results VDR expression was not significantly associated with clinicopathological characteristics. Although patients with low VDR expression showed longer overall survival than those with high expression, the difference was not significant ($p = 0.253$). Borderline associations were observed in early survival, with the 24-month landmark analysis ($p = 0.050$) and among patients with recurrent tumors ($p = 0.051$). VDR expression was not an independent prognostic factor.

Conclusion VDR gene expression alone has limited prognostic value in HPV-negative OSCC. However, the observed borderline associations with short-term survival and among patients with recurrent tumors suggest a potential context-dependent role in disease progression that warrants further investigation.

Keywords: oral carcinoma; vitamin D receptor; VDR; gene expression

INTRODUCTION

Oral squamous cell carcinoma (OSCC) represents the most common malignancy of the oral cavity and a significant cause of morbidity and mortality worldwide [1]. Despite advances in diagnostics and the implementation of multimodal therapeutic approaches, including surgery, radiotherapy, chemotherapy, and more recently immunotherapy [2], the prognosis remains unfavorable, with five-year overall survival rates of approximately 50–60%, largely due to frequent disease recurrence [1, 3]. Furthermore, the rising incidence among younger populations [4] and in regions of Southeastern Europe, including Serbia [5], highlights the growing public health significance of this malignancy.

The pathogenesis of OSCC is complex and multifactorial, involving an interplay of genetic and epigenetic alterations that disrupt the regulation of the cell cycle, differentiation, and apoptosis [2]. In addition to well-known etiological factors, such as smoking and alcohol consumption, there is growing interest in the influence of infections with human papillomavirus (HPV), which has led to the introduction of a distinct staging system and classification of oropharyngeal squamous cell carcinoma (OPSCC) and OSCC according to HPV status [6].

While the prognostic significance of HPV status is well established in OPSCC, in which HPV-positive tumors are associated with improved therapeutic response and better clinical outcomes, its role in OSCC remains insufficiently defined [6]. HPV-negative OSCC, which accounts for the majority of cases, is characterized by greater genomic instability, more aggressive clinical behavior, and poorer prognosis than HPV-positive tumors [7, 8]. These differences suggest distinct molecular and immunological pathways involved in tumor progression, including variations in antitumor immunity and tumor–host interactions. They also highlight the need for novel biomarkers to improve prognostic assessment and therapeutic decision-making [9, 10].

The vitamin D receptor (VDR) is a nuclear receptor that regulates the expression of a large number of genes involved in the control of cellular proliferation, differentiation, and apoptosis [11, 12]. Beyond its established role in calcium homeostasis, the active form of vitamin D (calcitriol), through VDR signaling, exerts pleiotropic effects, including modulation of

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immune responses, inflammatory processes, and tumor-related signaling pathways [13]. VDR signaling has been increasingly associated with modulation of innate and adaptive immune responses, regulation of inflammatory cytokine production, and maintenance of immune homeostasis within the tumor microenvironment, suggesting a possible role in immune-mediated processes and antitumor immunity [14].

VDR expression is regulated by genetic, epigenetic, hormonal, and environmental factors [11, 12], while calcitriol may induce its own expression through autoregulatory mechanisms [15].

Previous studies have demonstrated variable VDR expression in normal, premalignant, and malignant oral epithelium [16]. VDR showed a tendency toward increased expression in oral precancerous lesions compared to normal mucosa, whereas a relative decrease was observed in OSCC [17]. This pattern reflects dynamic changes in VDR signaling during disease progression [18, 19]. Furthermore, some studies suggest that VDR expression decreases with advancing tumor stage, implying its potential involvement in tumor progression and disease aggressiveness.

However, findings regarding VDR expression in oral cancer remain inconsistent, with some authors reporting increased VDR expression in malignant and premalignant lesions, while others describe reduced expression in tumor tissues. These discrepancies may reflect tumor heterogeneity, methodological differences, and variations in local vitamin D metabolism and VDR signaling within the tumor microenvironment [20]. Activation of oncogenic pathways, such as Ras signaling, and alterations in vitamin D metabolism, including increased CYP24-mediated degradation, may modulate VDR activity and its biological effects within the tumor microenvironment [21, 22].

Despite growing interest, the clinical and biological significance of VDR gene expression in HPV-negative OSCC has not yet been fully elucidated. Given the immunomodulatory and antiproliferative properties of vitamin D signaling, further investigation of VDR may contribute to a better understanding of the molecular mechanisms underlying disease progression and facilitate the identification of novel prognostic biomarkers and potential therapeutic targets. Clarification of the role of VDR signaling may also improve patient stratification and support the development of more personalized treatment approaches [9, 10, 18–20, 23].

The aim of this study was to evaluate whether VDR gene expression is associated with clinicopathological features and overall survival in patients with HPV-negative OSCC and to assess its potential prognostic value. We hypothesized that VDR expression may influence disease progression and clinical outcome through interactions within the tumor microenvironment.

METHODS

This study included 109 patients with OSCC treated surgically at the Clinic for Maxillofacial Surgery, Military Medical Academy, Belgrade. Clinical, histopathological, and follow-up data were collected from medical records. All surviving patients had a minimum follow-up period of two years. None received preoperative chemotherapy, while all underwent postoperative radiotherapy.

Tumor specimens were collected during surgery and stored at -20°C. Genomic DNA and total RNA were isolated using TRI Reagent according to the manufacturer's protocol. DNA quality was assessed by agarose gel electrophoresis, while nucleic acid concentration and purity were measured spectrophotometrically.

HPV status was determined using the HPV High Risk Screen Real-TM Quant kit targeting high- and intermediate-risk HPV types. All tumors were HPV-negative.

VDR mRNA expression was analyzed by reverse transcription quantitative PCR (RT-qPCR). Total RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit. Amplification was performed using TaqMan assays on a 7500 Real-Time PCR System. GAPDH was selected as the reference gene following stability assessment with BestKeeper and NormFinder software. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ and Pfaffl methods. All analyses were performed in triplicate.

Statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA) and JMP v17 (JMP Statistical Discovery LLC, Cary, NC, USA). Associations between categorical variables were assessed using χ^2 or Fisher's exact tests. Optimal expression cutoff values were determined using Cutoff Finder [24], enabling classification into low- and high-expression groups. Survival was analyzed using Kaplan–Meier curves and log-rank tests, while hazard ratios (HRs) were calculated using Cox proportional hazards models. Statistical significance was defined as $p < 0.05$.

RESULTS

The study population consisted predominantly of males (74%), with 61% of patients younger than 58 years. Smoking was reported by 75% of participants, whereas most reported low alcohol consumption (71%). Advanced-stage tumors (stage III–IV) accounted for 71% of cases, while nodal metastases and disease recurrence were observed in 64% and 58% of patients, respectively (Table 1).

No significant association was found between VDR expression and demographic variables. Although high VDR expression was more frequent among males, the difference was not statistically significant ($p = 0.191$). Similarly, age, smoking status, and alcohol consumption showed no significant relationship with VDR expression.

Analysis of histopathological characteristics revealed no significant association between VDR expression and histological grade or nuclear grade. A non-significant tendency toward higher expression in well-differentiated tumors was observed. Likewise, no statistically significant relationship was found between VDR expression and tumor stage, tumor size, nodal status, or recurrence (Table 1).

Table 1. Association of the vitamin D receptor (VDR) gene expression with demographic and clinicopathological features of oral squamous cell carcinoma patients' tumor samples

Variable	Total N = 109 (%)		VDR expression	
			Low	High
Sex	Male	81 (74)	34	47
	Female	28 (26)	16	12
	p		0.191	
Age (≥ 58)	<	67 (61)	31	36
	>	42 (39)	19	23
	p		1.000	
Smoking	Never	27 (25)	11	16
	Ever	82 (75)	39	43
	p		0.657	
Alcohol	Low	77 (71)	37	40
	High	32 (29)	13	19
	p		0.531	
Histological grade	H1	70 (64)	29	41
	H2/3	39 (36)	21	18
	p		0.234	
Nuclear grade	N1	59 (54)	24	35
	N2/3	50 (46)	26	24
	p		0.254	
Tumor stage	S I	5 (5)	4	1
	S II	26 (24)	10	16
	S III	59 (54)	29	30
	S IV	19 (17)	7	12
	p		0.284	
Tumor stage 1/2 *vs.* 3/4	1/2	31 (28)	14	17
	3/4	78 (72)	36	42
	p		1.000	
Tumor size	T1	46 (42)	20	26
	T2	25 (23)	14	11
	T3	28 (26)	13	15
	T4	10 (9)	3	7
	p		0.540	
Tumor size 1/2 *vs.* 3/4	1/2	71 (65)	34	37
	3/4	38 (35)	16	22
	p		0.687	
Nodal metastases	N-	39 (36)	17	22
	N+	70 (64)	33	37
	p		0.841	
Recurrence	No	46 (42)	22	24
	Yes	63 (58)	28	35
	p		0.846	

Table 2. Hazard risk analysis of prognostic factors in relation to overall survival (OS) according to Cox regression analysis in oral squamous cell carcinoma patients

Cox regression analysis	Variables	OS	
		HR [95% CI]	p
Univariate analysis	Sex	0.615 (0.325–1.162)	0.134
	Age (≥ median)	0.638 (0.365–1.116)	0.115
	Smoking	1.785 (0.902–3.532)	0.096
	Alcohol	1.447 (1.115–1.878)	**0.005**
	Histological grade 1 *vs.* 2/3	1.232 (0.947–1.603)	0.121
	Nuclear grade 1 *vs.* 2/3	1.210 (0.933–1.569)	0.152
	Tumor stage 1/2 *vs.* 3/4	1.581 (1.214–2.058)	**0.001**
	Tumor size 1/2 *vs.* 3/4	1.281 (1.078–1.524)	**0.005**
	Nodal metastases	3.009 (1.559–5.807)	**0.001**
	Recurrence status	11.436 (5.226–25.026)	**< 0.0001**
Multivariate analysis	Recurrence status	11.436 (5.26–25.026)	**< 0.0001**

Univariate Cox analysis identified alcohol consumption, advanced tumor stage, larger tumor size, nodal metastases, and recurrence as factors associated with poorer overall survival. Recurrence demonstrated the strongest effect on mortality risk (HR = 11.436, p = 0.0001). In multivariate analysis, recurrence remained the only independent prognostic factor (Table 2).

Patients with low VDR expression exhibited longer mean and median overall survival (58.6 and 42 months, respectively) than those with high expression (46.5 and 29 months). However, the difference was not statistically significant (p = 0.253) (Figure 1a). Consistent findings were obtained in Cox regression analyses, where VDR expression failed to emerge as an independent predictor of survival.

When survival was evaluated using a 24-month landmark analysis, the difference reached borderline statistical significance (p = 0.050) (Figure 1b). Stratification according to tumor stage or nodal status revealed no significant survival differences between expression groups. However, among patients with recurrent disease, elevated VDR expression showed a borderline association with poorer survival (p = 0.051), whereas no such relationship was observed in patients without recurrence (Figure 2).

Overall, VDR expression showed no significant association with clinicopathological characteristics or long-term survival. Nevertheless, the borderline findings observed in landmark and recurrence-stratified analyses suggest a potential role in early disease dynamics.

DISCUSSION

The present study demonstrated that VDR gene expression is not significantly associated with key clinicopathological parameters or overall survival in patients with HPV-negative OSCC. Although patients with higher VDR expression showed a trend toward shorter survival, the difference did not reach statistical significance, suggesting that VDR expression alone has limited prognostic value in this cohort. However, borderline significance observed in the 24-month cutoff analysis and among patients with recurrent disease suggests a time-dependent prognostic effect of VDR expression in OSCC, where VDR-related mechanisms may influence early disease dynamics rather than long-term survival and clinical outcomes. Specifically, VDR expression may influence early disease progression, potentially affecting initial tumor behavior and antitumor immune responses, treatment sensitivity, or early

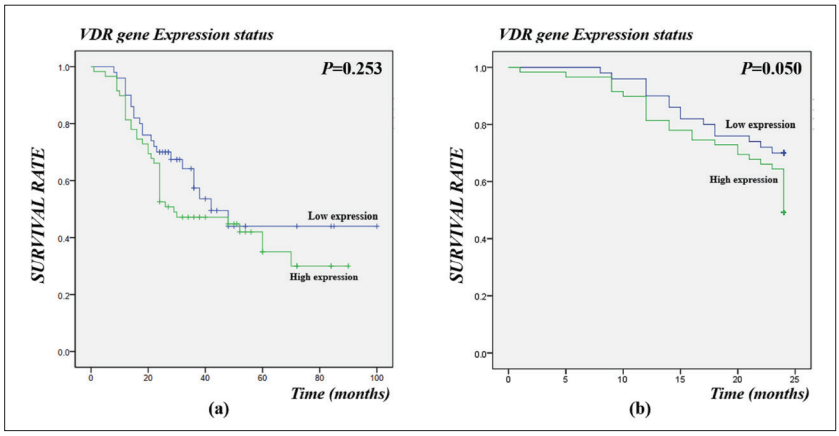


Figure 1. Kaplan–Meier survival curves with log-rank test according to dichotomized vitamin D receptor (VDR) gene expression (high *vs*. low) in the HPV-negative oral squamous cell carcinoma cohort: (a) overall survival; (b) two-year overall survival

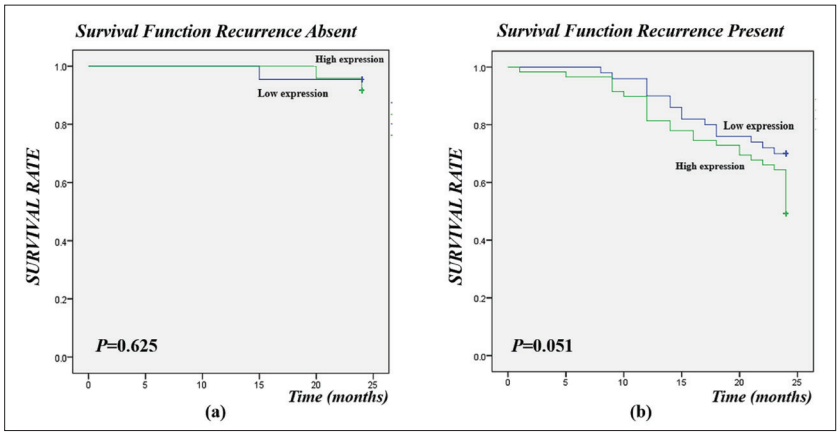


Figure 2. Kaplan–Meier survival curves according to dichotomized vitamin D receptor (VDR) gene expression (high *vs*. low) stratified by recurrence status in the HPV-negative OSCC cohort: (a) patients without recurrence; (b) patients with recurrence

recurrence dynamics. Although no significant survival differences were observed after stratification by tumor stage or nodal status, elevated VDR expression showed a borderline association with poorer survival in patients with recurrent disease, but not in those without recurrence. These findings suggest that VDR-related mechanisms may be more relevant in biologically aggressive or therapy-resistant tumors than in relation to anatomical disease extent, highlighting the complex and context-dependent role of VDR signaling in tumor biology [9, 18, 19, 20].

Biologically, this observation is plausible given the multifunctional role of the VDR in cancer biology. VDR signaling has been associated with antiproliferative, prodifferentiation, anti-inflammatory, and immunomodulatory effects in multiple malignancies, including head and neck cancers [25]. Experimental studies suggest that activation of the vitamin D_3/VDR axis may inhibit tumor cell proliferation, reduce epithelial–mesenchymal transition, modulate cytokine production, and influence antitumor immune responses [14]. Nevertheless, the consequences of VDR signaling appear to depend on tumor context, disease stage, and interactions within the tumor microenvironment. Although vitamin D/VDR signaling is generally considered protective, higher VDR expression in the present cohort was associated with a trend toward poorer survival during the earlier follow-up period. Similarly, several studies have suggested that elevated VDR expression in certain tumor settings may reflect compensatory, dysregulated, or non-functional signaling rather than effective antitumor activity. Paradoxically, in specific tumor contexts, VDR may promote pro-oncogenic phenotypes by creating immunosuppressive microenvironments or sustaining oxidative stress [14]. Therefore, elevated VDR expression may not necessarily reflect effective antitumor signaling, but could instead represent a compensatory or dysfunctional response within biologically aggressive tumors. The apparent short-term effect of VDR expression may diminish over time as clinical outcomes become increasingly influenced by stronger determinants of tumor progression, including recurrence, genetic instability, therapy resistance, and other molecular alterations. Alternatively, VDR-related effects may be restricted to specific patient subgroups or disease stages and may have been difficult to detect because of limited statistical power.

Another possible explanation is that VDR expression may be more relevant during the early stages of disease progression, reflecting tumor differentiation or initial biological behavior. As the disease advances, stronger drivers of tumor progression may predominate, diminishing the long-term prognostic impact of VDR expression. This may explain why the borderline association observed in the 24-month landmark analysis was not maintained throughout follow-up. The stronger association observed in patients with recurrent disease further suggests a potential role for VDR-related pathways in recurrence biology or treatment resistance. A critical aspect in interpreting these results is the distinction between quantitative gene expression and functional receptor activity. Importantly, VDR mRNA expression does not necessarily reflect functional receptor activity, which may be influenced by post-transcriptional regulation, altered receptor function, impaired interactions with co-regulatory proteins, such as retinoid X receptor, insufficient availability of its ligand, calcitriol, or dysregulated vitamin D metabolism [11, 12]. Consequently, increased VDR expression may represent compensatory or dysfunctional signaling rather than an indicator of effective tumor suppression [26]. This phenomenon, often referred to as "non-functional expression," may partly explain the absence of an expected correlation between VDR levels and clinical outcomes [20]. Further studies incorporating functional analyses and larger

cohorts are needed to clarify the biological and clinical significance of VDR signaling in OSCC.

Furthermore, the role of VDR in OSCC appears to be dynamic and stage-dependent. Previous studies [16, 17] have demonstrated a characteristic pattern in which VDR expression is increased in premalignant lesions and subsequently reduced in invasive carcinomas. This observation can be interpreted within the framework of clonal selection, whereby tumor cells that become less dependent on vitamin D-mediated growth regulation gain a selective advantage during disease progression. Given that the majority of tumors in the present cohort were at advanced stages (III–IV), it is plausible that VDR signaling was already functionally diminished, thereby limiting its impact on tumor behavior and patient prognosis. In such settings, VDR may no longer act as a regulator of proliferation and differentiation but instead reflect a residual or epiphenomenal feature of tumor evolution [20].

Another important consideration is that VDR operates within a broader vitamin D signaling axis, which includes key metabolic enzymes such as CYP27B1 and CYP24A1. While CYP27B1 is responsible for the activation of vitamin D, CYP24A1 mediates its degradation [27]. Overexpression of CYP24A1, frequently reported in aggressive tumors, may lead to reduced local concentrations of active vitamin D, effectively attenuating VDR signaling regardless of receptor expression levels [21]. This suggests that evaluating VDR expression alone may be insufficient and that a more comprehensive assessment of the vitamin D pathway, including key metabolic enzymes, may be necessary to fully capture the biological significance of vitamin D signaling in OSCC.

The tumor microenvironment represents an additional layer of regulation that may significantly influence VDR expression and function. Inflammatory cytokines, oxidative stress, and immune cell infiltration are known to modulate both VDR expression and vitamin D metabolism, while VDR signaling itself regulates cytokine production and immune responses. Notably, tumor and immune cells can locally synthesize or degrade vitamin D metabolites, establishing autocrine and paracrine signaling loops independent of systemic vitamin D levels [28]. This localized regulation may help explain the lack of correlation between VDR expression and clinicopathological parameters observed in this study, as key regulatory processes occur within the tumor microenvironment and may not be captured by measurements of gene expression in bulk tumor cells [20, 28]. Given the known immunomodulatory effects of vitamin D signaling, it is also plausible that VDR activity influences immune dynamics within the tumor microenvironment in a manner not detectable through isolated mRNA quantification alone.

Epigenetic mechanisms further contribute to the complexity of VDR regulation. Promoter methylation, histone modifications, and microRNA-mediated repression can all influence VDR expression and activity. These processes may result in discordance between mRNA levels and functional protein activity, thereby limiting the interpretability of transcription-based analyses [29]. In addition, alterations in chromatin accessibility may impair VDR binding to vitamin D response elements, further reducing its transcriptional efficacy [12]. Consequently, the lack of prognostic significance observed in this study may reflect underlying epigenetic dysregulation rather than the absence of biological relevance [26, 29]. Such mechanisms may be particularly relevant in HPV-negative OSCC, which is characterized by substantial molecular heterogeneity and genomic instability.

The exclusive inclusion of HPV-negative tumors in this study represents both a strength and a limitation. HPV-negative OSCC is characterized by greater genomic instability, a higher mutational burden, and frequent activation of oncogenic pathways such as Ras and PI3K/Akt [8]. Importantly, activation of these pathways has been shown to interfere with VDR-mediated transcriptional responses, thereby diminishing the biological effects of vitamin D signaling [7, 22]. This may provide an additional explanation for the lack of association between VDR expression and clinical outcomes in the present cohort, as dominant oncogenic drivers may override the regulatory influence of VDR. At the same time, restriction to HPV-negative tumors reduced biological heterogeneity associated with viral oncogenesis and enabled a more homogeneous evaluation of VDR expression within this clinically aggressive OSCC subgroup.

Despite the absence of significant findings, the present study provides important insights into the limitations of single-marker approaches in cancer prognostication. Future studies should therefore focus on integrative models incorporating multiple biomarkers, including CYP24A1, CYP27B1, and immune checkpoint molecules such as PD-1/PD-L1, in order to better capture the biological heterogeneity of OSCC [30]. The present findings also support the need for time-dependent survival analyses and functional studies evaluating not only receptor expression but also downstream signaling activity and tumor-specific vitamin D metabolism.

Several limitations of this study should be acknowledged. The sample size, although adequate for exploratory analysis, may limit the statistical power to detect subtle associations. Additionally, the analysis was restricted to mRNA expression levels without assessment of protein expression or receptor functionality. The lack of data on serum vitamin D levels and the absence of evaluation of key metabolic enzymes further constrain the interpretation of the results. Moreover, the number of survival events and the predominance of advanced-stage tumors may have influenced the ability to detect weaker prognostic effects. Future research should adopt a multidimensional approach combining transcriptomic, proteomic, and epigenetic analyses, as well as longitudinal monitoring of molecular changes during disease progression.

CONCLUSION

While VDR gene expression does not appear to have independent prognostic value in HPV-negative OSCC when assessed at the mRNA level, its role in tumor biology remains significant within a broader regulatory framework. The

findings underscore the complexity of vitamin D signaling in cancer and highlight the need for integrative approaches to fully elucidate its clinical relevance. Although the overall survival analysis did not demonstrate statistical significance, the observed borderline significance in the analysis at the 24-month time point suggests that VDR-related mechanisms may still contribute to early disease dynamics in OSCC. A deeper understanding of these mechanisms may ultimately contribute to the development of personalized therapeutic strategies and the identification of novel molecular targets in the management of oral cancer.

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Експресија гена за рецептор витамина *D* код *HPV*-негативних оралних карцинома – ограничени прогностички значај у оквиру сложене молекуларне мреже

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САЖЕТАК

Увод/Циљ Рецептор витамина *D* (*VDR*) учествује у регулацији ћелијске пролиферације, диференцијације, апоптозе и имунског одговора и представља потенцијални биомаркер у онкологији. Циљ ове студије био је да се испита експресија гена *VDR* у оралном сквамоцелуларном карциному (*OSCC*), агресивном малигнитету са неповољним исходом, посебно код *HPV*-негативних тумора.

Методе Експресија *VDR mRNA* анализирана је методом *RT-qPCR* у туморским узорцима 109 болесника са *HPV*-негативним *OSCC*, а релативна експресија израчуната је применом методе $2^{-\Delta\Delta Ct}$. Повезаност са клиничкопатолошким параметрима процењена је χ^2 тестом и Фишеровим егзактним тестом, док је преживљавање анализирано Каплан–Мајеровом методом и Коксовом регресионом анализом.

Резултати Није утврђена статистички значајна повезаност експресије *VDR* са клиничкопатолошким карактеристикама. Иако су болесници са ниском експресијом *VDR* имали дуже укупно преживљавање у односу на болеснике са високом експресијом, разлика није достигла статистичку значајност ($p = 0,253$). Граничне статистичке значајности уочене су у анализи раног преживљавања са пресеком на 24 месеца ($p = 0,050$), као и код болесника са рецидивом тумора ($p = 0,051$). Експресија *VDR* није представљала независан прогностички фактор.

Закључак Експресија гена *VDR* у ткиву оралних карцинома сама по себи показала је ограничен прогностички значај код *HPV*-негативних *OSCC*. Ипак, уочене граничне вредности статистичке значајности у анализи раног преживљавања и преживљавања болесника са рецидивом тумора указују на могућу улогу *VDR* у прогресији болести која зависи од контекста, што захтева даља истраживања.

Кључне речи: орални карцином; рецептор витамина *D*; експресија гена