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Image Segmentation-Based Analysis Reveals Correlation between IL-1RA Overexpression and Worse Survival Outcomes in Oral Squamous Cell Carcinoma

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Abstract

Background: The interleukin-1 (IL-1) signaling pathway plays a crucial role in mediating inflammatory responses by activating immune cells, with implications for various pathologies. In oral squamous cell carcinoma (OSCC), the role of IL-1 receptor antagonist (IL-1RA) and its isoforms remains poorly understood, with evidence suggesting context-dependent variations in their function. **Aim:** This study aimed to evaluate the immunoexpression of IL-1 signaling pathway in OSCC tissues and correlate these findings with clinicopathological prognostic factors. **Methods:** Clinicopathological data, including patient age, sex, tumor size, lymph node involvement, histological grade, and tumor stage, were evaluated. Hematoxylin and eosin-stained sections were reviewed for differentiation grade, tumor budding, perineural invasion, and the presence of adequate tumor tissue in the invasive front. Non-dysplastic adjacent epithelium (NDAE) from the same samples served as the control. Immunohistochemical analysis was performed against IL-1α, IL-1β, IL-1R1 and IL-1RA; staining was evaluated using the total tissue expression of each marker obtained with an ImageJ segmentation plugin. **Results:** This study analyzed 44 OSCC cases, with most tumors in advanced stages (63.6%) and a five-year overall survival (OS) rate of 54.4%. Immunohistochemical analysis showed significant overexpression of IL-1α, IL-1β, IL-1R1, and IL-1RA in OSCC compared to NDAE (p<0.0001). High IL-1RA expression was associated with tumor recurrence (p=0.030) and poorer OS (p = 0.018) and disease-specific survival (DSS) (p=0.028). Multivariate analysis identified IL-1RA overexpression (p=0.007) and lymph node metastasis (p=0.002) as independent risk factors for poor OS. **Conclusion:** High IL-1RA expression is associated with features of OSCC progression and adverse clinical outcomes. Further studies are needed to elucidate the mechanistic roles of IL-1RA and other IL-1 family members in OSCC.

1. Introduction

Oral cancer represents a major global health burden, ranking among the most prevalent malignancies and leading contributors to cancer-related mortality worldwide. Oral squamous cell carcinoma (OSCC) accounts for over 90% of oral cancer cases, underscoring its clinical significance within this category (1). According to recent data from the Globocan database, lip and oral cavity cancers collectively constituted the 16th most frequently diagnosed malignancies in 2022, with an estimated 389,485 new cases and 188,230 associated deaths reported globally (2). Key etiological factors include tobacco use, alcohol consumption, and betel quid chewing, particularly in South Asia (3). These epidemiological insights emphasize the critical need for advancements in preventive strategies, early detection protocols, and the development of targeted therapeutic interventions.

Neoplastic cells develop functional capabilities that enable their survival, proliferation, and dissemination. These traits are acquired through distinct mechanisms that differ across tumor types and arise at various stages of multistep tumorigenesis. The acquisition of these capabilities is often facilitated by enabling conditions, including a chronic inflammatory state associated with premalignant and malignant lesions via several mechanisms, including oncogenic mutation induction (4, 5). Inflammation, predominantly mediated by cells of the innate and adaptive immune system, plays a critical role in tumor progression by contributing to hallmark cancer traits (6). It enriches the tumor microenvironment (TME) with bioactive molecules, such as proinflammatory cytokines, which can drive inflammatory responses that ultimately promote carcinogenesis (4, 7, 8). In this context, an increasing body of evidence suggests a link between head and neck squamous cell carcinoma (HNSCC) and chronic inflammation (9, 10).

The interleukin-1 (IL-1) signaling pathway is essential in mediating inflammatory responses through the activation of immune cells (11). This pathway is initiated when IL-1 family members, such as IL-1α and IL-1β, bind to the IL-1 agonist receptor (IL-1R1), triggering a cascade of intracellular signaling events. IL-1 receptor antagonist (IL-1RA) is a critical negative regulator of this pathway. Four variants of IL-1RA are recognized, one secreted (sIL-1RA) and three intracellular (icIL-1RA1-3)

(12). icIL-1RA1 is the main intracellular isoform and is mainly expressed in keratinocytes and other epithelial cells, monocytes, tissue macrophages, fibroblasts and endothelial cells, whereas sIL-1RA is mainly produced by monocytes and neutrophils (13). Despite of the isoform, IL-1RA binds to IL-1R1 with high affinity but does not initiate the signaling cascade, thus acting as a competitive inhibitor of IL-1α and IL-1β (14, 15). IL-1α and IL-1β are consistently expressed in OSCC and have been implicated in critical roles in OSCC carcinogenesis and tumor progression (13, 16, 17). Regarding its antagonist form, reduced levels of IL-1RA, and specifically of icIL-1RA1, have been observed in several cancers, including OSCC (18, 19). However, other studies have reported elevated IL-1RA levels in specific cancer types, suggesting that its role may differ depending on the cancer context (20-22). This variability highlights the complex, context-dependent function of IL-1RA in tumor biology.

As discussed, there appears to be a relationship between inflammatory signaling pathways and the development of certain types of cancer. A limited number of studies have explored the connection between IL-1 signaling pathway and OSCC, yielding intriguing yet controversial findings, with insufficient data on its association with patient prognosis. Therefore, the present study aims to evaluate the immunoexpression of IL-1α, IL-1β, IL-1R1, and IL-1RA in OSCC and correlate these findings with prognostic factors.

2. Material and methods

Sample selection and clinicopathological data collection

This study was conducted using formalin-fixed paraffin-embedded (FFPE) tissue samples from the primary tumor site of patients diagnosed with oral squamous cell carcinoma (OSCC). The specimens were obtained from the archives of the Pathological Anatomy Unit of Hospital Carlos Van Buren, Valparaíso, Chile. Inclusion criteria were surgical resections specimens of primary untreated tumors. Exclusion criteria were cases without a complete clinical history or with insufficient tissue on the FFPE. Clinicopathological data, including patient sex, age at diagnosis,

history of alcohol and/or tobacco consumption, tumor location, TNM classification according to the American Joint Committee on Cancer (AJCC) 8th edition (23), locoregional recurrence, date of recurrence and, date and cause of death, were collected retrospectively from patient medical records to assess potential correlations with immunohistochemical markers. Non-dysplastic adjacent epithelium (NDAE) of each same sample, when available, was used as control (24, 25). All procedures were conducted in accordance with relevant guidelines and regulations and adhered to the ethical principles outlined in the Declaration of Helsinki. The requirement for informed consent was waived by the Bioethics Committee of the Valparaíso–San Antonio Health Service (Approval No. 54/2022) because many patients were deceased and close relatives could not be contacted. The study also received approval from the Bioethics Committee of Universidad Andrés Bello (Approval No. 032/2022).

Histomorphological analysis

All histological slides were reviewed by a specialist in oral and maxillofacial pathology (RM-F) and cases were categorized according to the degree of tumor differentiation, worst pattern of invasion (WPOI), tumor budding (TB), status of margins and the presence of perineural invasion (PNI). The differentiation grades considered were well, moderately, or poorly differentiated (26). TB was defined as the presence of single cancer cells or cluster of less than five cancer cells in the invasion front (27). Margin status was categorized as positive or negative depending on the presence or absence of cancerous cells at the resection margins, respectively (26). PNI evaluation was complemented with immunohistochemistry against AE1/AE3 and S100 proteins. PNI was considered positive when cancerous cells were observed within any of the three layers of the nerve sheath and/or were close to the nerve surroundings in more than a third of its circumference (28). All specimens stained with Hematoxylin and Eosin were revised for differentiation grade, tumor budding, perineural invasion, and identification of enough tumor tissue in the front of invasion.

Immunohistochemistry

For the immunohistochemical reactions, 3-μm sections were deparaffinized in xylene and rehydrated through graded ethanol. Antigen retrieval was achieved by heating the sections in a buffer (Reveal Decloaker, RTU; Biocare Medical) at 95ºC for 15 minutes to expose the antigenic epitopes. Endogenous peroxidase activity was blocked with 0.9% hydrogen peroxide for 5 min each. The tissue samples were incubated with rabbit polyclonal antibodies specific for IL-1α (Biorbyt, UK; catalog no. orb308737, diluted 1:200), IL-1β (Biorbyt, UK; catalog no. orb382131, diluted 1:200), IL-1R1 (Biorbyt, UK; catalog no. orb499639, diluted 1:200), and IL-1RA (Biorbyt, UK; catalog no. orb3662, diluted 1:100) for 60 min and then incubated with a biotinylated anti-mouse/anti-rabbit antibody and a streptavidin-horseradish peroxidase complex for 40 min each (mouse/rabbit ImmunoDetector Biotin Link and HRP Label; Bio SB). For the negative control samples, the primary antibody was omitted, and for positive controls, testicle, tonsil, and liver tissues were used. The reaction was visualized using the 3,3'- diaminobenzidine-H_2O_2 substrate (Biocare Medical), and the sections were counterstained with Harris hematoxylin.

Evaluation of immunohistochemical staining

All slides were digitized using a digital scanner (Motic Asia, MoticEasyScan One, Hong Kong) with a x40 objective in standard mode and analyzed digitally with Pathomation software (Pathomation BV, Belgium, PMA.start). Two regions of interest (ROI) were defined: the invasion front and the superficial area of each tumor. The superficial area was defined as the tumor region immediately beneath the epithelium, and the invasion front as the deepest are of the tumor. There was no standardized proportion of tumor tissue across patients. For each ROI, 3 representative images of different fields per OSCC case were taken. Each RGB image corresponded to a field of view of 475x253 μm (x20 magnification). After capturing all sections, a stack was created, where each image represented a different sample. ImageJ plugin Color Segmentation was used to quantify staining intensities across each sample analyzing immunohistochemical results (29). Eight clusters of color were determined manually by the operator, where four clusters were

determined to represent IHC stain, two clusters for artifacts and two for background. The expression of each cluster was represented as percentage (Figure 1). The regions containing the IHC product were segmented by color pixels clustering using K-means algorithm. Total tissue expression (TTE) of a marker was defined as the addition of all clusters together (B+C+D+E) per field and was expressed as a proportion (total percentage of brown in the area) and included both tumor epithelial cells and tumor-associated inflammatory and stromal cells. This was calculated for both NDAE and OSCC. To assess if the expression of the IL-1 signaling pathway changed within the tumor, TTE was independently analyzed in the superficial and invasion front of the tumors and comparisons were made. As all markers were expressed at some levels by all tumors, we also divided the tumors into two categories: high expression and low expression (this was done considering only the expression of the markers at the invasion front). Clusters B, C and D were grouped together and considered as "high intensity" (as represented the darkest shades of brown), while cluster E was considered as "low intensity" (as was the lightest shade of brown). When the expression of B+C+D in a tumor was $\geq$ 30%, that tumor was considered to have high expression, and when B+C+D was < 30%, that tumor was considered to have low expression. The cutoff value of $\geq$ 30% was chosen as this threshold resulted in a relatively balanced distribution of the samples per group.
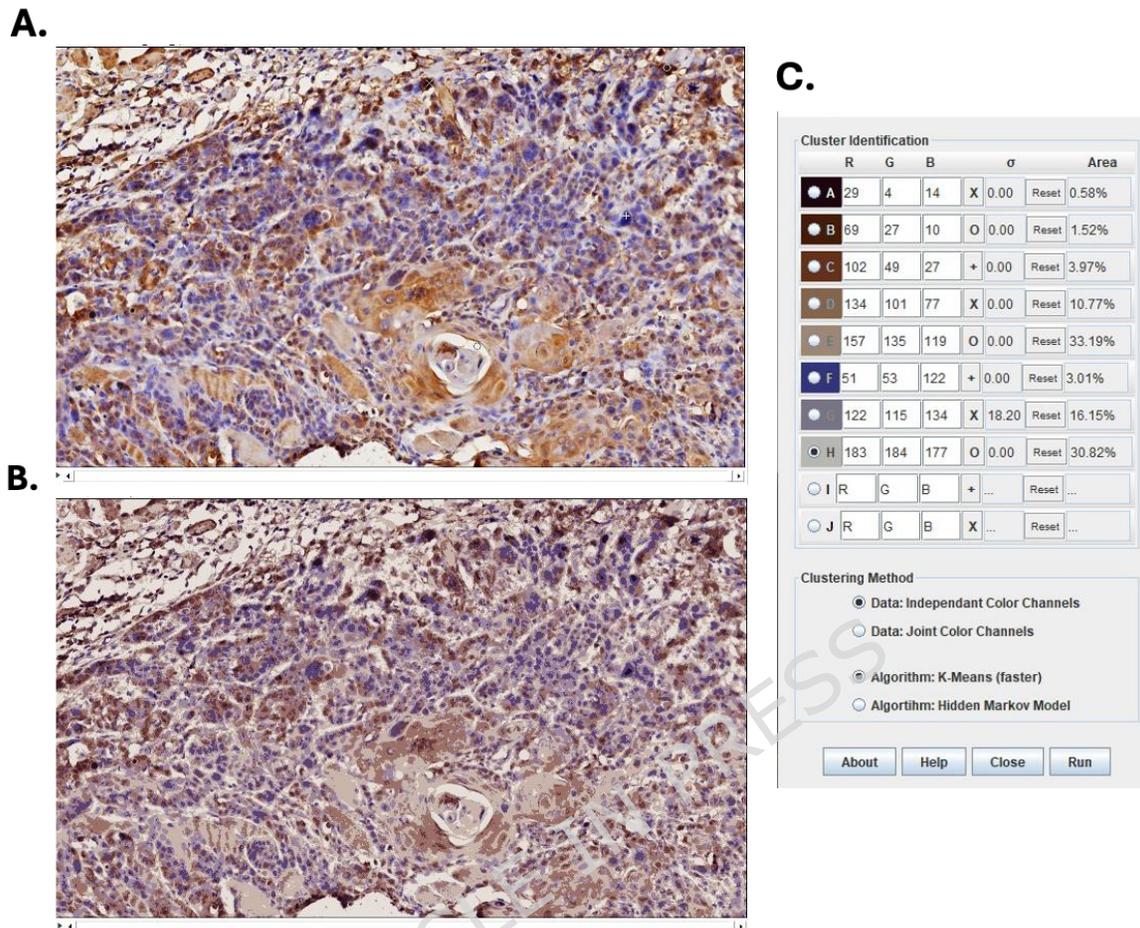
A.

B.

C.

Figure 1. A) Immunohistochemical expression of a member of the IL-1 signaling pathway (IL-1α) at the invasion front in oral squamous cell carcinoma. B) Result of the color segmentation applied to the image in (A). C) Output of the Color Segmentation plugin showing eight distinct channels and the corresponding percentage contribution of each channel.

Statistical Analysis

The data were summarized using crosstabs. Association between variables was performed using the chi-square test or Fisher's exact test. To compare the expression of each marker between the invasion and the superficial zone, the T student test was used. Survival analysis considered 5-year overall survival (OS) and 5-year disease-specific survival (DSS). OS was defined as the time between the date of primary surgery and the date of death due to any cause. DSS was defined as the time between the date of primary surgery and the date of death due to OSCC. Survival curves were constructed according to the Kaplan-Meier method and were

compared with the log-rank test. To identify independent risk factors related to poor survival, the Cox proportional hazard model was used. Backward elimination was used to select the covariates included in the regression model. Data were processed using the IBM SPSS Statistics™ software (Version 23) and the level of statistical significance was set at 5% (P ≤ 0.05).

3. Results

Sample characteristics

A total of 44 OSCC patients were included in the data analysis. The mean patient age was 62.3 years (range: 28–88 years), with an equal distribution between males (n = 22) and females (n = 22). Most tumors were located on the tongue (61.4%), followed by the floor of the mouth (11.4%) and the alveolar ridge (11.4%). Most patients (63.6%) were classified as stage III or IV, and 66% died within the first five years of the disease. Additional case details are provided in Table 1.

Table 1. Demographics and clinicopathological characteristics of the study sample

Variable	n	%
Sex		
Male	22	50
Female	22	50
Tobacco consumption		
No	24	54,5
Yes	20	45,5
Alcohol consumption		
No	26	59
Yes	18	41
Tumor site		
Oral tongue	27	61,4

Floor of the mouth	5	11,4
Cheek	3	6,8
Retromolar pad	2	4,5
Alveolar ridge	5	11,4
Hard palate	2	4,5
Differentiation grade		
Well differentiated	24	54,5
Moderately differentiated	20	45,5
Tumor budding		
Negative	11	25
Positive	33	75
PNI		
Negative	19	43
Positive	25	57
T		
T1-T2	22	50
T3-T4	22	50
Lymph node metastasis		
Negative	23	52,3
Positive	21	47,7
Margins		
Negative	40	91
Positive	4	9
TNM stage		
I-II	16	36,4

III-IV	28	63,6
Locoregional recurrence		
No	22	50
Yes	22	50
Patient status		
Alive	15	34
Dead	29	66

The IL-1 signaling pathway is overexpressed in OSCC

Immunohistochemical expression of IL-1α, IL-1β, IL-1R1, and IL-1RA was quantified in both OSCC tissue (n = 44) and NDAE, when available (n = 15), obtaining 935 images. IL-1α was constitutively expressed in all NDAE samples, primarily in the cytoplasm but also in the nucleus, with a TTE of 50.3%. In contrast, IL-1β, IL-1R1, and IL-1RA were expressed in most NDAE samples (IL-1β in 93%, IL-1R1 in 94%, and IL-1RA in 87%), but at lower levels, with TTEs of 15.6%, 13.1%, and 23%, respectively. All OSCC cases constitutively expressed all four markers, except for one case that lacked IL-1β expression, with TTEs of 77.8%, 79.4%, 78.2%, and 80.2% for IL-1α, IL-1β, IL-1R1, and IL-1RA, respectively. All markers were significantly overexpressed in OSCC compared to NDAE (p < 0.0001) (Figure 2).
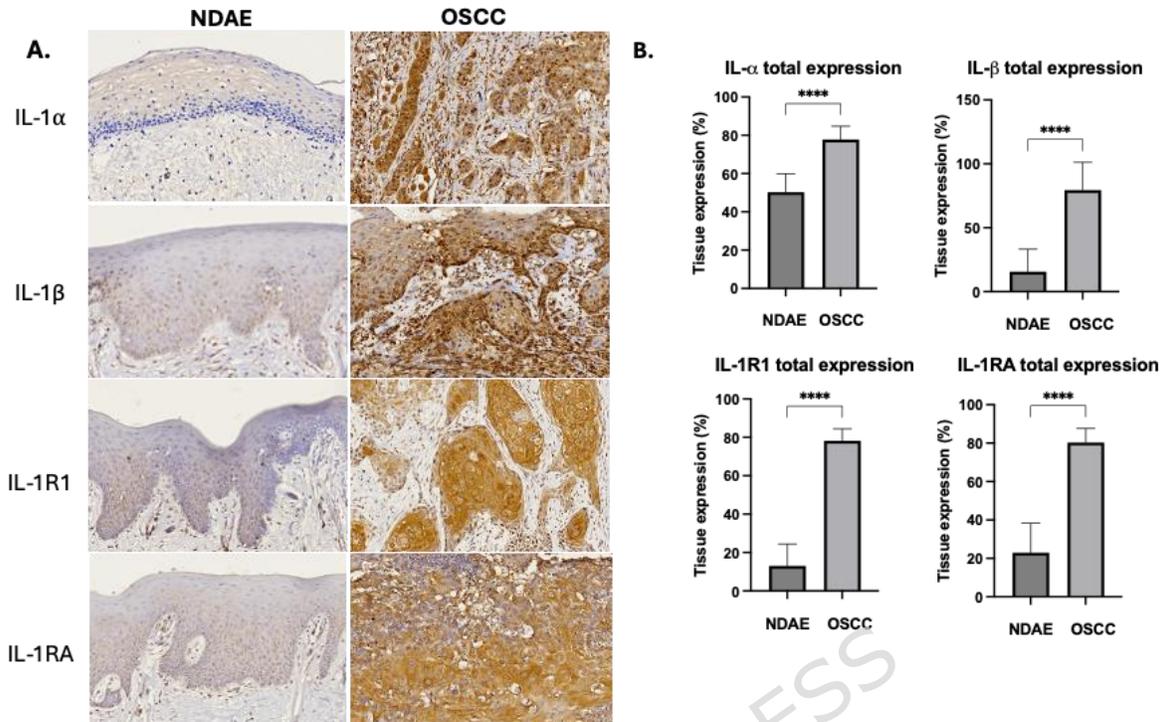
Figure 2. A) Immunohistochemical expression of the different members of the IL-1 signaling pathway and B) quantification of expression. Magnification is 20X. The images correspond to related specimens.

**** p < 0.0001

The overexpression of IL-1 signaling pathway proteins was not associated with invasion

To determine whether the overexpression of IL-1 pathway proteins in OSCC confers a phenotypic advantage to cancerous cells at the tumor front, we compared the TTE of IL-1α, IL-1β, IL-1R1, and IL-1RA between the invasive front and the superficial area of all OSCC samples. No significant differences were observed (p > 0.05) (Sup. Fig. 1).

The IL-1 signaling pathway is associated with clinicopathological variables

Smoking was significantly associated with lower IL-1α levels (p = 0.037) and higher IL-1R1 levels (p = 0.042). Increased IL-1R1 expression was also correlated with tumor budding (p = 0.031), while high IL-1RA levels were associated with tumor

recurrence (p = 0.030). No significant associations were found between IL-1α, IL-1β, IL-1R1, or IL-1RA expression and tumor grade, WPOI, PNI, margin status, stage, tumor size, or lymph node metastasis (p > 0.05) (Table 2).

Table 2. Association of clinicopathological variables with IL-1 signaling expression

Variables	IL-1RA		p	IL-1R1		p	IL-1α		p	IL-1β		p
	Low	High		Low	High		Low	High		Low	High	
Age (years)												
≤ 64	11	12	0.19	16	7	0.242	12	8	0.44	10	13	0.555
> 64	6	15		11	10		15	6		11	10	
Sex												
Male	5	17	**0.03**	11	11	0.122	15	6	0.44	9	13	0.365
Female	12	10		16	6		12	8		12	10	
Smoking												
No	12	12	0.09	18	6	**0.042**	12	11	**0.037**	12	12	0.741
Yes	5	15		9	11		15	3		9	11	
Alcohol												
No	12	14	0.218	16	10	0.977	17	7	0.424	15	11	0.112
Yes	5	13		11	7		10	7		6	12	
Tumor grade												
WD	12	12	0.09	17	7	0.158	14	9	0.447	10	14	0.378
MD	5	15		10	10		13	5		11	9	
WPOI												
Types 1, 2 or 3	1	0	0.386	1	26	0.614	0	1	0.341	1	0	0.477
Types 4 or 5	16	27		0	17		27	13		20	23	
TB												
No	6	11	0.22	10	17	**0.031**	6	4	0.653	4	7	0.494
Yes	5	55		1	16		21	10		17	16	
PNI												
No	8	9	0.68	13	6	0.402	11	7	0.742	7	12	0.208
Yes	11	16		14	11		16	7		14	11	
Margins												
Compromised	1	3	0.557	3	1	0.557	2	2	0.596	1	3	0.34
Free	16	24		24	16		25	12		20	20	
T												
T1-T2	10	12	0.537	15	7	0.353	14	7	0.91	10	12	0.763
T3-T4	7	15		12	10		13	7		11	11	

N											
No	8	9	0.583	13	10	0.548	13	9	0.326	9	14	0.365
Yes	15	12		14	7		14	5		12	9	
Stages											
I-II	6	10	0.97	11	5	0.447	11	5	0.754	8	8	0.82
III-IV	11	17		16	12		16	9		13	15	
Recurrence											
No	12	10	**0.03**	11	16	0.122	14	6	0.585	13	9	0.131
Yes	5	17		11	6		13	8		8	14	

WD: Well-differentiated; MD: Moderately differentiated; WPOI: Worst pattern of invasion; TB: Tumor budding; PNI: Perineural invasion; T: Tumor size; N: Lymph node involvement

High IL-1RA expression is associated with worse survival

The follow-up period for all patients ranged from 4 to 200 months. The 5-year OS rate was 54.4%, which was significantly lower in tumors with high IL-1RA expression (p = 0.026). In contrast, high tumor expression of IL-1R1 (p = 0.827), IL-1α (p = 0.543), or IL-1β (p = 0.321) did not significantly impact 5-year OS. Regarding 5-year DSS, lower survival was observed in tumors with high IL-1RA expression (p = 0.038), whereas tumor expression of IL-1R1 (p = 0.707), IL-1α (p = 0.533), and IL-1β (p = 0.782) did not significantly affect DSS (Figure 3; Table 3).
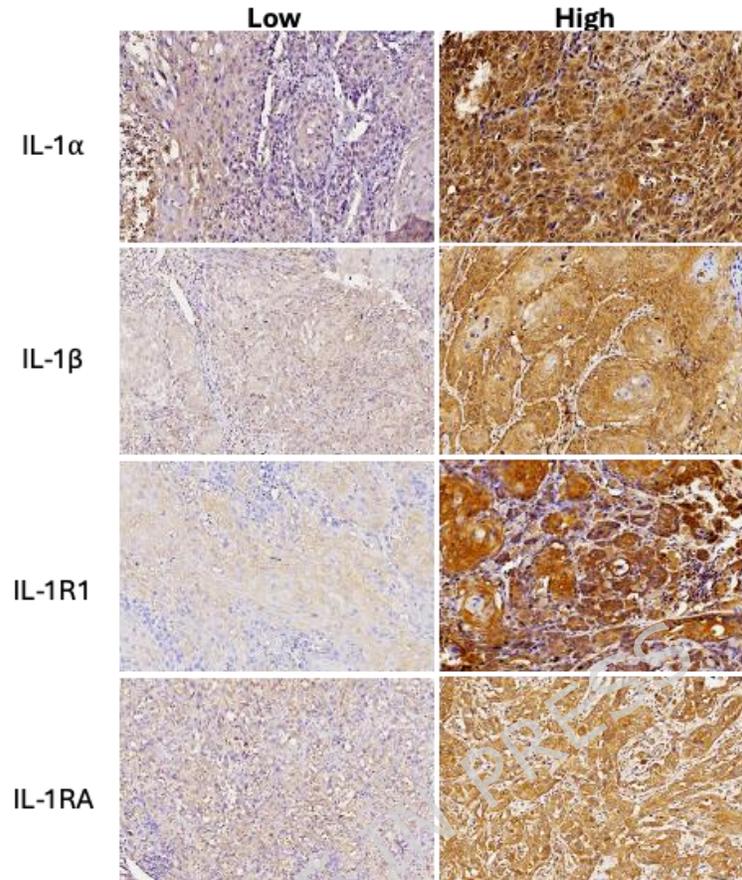
Figure 3. Representative images of tumors with low or high expression levels of each cytokine. Magnification is 20X.

Table 3. Univariate analysis of Cox regression for 5-year OS and DSS.

Variable	5-year OS HR (95% CI)	p	5-year DSS HR (95% CI)	p
Age (years)				
≤64	Ref		Ref	
>64	1.151(.531-2-494)	0.721	1.183 (.510-2.745)	0.695
Tumor grade				

WD	Ref		Ref	
MD	.614 (.278-1.355)	0.227	.680 (.290-1.596)	0.376
Tumor budding				
no	Ref		Ref	
yes	1.257 (.504-3.134)	0.624	1.283 (.473-3.485)	0.624
PNI				
no	Ref		Ref	
yes	2.163 (.939-4.982)	0.070	2.027 (.825-4.981)	0.123
Margin status				
Compromised	Ref		Ref	
Free	.511 (.174-1.499)	0.221	.573 (.167-1.962)	0.375
T				
T1-T2	Ref		Ref	
T3-T4	1.791 (.820-3.908)	0.44	2.748 (1.118-6.758)	**0.028**
N				
no	Ref		Ref	
yes	2.780 (1.255-6.157)	**0.012**	2.998 (1.250-7.192)	**0.014**
TNM stage				
I-II	Ref		Ref	
III-IV	2.807 (1.122-7.023)	**0.027**	3.683 (1.240-10.938)	**0.019**
Locoregional recurrence				
no	Ref		Ref	
Yes	2.930 (1.268-6.774)	**0.012**	3.462 (1.346-8.901)	**0.006**
IL-1RA expression				
Low	Ref		Ref	
High	2.844 (1.134-7.132)	**0.026**	2.906 (1.063-7.946)	**0.038**
IL-1R1 expression				
Low	Ref		Ref	
High	.916 (.415-2.020)	0.827	.846 (.355-2.021)	0.707
IL-1α expression				
Low	Ref		Ref	
High	1.297 (.561-3.002)	0.543	1.330 (.543-3.258)	0.533
IL-1ß expression				

Low	Ref		Ref	
High	1.494 (.676-3.300)	0.321	1.126 (.485-2.614)	0.782

OS, overall survival; DSS, disease-specific survival; HR, hazard ratio; CI, confidence interval; Ref, reference; WD, well differentiated; MD, moderately differentiated; PNI, perineural invasion; T, tumor size/stage; N, lymph node involvement; TNM, tumor-node-metastasis

Univariate analysis of clinicopathological variables

In the Cox univariate analysis, cervical lymph node metastasis (p = 0.012), stage III-IV disease (p = 0.027), locoregional recurrence (p = 0.012), and high tumor expression of IL-1RA (p = 0.026) were identified as risk factors for lower 5-year OS. Similarly, alcohol consumption (p = 0.037), T3-T4 tumors (p = 0.028), lymph node metastasis (p = 0.014), stage III-IV disease (p = 0.019), locoregional recurrence (p = 0.006), and high tumor expression of IL-1RA (p = 0.038) were associated with lower 5-year DSS (Table 3).

Multivariate analysis

A Cox multivariate regression analysis was performed to identify independent risk factors for 5-year OS and DSS, including lymph node metastasis, locoregional recurrence, and IL-1RA tumor expression as covariates. Lymph node metastasis (p = 0.002) and high tumor expression of IL-1RA (p = 0.007) were found to be independent risk factors for poor 5-year OS. In contrast, only lymph node metastasis (p = 0.012) and locoregional recurrence (p = 0.009) were independent risk factors for poor 5-year DSS (Table 4) (Figure 4).

Table 4. Cox multivariate regression analysis for 5-years OS and DSS survival

	5-year OS		5-year DSS	
Variable	HR (95% CI)	p-value	HR (95% CI)	p-value

Lymph node metastasis				
no	Ref		Ref	
yes	3.487 (1.552-7.836)	**0.002**	3.077 (1.283-7.377)	**0.012**
Locoregional recurrence				
no	Ref		Ref	
yes	2.008 (.797-5.062)	0.139	3.539 (1.378-9.090)	**0.009**
IL-1RA expression				
Low	Ref		Ref	
High	3.627 (1.425-9.231)	**0.007**	2.407 (.791-7.329)	0.122
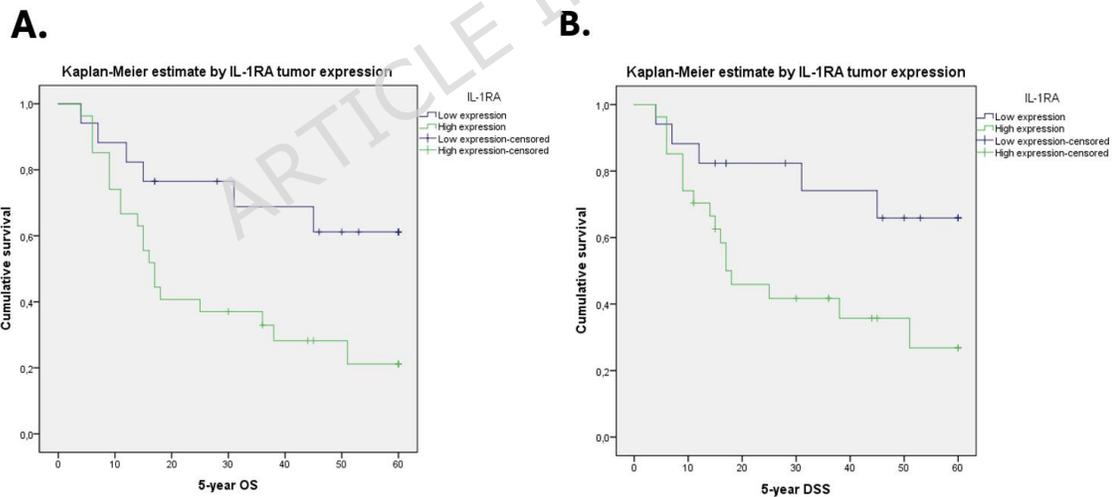
Figure 4. Five-year OS and DSS survival in tumors with high and low IL-1RA expression. P < 0,05.

Discussion

This study included OSCC patients with a mean age of 62.3 years and an equal distribution between males and females, with most tumors located on the

tongue. Most patients presented with advanced-stage disease, and the five-year OS rate reflected the aggressive nature of OSCC, aligning with the most reported clinicopathological characteristics of this condition (2). These findings underscore the severity of OSCC and highlight the need for further studies to elucidate its molecular implications in tumor development and progression. In this context, the present study revealed a significant overexpression of IL-1α, IL-1β, IL-1R1, and IL-1RA in OSCC compared to NDAE. Notably, high IL-1RA expression was associated with tumor recurrence and poorer five-year OS and DSS. Furthermore, multivariate analysis identified IL-1RA overexpression and lymph node metastasis as independent risk factors for poor overall survival, suggesting that IL-1RA may exert a modulatory and potentially adjuvant role in OSCC progression.

In the interplay between inflammation and cancer, pro-inflammatory mediators modulate the inflammatory response, either suppressing tumor progression or promoting tumor growth and metastasis (8, 11). The IL-1 family comprises both pro- and anti-inflammatory proteins and is recognized as a key cytokine within the tumor microenvironment, where it plays a possible role in carcinogenesis and tumor progression (30). The IL-1 signaling pathway is constitutively expressed in OSCC, suggesting its involvement in OSCC development and progression (13). Mechanistically, IL-1 signaling is known to promote inflammation, angiogenesis, and immune evasion by inducing the secretion of pro-inflammatory cytokines, stimulating neovascularization, and modulating immune cell infiltration, ultimately fostering a pro-tumorigenic environment (31). In this context, immunohistochemical analysis in the present study revealed a significant overexpression of IL-1α, IL-1β, IL-1R1, and IL-1RA in OSCC compared to NDAE, suggesting an involvement of IL-1 signaling in OSCC pathogenesis. However, no significant differences in expression were observed between the superficial area and the invasive front, although increased IL-1R1 expression was associated with tumor budding. This finding suggests that while IL-1 signaling may not drive invasion, it could modulate localized invasive phenotypes such as budding.

Other inflammatory pathways have been implicated in OSCC invasion. For instance, IL-8 inhibition has been reported to decrease OSCC cell invasion and

neutrophils have been shown to promote invasion by inducing matrix degradation and invadopodia formation through TNF-α signaling (10, 32, 33). Tumor invasion is a multifactorial process influenced not only by inflammatory cytokines but also by factors such as matrix metalloproteinases, epithelial–mesenchymal transition, and tumor–stromal interactions. These elements may overshadow the contribution of IL-1 signaling in certain OSCC contexts. Thus, while IL-1 signaling appears active in OSCC pathogenesis, its role in invasion remains unclear and likely context-dependent.

When analyzing the association between the immunoexpression of each protein and the clinicopathological variables in the present study sample, it was observed that smoking was significantly associated with lower IL-1α levels and increased IL-1R1. IL-1α is a key alarmin cytokine that initiates and amplifies inflammatory responses. It plays a crucial role in the IL-1 signaling pathway, which is activated when IL-1α binds to IL-1R1 (34). In this context, studies in pulmonary models indicate that cigarette smoke can increase IL-1β production and activate IRAK4, a key mediator of IL-1β signaling (16). However, little is known about the effects of smoking on the IL-1 signaling pathway in relation to OSCC. In a study from Lee et al. (16), increased IL-1ß and IL-1R1 expression was reported in both tobacco-related mouse models and OSCC cell lines derived from smokers, suggesting that smoking may contribute to tumor progression through IL-1 signaling dysregulation. Based on the present findings, it may be inferred that IL-1α and IL-1β dynamics differ in the context of tobacco exposure, and that IL-1R1 upregulation may enhance tumor cell sensitivity to IL-1 signaling, thereby contributing to pro-tumorigenic effects. Together, these findings suggest a broader impact of smoking on IL-1 signaling components.

In the present study, high IL-1RA expression was significantly associated with reduced 5-year overall survival. Moreover, univariate and multivariate analyses identified high IL-1RA expression as an independent risk factor for poorer 5-year OS, alongside lymph node metastasis. Different studies have reported discordant results regarding the pro- and anti-tumorigenic roles of IL-1 family members in cancer (35-37). Although several studies have suggested a potential role for IL-1 signaling in

head and neck squamous cell carcinoma, the findings remain inconsistent, and few have specifically examined its role in OSCC (16, 38). In the context of OSCC, it has been shown that IL-1β can induce epithelial-mesenchymal transition in OSCC cells, leading to increased migration and invasiveness (16), and that elevated IL-1β expression has been associated with lymph node metastasis in OSCC (38), which contrasts with the findings observed in the sample of the present study. In addition, recent evidence suggests that IL-1RA may also play a relevant role in OSCC progression. A previous study demonstrated that elevated IL-1RA expression in OSCC tumor tissues was associated with larger tumor size, advanced stage, and reduced survival, particularly among patients receiving adjuvant radiotherapy (39). These findings align with the present study, in which high IL-1RA expression was independently associated with poorer 5-year OS. However, on the other hand, Ding et al. (40) demonstrated that IL-1RA expression is reduced in OSCC tissues and cell lines CAL27 and HN6, and that *in vivo* overexpression of IL-1RA significantly reduced tumor growth. In this context, it is important to consider that the biological behavior of IL-1 family members may vary depending on whether they are expressed intracellularly or secreted by tumor cells, as well as according to the tumor stage and microenvironment. There are no antibodies available to identify secreted or intracellular IL-1RA on tissue samples, thus we could only assess total IL-1RA expression. Differences in the tumor microenvironment may significantly influence the functional outcome of IL-1 signaling. It is also possible that IL-1RA and IL-1RA variants exerts context-dependent effects, particularly in the activation of stroma on invasion, functioning as either a tumor suppressor or promoter depending on the molecular landscape of the tumor. These discrepancies underscore the context-dependent roles of IL-1RA in OSCC and highlight the need for further research to elucidate the regulatory mechanisms and prognostic relevance of IL-1RA across different tumor settings and patient subgroups.

Another important point to mention is that in this study, the expression of the proteins analyzed was statistically significant in the multivariate analysis for OS, but not for DSS. This may be because these proteins are nonspecific markers of inflammation, present in various disease contexts. Although they play a role in

cancer, assessing inflammation requires a broader panel of interleukins and cytokines to achieve predictive value. Finally, there are some limitations inherent in our current data that should be taken into consideration when interpreting the results. First, the OSCC samples were selected by convenience from a specific case database, which may limit the generalizability of the findings. Second, although immunohistochemistry remains a widely used technique for protein expression analysis, it is a multi-step procedure subject to variability at different stages—such as tissue processing, antigen retrieval, and antibody performance—which can compromise reproducibility and hinder comparison across studies. Additionally, the quantification of immunostaining in this study was based on digital image analysis. While this approach allows for standardized and objective measurements, it also depends on the quality of image acquisition and the parameters defined for analysis, which may introduce technical bias. Moreover, due to the scope and design of this study, other important components of the tumor microenvironment were not assessed. Regarding the use of NDAE as a control, it is acknowledged that while often utilized for comparison and potentially for identifying early biomarkers, the concept of field cancerization introduces a caveat, considering that NDAE may harbor occult molecular alterations not visible histologically, meaning it might not represent a truly 'normal' baseline, and thus, comparative findings should be interpreted with this consideration.

In conclusion, this study highlights the significant overexpression of IL-1α, IL-1β, IL-1R1, and IL-1RA in OSCC compared to NDAE, reinforcing the possible role of IL-1 signaling in OSCC pathogenesis. Among these markers, IL-1RA emerged as a potential prognostic biomarker, with its overexpression independently associated with reduced 5-year survival. These findings suggest that IL-1RA may contribute not only to OSCC progression but also to adverse clinical outcomes. Nevertheless, these findings are preliminary and further validation in larger, independent cohorts is required. Further studies are needed to elucidate the mechanistic roles of IL-1RA and other IL-1 family members in OSCC and to assess their potential as therapeutic targets. In addition, research involving larger, well-characterized cohorts and integrative analyses of the tumor microenvironment is warranted to validate these

findings and provide a more comprehensive understanding of IL-1 signaling in OSCC.

Data availability statement:

The data supporting the results is available upon request to the corresponding authors.

Authors contribution:

Study conceptualization: RMF, KDH, SEN, RBM, MM; data collection: RMF, WGA; immunohistochemical staining: FM, VP; data analysis, FM, VP, WGA, RMF, SEN, RBM, manuscript writing: FM, VP, SEN, RMF, RBM, KDH. MM. All authors approved the final version of the manuscript.

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