Histological and immunohistochemical analysis of oral squamous cell carcinoma samples from 2018-2022

FATIN L. KHAPHI, GHAYDAA HASHIM AL QUDSI, OULA FOUAD HAMEED and HANAA ALI HUSSEIN

College of Dentistry, University of Basrah, Basrah 61001, Iraq

Received December 23, 2024; Accepted April 8, 2025

DOI: 10.3892/wasj.2025.347

Abstract. Oral squamous cell carcinoma (OSCC) is a critical public health issue that is becoming more common, with an increasing incidence and mortality rate due to a low quality of life. The aim of the present study was to investigate the prevalence of OSCC in relation to the demographic parameters of patients between 2018 and 2022, and to analyze the association with histopathological grades. Additionally, the present study aimed to evaluate the immunoexpression of biomarkers, such as p63, cluster of differentiation 44 (CD44) and epidermal growth factor receptor (EGFR) in different grades of OSCC. For this purpose, a total of 58 patient samples were retrieved and a biopsy sample was obtained for histopathological analysis and immunohistochemistry (IHC). Hematoxylin and eosin-stained sections were graded using Broder's criteria. A manual tissue microarray technique was employed for the analysis of expression of IHC markers, such as p63, CD44 and EGFR. The results revealed that as regards histological grading, 35 cases (60.34%) were classified as well-differentiated squamous cell carcinoma (WDSCC, grade I), while 23 cases (39.33%) were identified as moderately differentiated squamous cell carcinoma (MDSCC, grade II). An increased immunoexpression of CD44 and EGFR was noted in WDSCC, while there was a lower immunoexpression in MDSCC. All cases of WDSCC and MDSCC exhibited a strong positive nuclear immunoexpression of p63. On the whole, the present study demonstrates that the majority of the OSCC cases were WDSCC, followed by MDSCC. Thus, OSCC histopathological grades can be strong prognostic indicator. A low expression of CD44 in OSCC tissues may indicate tumor invasion and a higher risk of developing metastasis. Patients with OSCC exhibiting EGFR immunoexpression may benefit from specific targeted therapy.

Correspondence to: Dr Hanaa Ali Hussein, College of Dentistry, University of Basrah, Al-Bara'iyah Street, Basrah 61001, Iraq
E-mail: hanaahussein@uobasrah.edu.iq; hanaazahraa85@gmail.com

Key words: oral squamous cell carcinoma, histological grade, immunohistochemistry, p63, CD44, EGFR

Introduction

One of the most prevalent types of cancer worldwide is oral cancer, with an estimated 53,260 new cases and 10,750 related deaths in 2020 (1). Almost 90% of all malignant tumors of the oral cavity are oral squamous cell carcinomas (OSCC) and are mainly treated using surgery, radiotherapy and adjuvant chemotherapy (1-3). OSCC is uncommon among individuals <40 years of age and more frequently affects males (male to female ratio, 1.5:1), as a greater number of males than females engage in high-risk behaviors (4,5). All parts of the oral cavity can be affected by squamous cell carcinoma; however the tongue, floor of the mouth and lower lip are the most frequently reported sites (6). The essential risk factors for developing OSCC are chewing areca nut, using narcotics or marijuana, and regularly consuming processed foods. Additionally, deficiencies in vitamins such as folate, A, C, E and B12, as well as fungal infections (caused by *Candida* species), premalignant lesions, ultraviolet light, inorganic acids, sulfur dioxide, syphilis and poor oral hygiene are also potential risk factors for the disease (7).

OSCC presents with distinct early-stage symptoms, including a painless, non-healing ulcer with raised, rolled edges and irregular margins. Other signs may include leukoplakia and erythroplakia (indicating potential early lesions). Patients often experience oral pain, discomfort, a burning sensation, or unexplained tooth mobility. In the case that the cancer affects the base of the tongue or oropharynx, symptoms such as a persistent sore throat or hoarseness may occur (8). While patients with early-stage OSCC now have significantly improved outcomes (9,10), there has been minimal improvement in the overall survival rate of patients with advanced-stage OSCC over the past 40 years (11).

In order to increase the survival rate of patients with OSCC, new methods for early detection, risk assessment and intervention are regularly being developed. Consequently, there is a need for studies that clarify the oncological behavior of OSCC. Some researchers now acknowledge that relying solely on microscopic classification is not strongly associated with clinical outcomes and treatment responses, due to the limited prognostic value of the histological classification of conventional OSCC (well, moderate and poorly differentiated types) (12). The cumulative effect of mutations in the genes responsible for tumor suppression, cellular proliferation and

cell differentiation outcomes in the development of OSCC is a continuous multi-stage process (13).

Immunohistochemistry (IHC) is used to analyze OSCC for a better understanding of its biology, diagnosis, prognosis and treatment. Notable progress has been achieved in recent years in clarifying the underlying regulatory and molecular mechanisms, and cell cycle progression in mammalian cells (14). Tumor biomarkers are molecules produced by the tumor or surrounding tissues in response to tumorigenesis. They have various clinical applications, including risk assessment, cancer diagnosis, staging, prognosis and treatment selection (15). The most frequently mutated oncogenes in OSCC include p16, p53, cyclin D1, PTEN, Rb, p63 and the epidermal growth factor receptor (EGFR) (7).

The p63 gene is located on chromosomes 3q27-29 and is considered to represent a novel IHC marker for basal cells. It has been proven that the p63 protein plays a dual role in oncogenesis and tumor suppression, depending on the context and type of cancer (16,17). In the normal oral mucosa, the proliferative layer of cells close to the basement membrane expresses p63 protein, which inhibits basal cell differentiation and preserves basal cell status (18,19). Dysplastic changes in the oral mucosa can result in dysplastic keratinocytes, which exhibit characteristics akin to those observed in embryogenesis. Notably, these cells can continue to express p63 protein to enhance the proliferative ability of dysplastic cells within the oral dysplastic mucosa. Additionally, p63 plays a crucial role in the progression of epithelial dysplasia by altering stem cell function in the basal layer, leading to an increased number of proliferating cells. Consequently, this contributes to noticeable changes in the distribution of cells, both in the basal and suprabasal layers during the development of oral epithelial dysplasia (14). p63 mRNA and protein expression has been observed in a variety of human tumors, including OSCC. This expression results from the amplification of genes. The morphogenesis of epithelium-derived structures, such as the salivary glands, dental structures and oral mucosa, is significantly influenced by p63 (20-22).

Cluster of differentiation 44 (CD44) is a cell surface glycoprotein that controls and plays a role in cell adhesion, proliferation and migration; it is a common marker for highly oncogenic cancer stem cells in squamous cell carcinomas of the head and neck (21). In OSCC, the role of CD44 in predicting diagnosis is debatable due to inconsistent findings that have been reported (21,22). CD44 is involved in controlling cell survival, proliferation and migration, which are essential for tumor growth and invasion. Several studies have reported an association between CD44 expression and poor survival rates (23-25), while others have associated the expression of CD44 with epithelial-mesenchymal transition. However, the impact of CD44 expression on the prognosis of patients with OSCC remains unclear with conflicting results regarding its prognostic value (23).

The majority of OSSC tumors exhibit an overexpression of EGFR (ErbB1/HER1), and associations have been observed between high EGFR expression levels and an aggressive phenotype, a poor prognosis and resistance to anticancer therapy (26,27). EGFR signaling plays a role in regulating the division and differentiation of cells during development. In neoplastic cells, EGFR contributes to cell proliferation, invasion and metastasis (28). The dysregulation of EGFR expression and signaling in oral epithelial cells plays an essential role in the development and progression of OSCC. As a result, EGFR levels, assessed using quantitative and semi-quantitative IHC, are often considered an independent prognostic factor for tumor recurrence in patients with OSCC (29). EGFR is not only a prognostic biomarker, but also a promising therapeutic target for OSCC. Cetuximab (monoclonal antibody which blocks EGFR signaling) has demonstrated clinical efficacy when combined with radiotherapy or chemotherapy for the treatment of locally advanced OSCC, improving survival rates. Recent research has demonstrated that EGFR-targeted therapies can also sensitize tumors to various therapies, enhance chemotherapy and radiotherapy efficacy, and help overcome treatment resistance (30).

The prognostic value of EGFR expression is inconsistent and controversial, as numerous tumors in the head and neck region exhibit a minimal or no EGFR expression. However, several studies have reported no clear prognostic significance (26-29). Thus, EGFR expression is not routinely tested in the clinical management of OSCC, despite its established role in tumor aggressiveness (29). However, additional research is required to address resistance mechanisms, optimize treatment strategies and enhance patient selection criteria to fully harness the therapeutic potential of EGFR inhibition in OSCC (8).

The present study was carried out to determine the prevalence of OSCC and patient demographic parameters (age, sex and site of the lesions) in Basrah, Iraq between 2018 and 2022, as well as to analyze the association with histopathological grades of OSCC. Furthermore, the immunohistochemical expression of biomarkers, such as CD44, EGFR and p63 was also evaluated in different grades of OSCC.

Patients and methods

Patients. The present study involved 58 patients, aged between 15 and 85 years. Among these patients, 32 (55.17%) patients were male and 26 (44.83%) patients were female with oral tumor lesions; samples were collected from the Teaching Dental Clinic/College of Dentistry/University of Basrah and private laboratories in Basrah, Iraq between 2018 and 2022. Following the clinical examination of the patients, a biopsy sample was obtained for histopathological analysis and the IHC of p63, CD44 and EGFR as prognostic markers. The present cross-sectional study was carried out at the College of Dentistry, University of Basrah; the Institutional Ethical Committee granted ethical clearance and written informed approval was obtained from each patient or from their parents/legal guardians.

Histopathological analysis. In the histopathological analysis, selected cases of hematoxylin and eosin (H&E)-stained slides were examined. For the histopathological analysis, the tissues were sectioned using a rotary microtome at a thickness of 1-3 μm of paraffin-embedded tissue blocks that were previously fixed with 10% neutral buffered formalin (Merck KGaA) at room temperature for 24 h. The sections were mounted on glass slides and stained with hematoxylin and eosin (H&E, Leica Microsystems GmbH) for 5-7 min and examined under

a light microscope (Light leica UCC50; Leica Microsystems GmbH); the diagnosis was confirmed according to the revised criteria suggested by the World Health Organization (31) by senior histopathologists (10,32). The grades of OSCC cases were 35 cases of well-differentiated and 23 cases are moderately differentiated squamous cell carcinoma the blocks removed from the archives and the diagnosis was confirmed according to the revised criteria suggested by the World Health Organization (31) by senior histopathologists (10,32). The grades of OSCC cases were 35 cases of well-differentiated and 23 cases are moderately differentiated squamous cell carcinoma.

IHC. For IHC analysis, the most appropriate tissue block from OSCC cases was selected. For the examination of p63, CD44 and EGFR primary antibodies, a manual tissue microarray technique was used in each instance, with one tissue core removed from each selected OSCC block (33). To prepare serial sections, each specimen was first fixed in 10% buffered formalin at room temperature (20-25˚C) for 24 h and then embedded in paraffin. The primary antibodies used were p63 (monoclonal mouse anti-human p63 protein; dilution, 1:100; cat. no. sc-8431, Santa Cruz Biotechnology, Inc.), EGFR (dilution, 1:200; cat. no. AR335-5RE, BioGenex Laboratories) and CD44 (anti-CD44 antigen; dilution, 1:100; NCL-CD44-2, Novocastra Laboratories). Antigen retrieval was carried out via incubation, following the manufacturer's recommendations (Dako, Agilent Technologies, Inc.). Briefly, the sections were washed in phosphate-buffered saline (PBS; Merck KGaA) and deparaffinized. Using a 0.3% hydrogen peroxide solution at room temperature for 5 min, endogenous peroxidase activity was inhibited. Following antigen retrieval via microwave treatment (95-100˚C for 20 min)., primary antibodies were applied, and incubated at room temperature for 60 min, and then washed in PBS and left in a humidified chamber for 30 min at room temperature to be incubated with the HRP-conjugated secondary antibody Signal Stain Boost detection reagent (rabbit; 1:1 dilution; cat no. 8114, Cell Signaling Technology, Inc.). The samples were washed, and visualization was completed using the Signal Stain DAB Substrate kit (cat. no. 8059, Cell Signaling Technology, Inc.) and counterstaining lightly with Mayer's hematoxylin solution for 2 min at room temperature.

A light microscope (Olympus BX53, Olympus Corporation) was used to capture images of the tissue sections that had been immunohistochemically stained. For each sample, five images were obtained at a magnification of x20. Under x40 magnification, the immunostaining intensity of each marker was examined by two calibrated investigators using the following criteria: 0, no staining; 1, light staining (≤10% stained cells); 2, moderate staining (10-50% stained cells); and 3, intense staining (strong immunolabeling; ≥50% stained cells) (34).

Statistical analysis. All statistical analyses were performed using SPSS software (version 20.0, IBM Corp.). For categorical data, percentages and frequencies were determined. Data obtained were statistically analyzed using Fisher's exact test and Spearman's correlation analysis. A P-value <0.01 was considered to indicate a statistically significant difference.

Results

The present study population consisted of 58 patients with OSCC; samples were collected in Basrah, Iraq from 2018 to 2022. Among these, 32 (55.17%) patients were male and 26 (44.83%) patients were female (Fig. 1 and Table I). The age of all the patients in the present study ranged between 15 and 85 years. The age group of 66-75 years included the highest percentage of patients (24.1%), followed by the age groups of 36-45 and 56-65 years (20.7), and the age group of 46-55 years with 197% (Fig. 1 and Table I); the younger age groups, i.e., those of 15-25 and 26-35 years included the lowest percentage of patients (3.4 and 5.2%, respectively). As regards the sites of OSCC (Fig. 1 and Table I), the most commonly affected sites were the tongue (50%), followed by the lower jaw (13.8%), lower lip (10.3%), buccal mucosa and palate (6.9%). While the less affected sites were the floors of the mouth (1.7%) (Table I).

As regards the years of samples collected (Fig. 2), 37.93% of the samples were initially collected in 2019, followed by 2018, while the least amount of collected samples was in 2020 due to the COVID-19 pandemic. For the histological grade of OSCC (Fig. 2), 35 (60.34%) cases of OSSC were recognized as well-differentiated (WDSCC, grade I) and 23 (39.33%) cases were recognized as moderately differentiated (MDSCC, grade II) according to Broder's criteria (Fig. 3). The results of the bivariate analyses of variables associated with the histopathological grades of OSCC are presented in Table II. All variables exhibited statistically significant correlations with the grades of OSCC, with a P-value of <0.001. Fig. 4 illustrates the heatmap Spearman's correlation coefficients (ρ) between variables (age, sex, site, and year) and histopathological grades of OSCC. The color scale indicates the strength and direction of these correlations, with red signifying positive correlations and blue representing weaker or negative correlations. Statistically significant correlations (P<0.001) exhibit strong associations between these variables.

IHC results. The statistical analysis of the data illustrated a significant correlation at a P-value of 0.001, between the immunostaining intensity of all immunomarkers (p63, CD44 and EGFR) and the grades of OSCC (Fig. 5), where the results indicate a highly significant association (P=0.003) between the staining intensity of all these markers and different grades of OSCC (Table III). All cases of WDSCC and MDSCC exhibited a positive expression of p63 (Fig. 6). Among the WDSCC cases, 4% exhibited low-intensity staining, 35% exhibited moderate staining and 60% exhibited strong staining. Of note, 75% of cases of MDSCC exhibited a strong staining intensity pattern. The results revealed that 71% of cases of WDSCC exhibited a strong staining intensity for CD44, whereas 54% of cases of MDSCC demonstrated a less intense immunoexpression (Fig. 7). EGFR immunoreactivity exhibited a high rate in WDSCC, with 76% of cases exhibiting a strong staining intensity, with the intensity of staining became lower in MDSCC (Fig. 8).

Discussion

The most common type of cancer in the head and neck region is oral cancer. The prevalence of OSCC varies worldwide (35).

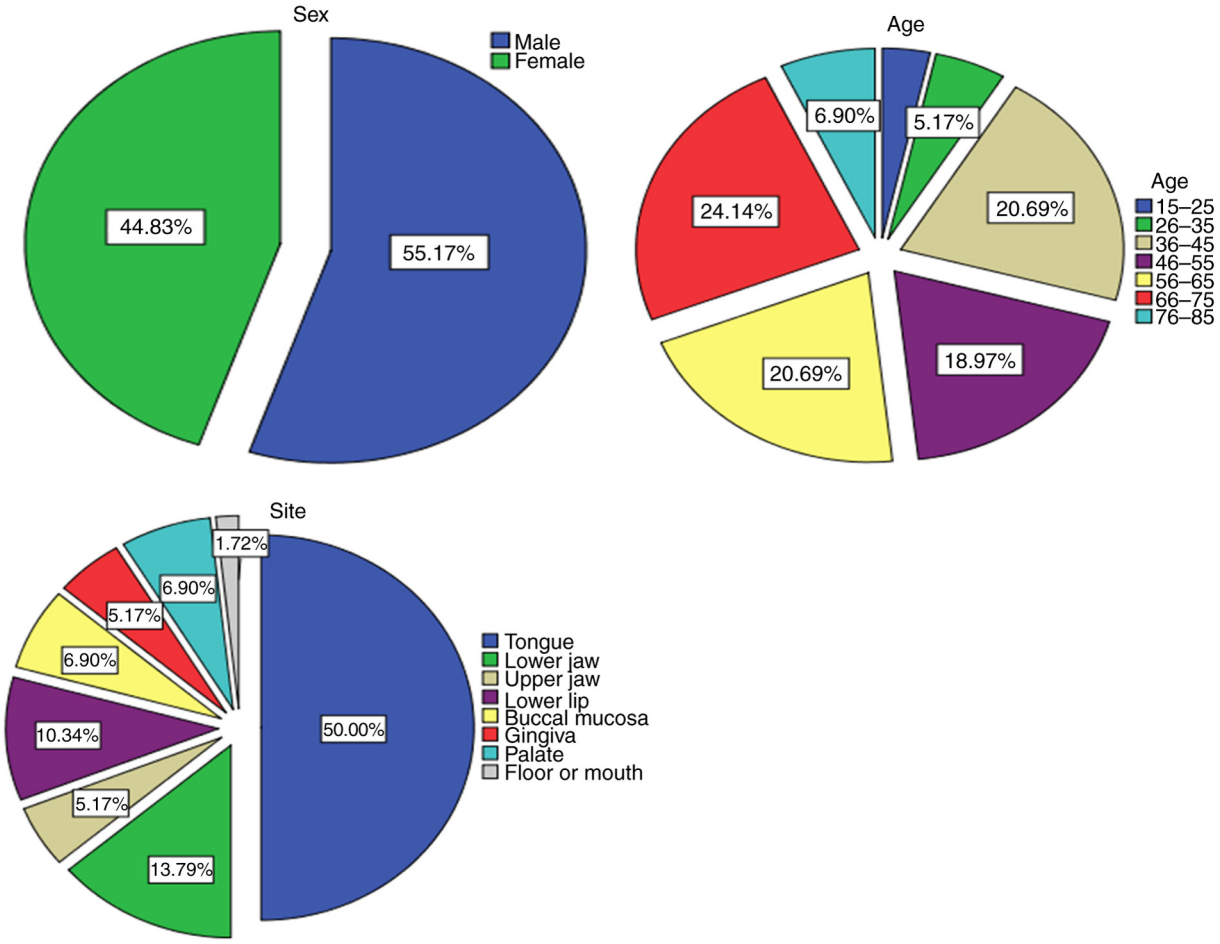


Figure 1. Socio-demographic characteristics of the patients, including sex, age and tumor site (n=58).

In the present study, the majority of patients with OSCC were males. This may be due to the fact that the majority of males smoke tobacco compared to females; the prevalence of this habit varies depending on the region, whereas women in Iraq are less exposed to this habit than they are in other countries (36). This result is in agreement with the findings of other studies that have reported that OSCC is more common in males in their 5 to 7th decade of life (37,38). By contrast, a previous study reported a higher incidence of OSCC in females (51.4%) compared to male patients (48.5%) (39). The results from the present study revealed a high percentage (24.1%) of aged patients (66-75 years), while younger patients constituted a low percentage (age groups of 15-25 and 26-35 years, 3.4 and 5.2%, respectively). In a previous study, the number of OSCC cases increased with age, with the majority of OSCC cases occurring in patients >40 years of age (40). Thus, the dimension of utagenic and epigenetic changes associated with aging is further enhanced by the probability of developing a period of exposure to risk factors and growing older. This finding is in contrast with that of another study that reported a higher incidence of OSCC in young patients than in adult patients (39).

In the present study, the most common site of OSCC affected was the tongue (50%), followed by the lower jaw (13.8%), Similar studies have reported the tongue as the predominant site in patients with OSCC (1,37,41). Comparable results have also been found in investigations conducted in the USA and Europe, where OSCC affects the tongue in 20 to 40% of cases. Additionally, the floor of the mouth is affected in 15 to 20% of cases (42,43). Together, these locations account for ~50% of all OSCC cases (4). By contrast, other studies have reported that the buccal mucosa was the most common site in patients with OSCC (38,44,45). The majority of the cases grades were WDSCC followed by MDSCC. This finding was similar to the studies reported by Bernardes *et al* (41), Nayak *et al* (44), Bunget *et al* (40), Tran *et al* (1), Khan *et al* (38) and Zahir *et al* (45), without poorly differentiated grade due to limited cases during the study period (2018-2022) and coinciding with the COVID-19 epidemic. When a tumor grows larger than its blood supply, it can lead to ulceration and necrosis. In a previous study, the most common presenting symptom was ulceration, accompanied by nests of malignant squamous cells (37).

The statistical analysis of the correlation between the histological grades of OSCC and the patients' sex, age, site, and year of involvement revealed highly significant associations with grades of OSCC at a P-value of <0.001. By contrast, other studies have reported an insignificant correlation between sex and OSCC grades (37,38), while Shaikh *et al* (46) and Yasin *et al* (47) reported correlated histopathological patterns of OSCC with age and site, with a statistically significant value between these two correlations.

Table I. Demographics and characteristics of the patients with oral squamous cell carcinoma in the present study.

Characteristic	Category	Frequency (no. of patients)	Percentage	Cumulative percentage
Sex	Male	32	55.2	55.2
	Female	26	44.8	100.0
Age (years)	15-25	2	3.4	3.4
	26-35	3	5.2	8.6
	36-45	12	20.7	29.3
	46-55	11	19.0	48.3
	56-65	12	20.7	69.0
	66-75	14	24.1	93.1
	76-85	4	6.9	100.0
Site of tumor	Tongue	29	50.0	50.0
	Lower jaw	8	13.8	63.8
	Upper jaw	3	5.2	69.0
	Lower lip	6	10.3	79.3
	Buccal mucosa	4	6.9	86.2
	Gingiva	3	5.2	91.4
	Palate	4	6.9	98.3
	Floor of mouth	1	1.7	100.0
Year	2018	13	22.4	22.4
	2019	22	37.9	60.3
	2020	5	8.6	69.0
	2021	7	12.1	81.0
	2022	11	19.0	100.0
Grade	Moderately differentiated	23	39.7	39.7
	Well differentiated	35	60.3	100.0

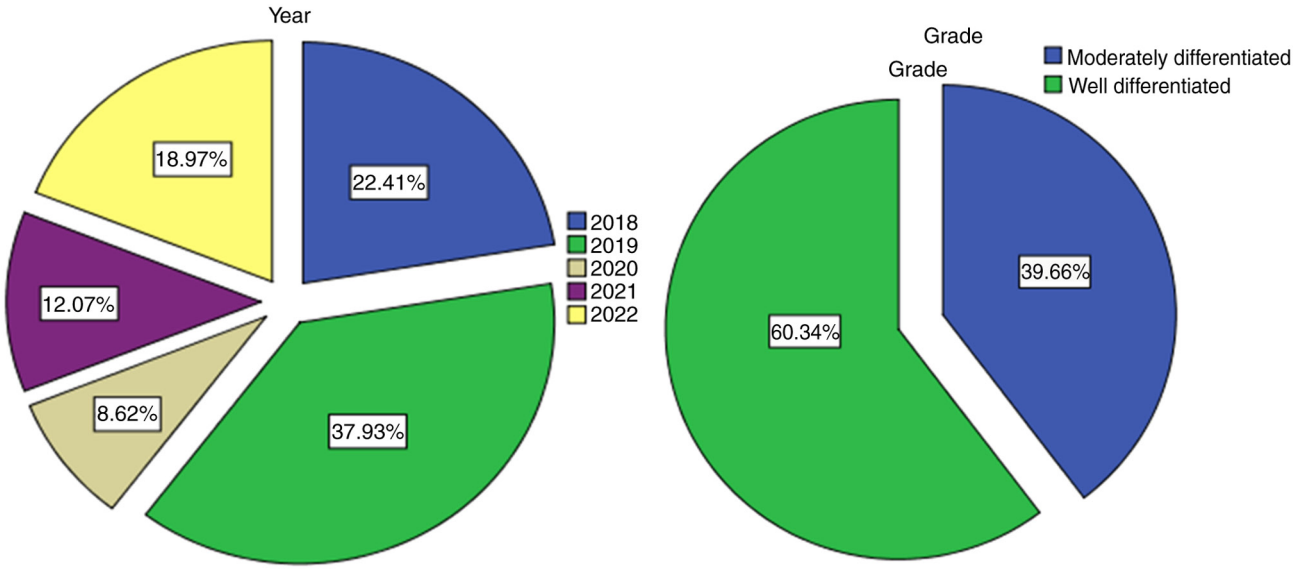


Figure 2. Distribution of years of collected sample and histopathological grades of oral squamous cell carcinoma.

The present study examined the IHC results for p63, CD44 and EGFR in different grades of OSCC. It was found that all cases of WDSCC and MDSCC displayed a positive immunoexpression of nuclear p63. The percentage of stained cells, which was lower in WDSCC than in MDSCC, reflects this phenomenon. Of note, ~75% of MDSCC cases had a strong staining intensity pattern, and the other cases had a moderate staining pattern. Therefore, the staining intensity

Table II. Spearman's correlation analysis variables and histopathological grades of OSCC.

Variables	Spearman's Rho (ρ)	P-value	Histopathological grade correlation
Age	0.791	<0.001	0.75 (strong positive correlation)
Sex	0.698	<0.001	0.60 (moderate positive correlation)
Site	0.602	<0.001	0.55 (moderate positive correlation)
Years	0.678	<0.001	0.65 (moderate positive correlation)

Data were analyzed using Spearman's correlation coefficient. The correlation is significant at the 0.01 level (two-tailed). OSCC, oral squamous cell carcinoma.

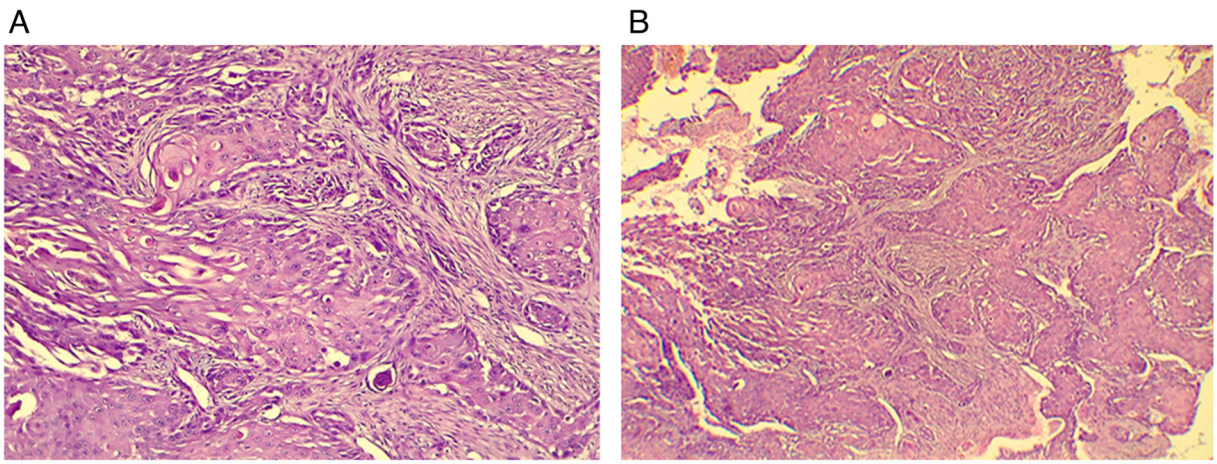


Figure 3. Hematoxylin and eosin-stained sections (x10 magnification). (A) Nest of Moderately differentiated squamous cell carcinoma invading submucosal tissue. (B) Well differentiated squamous cell carcinoma. Image illustrates an ulcerated squamous epithelial lining with nests of malignant squamous cells, with keratinization invading the underlying tissue.

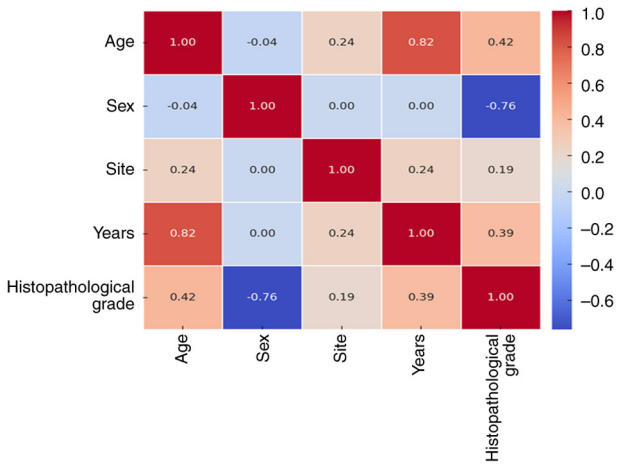


Figure 4. Spearman's correlation heatmap for correlation analysis of variables and histopathological grades of oral squamous cell carcinoma.

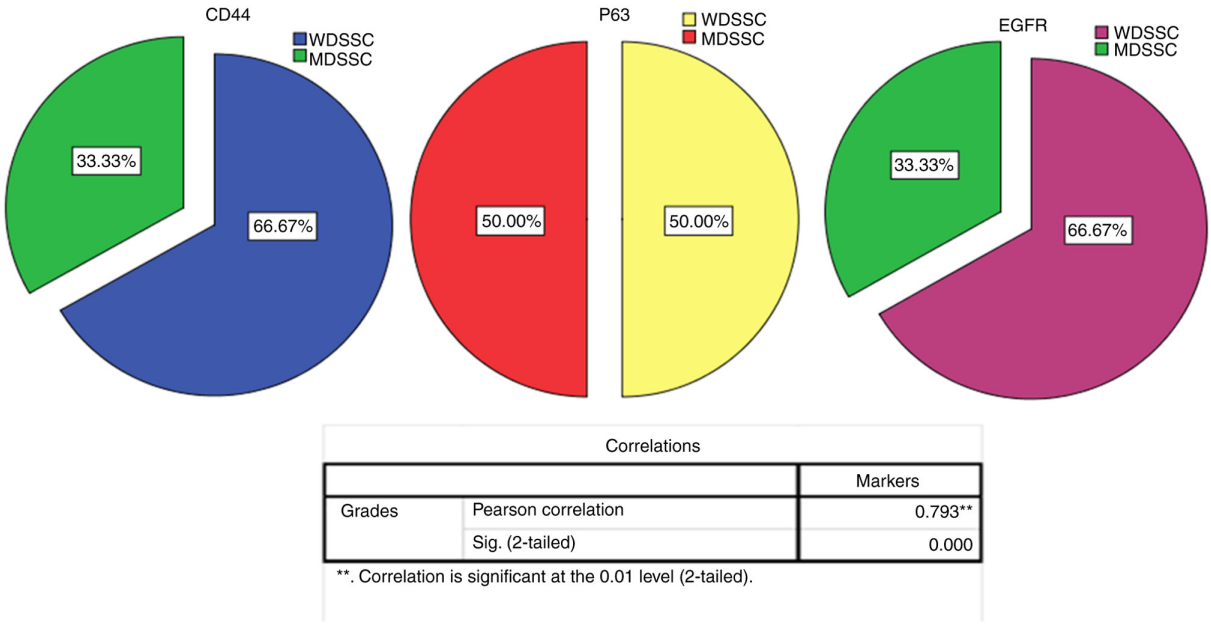
increased with the increasing grade in the present study. This is consistent with previous research by Matsubara *et al* (48), Lo Muzio *et al* (49), Saghravanian *et al* (50), and Sri Gowri *et al* (51), which also reported a positive correlation between the histologic grade and ΔN p63 positivity. ΔNp63 binds to DNA using its DNA binding domain or interacts directly with P53/TAP63. This interaction helps ΔNp63 prevent apoptosis and acts as an oncogene that causes the development of oral tumors (52). ΔNp63 helps regenerate the basal keratinocytes in squamous epithelium by reducing the levels of CDKN2A and NOTCH1. In response to genotoxic stress, squamous epithelium can also undergo terminal differentiation. This process is induced by p53 activating NOTCH1; however, ΔNp63 prevents this activation (53-55). The study of p63 is complex as there are six known isoforms, TA p63α, TA p63β, TA p63γ, ΔN p63α, ΔN p63β and ΔN p63γ, that have opposing functions (56). Analyzing p63 expression in human malignancies is challenging due to the numerous antibodies needed to distinguish between these different isoforms. While a number of studies have shown that human malignancies overexpress p63, others have reported a decrease in its expression (57).

However, de Oliveira *et al* (58) found that neither the staining intensity pattern nor the quantity of stained cells was beneficial in significantly differentiating the degree of differentiation of the studied lesions, despite the fact that p63 positivity was observed in 87.8% of the tumors. Similar findings were noted by Dayakar *et al* (59), who found that 96.15% of the cases had a positive p63 expression. According to Patel *et al* (14), 100% of cases exhibited p63 immunopositivity; these findings are relatively similar to those of the present study. An increased expression of p63 with

Table III. Tabular analysis for intensity of p63, CD44 and EGFR staining, and grade of OSCC.

Marker	Histological grade	Staining intensity				Total no. of cases	P-value
		0 (%)	1 (%)	2 (%)	3 (%)		
p63	WDSSC	0 (0)	6 (4)	11 (35)	18 (60)	35	0.010 (significant)
	MDSSC	0 (0)	0 (0)	3 (25)	20 (75)	23	
CD44	WDSSC	0 (0)	0 (0)	6 (29)	29 (71)	35	0.00000594 (highly significant)
	MDSSC	0 (0)	1 (2)	10 (44)	12 (54)	23	
EGFR	WDSSC	0 (0)	1 (4)	9 (20)	25 (76)	35	0.773 (not significant)
	MDSSC	0 (0)	1 (2)	7 (41)	15 (57)	23	

Data were analyzed using Fisher's exact test.



Correlations

		Markers
Grades	Pearson correlation	0.793**
	Sig. (2-tailed)	0.000

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 5. Correlation between grades of oral squamous cell carcinoma and immunostaining intensity of all immunomarkers.

the increased severity of OSCC may reflect the main role of this protein in the development of OSCC, according to the present study and all previous investigations (16,48,53). The p63 proteins play a crucial role in the development of the oral mucosa, and the six proteins that comprise the p63 family are balanced in the healthy oral mucosa. On the other hand, squamous cell carcinomas in the same region exhibit an imbalance in the levels between them (60). The clonal growth of specific cell populations and the successive accumulation of genetic and epigenetic abnormalities cause cancer in a multi-step process (61).

Alkhatib *et al* (62) confirmed that the stepwise activation of p63 and MEK/ERK-MAPK plays a key role in the growth of OSCC by regulating ARL4C expression. Notably, p63 expression was upregulated, and the MEK/ERK-MAPK pathway was activated in both carcinoma *in situ* and invasive carcinoma lesions (ICLs). IHC revealed that p63 expression was initially increased in carcinoma *in situ*, while ERK activation was observed in ICL. ARL4C is a gene known to promote oncogenic and can induced by p63 and/or the MEK/ERK-MAPK pathway. In OSCC tissue, ARL4C was more commonly detected in ICL than in carcinoma *in situ*. Additionally, ARL4C and phosphorylated ERK were often co-expressed in ICL. Loss-of-function experiments demonstrated that both p63 and MEK/ERK-MAPK pathways cooperatively promote ARL4C expression and cell growth in OSCC (62).

In the present study, CD44 exhibited a strong immunoexpression in WDSCC and a less intense expression in MDSCC cases (71% of cases of WDSCC exhibited a strong staining intensity of CD44, whereas 54% of cases of MDSCC demonstrated a less intense immunoexpression). This is in accordance with the findings in the studies by Fonseca *et al* (63), Kanke *et al* (64) and Poothakulath Krishnan *et al* (65). When the tumor grade increases, the intensity of CD44 expression decreases, indicating a reduction in cell-to-cell adhesion and

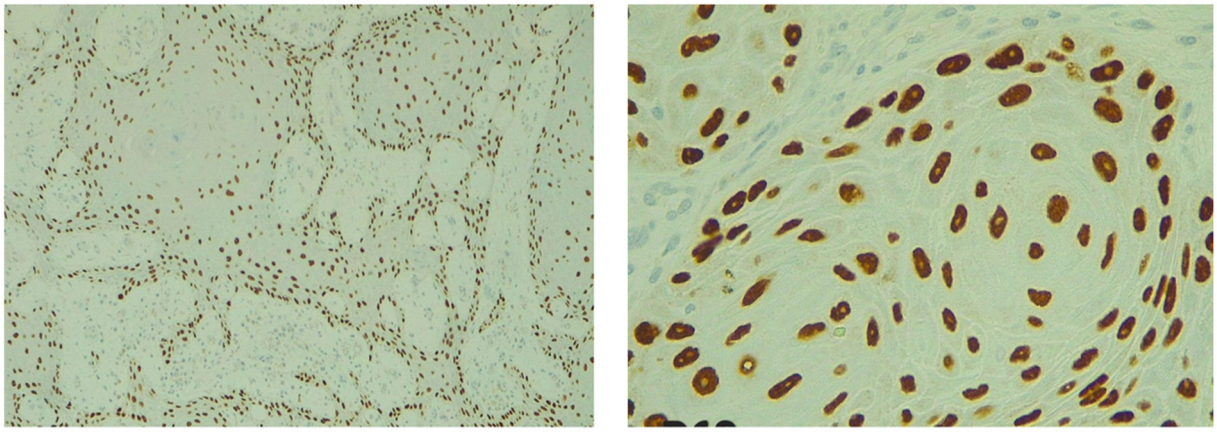


Figure 6. Strong nuclear immunoexpression of p63 in oral squamous cell carcinoma in all cases (immunohistochemistry; magnification: Left panel, x10; right panel, x40).

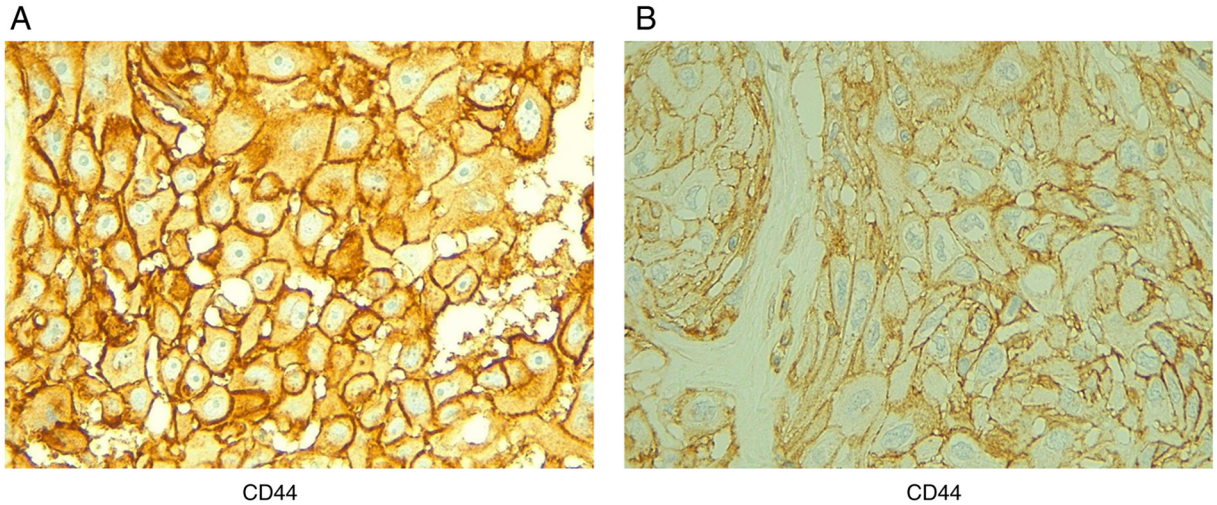


Figure 7. (A) Strong expression of CD44 in well-differentiated squamous cell carcinoma. (B) Less intense immunoexpression of CD44 in moderately differentiated squamous cell carcinoma. Immunohistochemistry (magnification, x40).

an easier time for cells to separate from a rigid. A low CD44 expression in OSCC tissues may be associated with lymph node metastasis and a sign of high metastatic potential. Thus, a poor prognosis may be associated with a decreased expression of CD44 (66,67).

The CD44 family consists of transmembrane glycoproteins that are widely expressed and can bind to extracellular matrix proteins, growth factors and hyaluronic acid. This family includes the standard form of CD44 (CD44s) as well as various alternative splice variants (CD44v). The overexpression of certain CD44 isoforms may be a key factor in the differences observed in the recurrence, locoregional or distant metastasis, and radioresistance of OSCC cells. Additionally, these distinct isoforms are often associated with lymph node metastasis and resistance to chemotherapy. Specifically, the overexpression of CD44v isoforms, such as v3 and v6, appears to be associated with increased cellular invasiveness and contributes to the heightened aggressiveness of some head and neck squamous cell carcinomas and OSCCs (68).

Yuan *et al* (69) reported a novel mechanism, demonstrating that MRE11 overexpression increased CD44 expression and tumor formation in OSCC cells, while MRE11 knockdown had the opposite effect. In addition, CD44 blockade using siRNA reduced MRE11-induced tumor formation, cell migration and AKT phosphorylation (69). Their study demonstrated an association between a high expression of CD44 and lymph node metastasis, as well as between MRE11, CD44 and phosphorylated AKT in OSCC tissues. In a mouse model, MRE11-overexpressing OSCC cells exhibited higher CD44 levels in metastatic lung nodules, with a positive association between CD44 and phosphorylated AKT. This mechanism provides a potential novel therapeutic strategy which can be used to target this pathway (69).

Qiao *et al* (70) reported that CD44 may play a role in the progression of OSCC through upregulation and was associated with the WNT, EGFR, NF-κB and ErbB signaling pathways. Previous studies have shown that ErbB signaling influences OSCC progression by activating the PI3K/Akt pathway. Essa and Deraz (71) reported that CD44 interacts with both

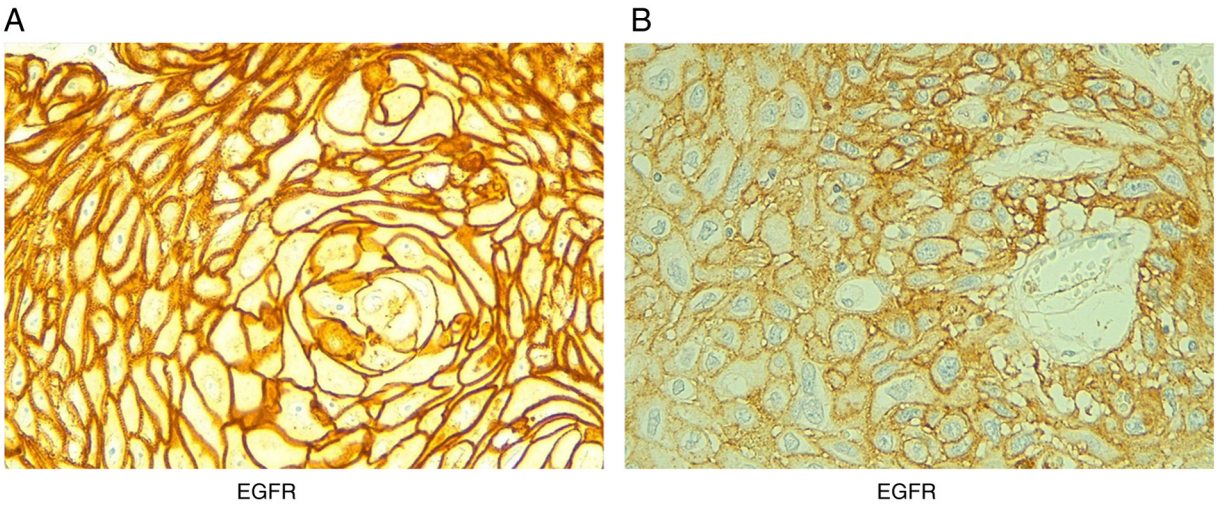


Figure 8. (A) Strong immunoexpression of EGFR in WDSCC; (B) less intense immunoexpression of EGFR in MDSCC. Immunohistochemistry (magnification, x40).

transforming growth factor-β and matrix metalloproteinase-9 in OSCC cells, which is essential for promoting tumor growth and invasion.

Cytoplasmic and nuclear-localized EGFR may be associated with increased proliferation rates in cancer cells (72,73). In the present study, by analyzing the immunomarker for EGFR, strong positive staining for EGFR expression was identified in WDSCC and less intense in MDSCC (in WDSCC, 76% of cases exhibited a strong staining intensity, whereas the intensity of staining was lower in MDSCC, namely 57%). There was a significant positive correlation between the intensity of EGFR expression and the grade of OSCC; this finding was similar to that in the study by Verma *et al* (74). There are conflicting reports of preferential expression of EGFR in either well or moderately differentiated tumors documented in the literature. Similar results were also observed in the study by Singla *et al* (34), which demonstrated a significant correlation between poor tumor differentiation and EGFR overexpression. According to previous research, the co-expression of EGFR is linked to a poorer prognosis and an invasive growth pattern (52,53). Another study investigated the prognostic significance of nuclear EGFR expression in patients with OSCC. The researchers identified nuclear 28% EGFR staining (23 patients), with no association between EGFR and patient outcomes or clinicopathological factors (75). Kappler *et al* (73) reported that the unclear prognostic impact of EGFR in patients with OSCC may be due to the presence of alternative forms of EGFR, which can activate different signaling pathways than the full-length form of EGFR.

The activation of EGFR leads to the induction of the phosphorylation of glycogen synthase kinase 3β (GSK3β) protein [breakdown of programmed cell death ligand 1 (PD-L1)], resulting in the prevention of the breakdown of PD-L1 by preventing the binding of GSK3β with PD-L1. PD-L1 helps cancer cells to escape from the immune system; thus, its breakdown can promote the improved function of the immune system against cancer. Thus, this phosphorylation can stop the activity of β-TrCP (degrade PD-L1), promoting PD-L1 stability in OSCC (76,77).

The primary method for acquiring one of the key characteristics of oral cancer, namely the capacity of tumor cells to sustain a continuous proliferation, is the constitutive oncogenic activation of EGFR (78). This then causes the cells to experience genomic instability, which makes it easier for them to acquire new additive oncogenic changes and distinctive characteristics that will be passed down clonally to their offspring. As in other human neoplasms where, on average, 50-70% of malignant cells overexpress EGFR, the oncogenic process associated with constitutive activation of EGFR is pertinent to oral carcinogenesis (79,80).

Although some key findings were reported in the present study, some limitations still need to be addressed. First, the addition of outcome and follow-up data would have provided a clearer image of the clinical relevance of these biomarkers. Secondly, the addition of more biomarkers, such as Ki-67 or VEGF, could enhance the understanding of OSCC. In the present study, the authors selected p63, CD44 and EGFR, as they are cortical in OSCC, and play a role in tumor growth and potential outcomes. To the best of our knowledge, the present study is the first to report these three biomarkers together. Previous studies have usually focused on only one or two biomarkers (14,23,50,51,59,81). Overall, further studies with long-term patient follow-up and outcome data are required in order to better understand the predictive value of these biomarkers, and their role in patient management and treatment decisions. Additionally, further research is required to investigate more sets of biomarkers. Expanding the biomarker panel to gain a clearer understanding of OSCC, may lead to the development of novel treatment options or indicators for prognosis.

In conclusion, for the prognosis and patient survival rates to improve, the early detection of malignancies is critical. Regrettably, the majority of cases are discovered when they are already in the advanced stages. In the present study, from a demographic point of view, it can be concluded that males in Basrah, Iraq are more likely to develop OSCC, with the tongue, lower jaw and buccal mucosa being the most common sites. The majority of cases of OSCC were

well-differentiated, with moderate cases following without poorly differentiated OSCC due to the limited case prevalence in Basrah city during the period of the study. Herein, a statistically a significant result was obtained between histological grades of OSCC and all variables (age, sex, site and year). In addition, the results revealed a highly significant positive correlation between the immunostaining intensity of all immunomarkers (p63, CD44 and EGFR) and the histological grades of OSCC. It was found that all cases of WDSCC and MDSCC displayed a positive immunoexpression for p63. However, the percentage of staining intensity was lower in WDSCC than in MDSCC. Thus, the tumor behavior in different grades of oral squamous cell carcinoma can be ascertained with the use of p63. Moreover, an increased immunoexpression of CD44 was more common in WDSCC and a decreased expression in MDSCC cells may be due to the reduced cell-to-cell and cell-to-matrix adhesion, resulting in easy detachment from the rigid constitution. It can be concluded that the immunoexpression of CD44 may be useful in predicting tumor stage, the ability to invade and the potential for metastatic spread. The immunoexpression of EGFR may be regularly included in surgical pathology reports as a prognostic marker to aid in improved patient management. Future research is required however, to explore and integrate advanced technologies (advanced genomic sequencing, single-cell RNA sequencing and new bioinformatics tools), to further investigate the molecular mechanisms underlying OSCC. In addition, novel therapeutic strategies and biomarkers to improve prognosis and treatment outcomes should be used. Future studies examining the association between these markers and treatment outcomes may help validate their potential in clinical settings.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

All authors (FLK, GHAQ, OFH and HAH) contributed to the design of the study, in the writing of the manuscript, in the statistical analyses and in data collection. GHAQ and OFH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval was obtained from the College of Dentistry at the University of Basrah, Basrah, Iraq after obtaining informed consent from the patients of the legal guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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