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Original Article

Correlation between clinicopathological indices and expression of cluster of differentiation 24 and cluster of differentiation 44 biomarkers in oral epithelial dysplasia and oral squamous cell carcinoma patients: A follow-up study

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is the most common oral cavity cancer and may occur following oral epithelial dysplasia (OED). Cancer stem cells (CSCs) can self-renew and multi-directionally differentiate to promote tumorigenesis with high expression of cluster of differentiation (CD) 24 and CD44 markers. CSCs play a pivotal role in tumor development, drug resistance, and relapse after treatment. We aimed to evaluate the correlation between both marker expressions and clinicopathological indices in OED and OSCC patients.

Materials and Methods: In this follow-up study, we could access 37 patients, including 12 OEDs and 25 OSCCs (Grade I: n = 9, Grade II: n = 8, and Grade III: n = 8). Data were analyzed using SPSS software (version 26) and log-rank tests, Fisher's exact test, Chi-square, and one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results: There was no significant difference in the expression of CD24 and CD44 markers between the study groups ($P > 0.05$) and the expression of both markers and clinicopathological indices in the study groups ($P > 0.05$). The mean and standard deviation of overall survival (OS) were 54.46 ± 43.08 with a range of 6–193 months, and they were 8.24 ± 15.34 months with a range of 0–70 months for disease-free survival (DFS) in patients, respectively. The average of DFS in Grade I was significantly lower than the OED ($P = 0.002$) and Grade II ($P = 0.039$) groups. The OS average in the Grade I ($P = 0.014$) and Grade III ($P = 0.004$) groups was statistically lower than the OED group.

Conclusion: Although more than half of the patients demonstrated high expression of both markers, there was no statistically significant difference between them and clinicopathological indices.

Key Words: Cluster of differentiation 24, cluster of differentiation 44, dysplasia, neoplastic stem cells, squamous cell carcinoma of head and neck

INTRODUCTION

Oral squamous cell carcinomas (OSCCs) are an oral cavity malignancy that originates from the

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epithelial cells of buccal mucosa, the floor of the mouth, the anterior tongue, alveolar ridges, retromolar trigone, the hard palate, and inner part of lips. OSCC comprises more than 90% of all oral cavity malignancies, the most frequent SCC of the head-and-neck region. OSCC is manifested in the tongue as the most common, and after that, the floor of the mouth is more frequent in males than females.[1] According to the International Agency for Research on Cancer report, oral cancer comprised more than 377,000 new patients and approximately 177,000 new deaths in 2020, with around 264,000 cases of incidence, which ranked it 16th in incidence and mortality worldwide.[2] It was reported that OSCC is the most common cancer in Southeast Asian countries, while it is the 16th most frequent cancer in Finland. One of the leading causes of the difference in global prevalence is related to the variations in lifestyle and cultures that exposure to carcinogenic risk factors such as tobacco utility and alcohol drinking.[3] Oral epithelial dysplasia (OED) is differentiated oral epithelial changes that affect normal cell growth and is known as an oral potentially malignant disorder (OPMD) that can transform into a malignant lesion such as OSCC.[4] Leukoplakia and erythroplakia are two types of OPMD highly associated with the manifestation of OED at first biopsy. However, lesions of submucous fibrosis develop OED after being present for years.[5]

The tumor microenvironment (TME) encompasses the tumor surrounding and comprises a complicated network, including the immune cells, cancer-associated fibroblasts, stromal cells, blood vessels, and signaling molecules.[6] These variabilities, besides genetic heterogeneity and diversity of OSCC, affect the biological behavior, tumor progression, and resistance to therapeutic approaches that result in poor prognosis. Characterization of tumor-specific molecular signature profile and TME can promote personalizing anti-cancer treatment for patients in addition to the early diagnosis.[7] Cancer stem cells (CSCs) are one of the rare heterogeneous subpopulations of tumor cells with the ability to self-renewal, proliferation, invasion, metastasis, angiogenesis, and multi-directional differentiation. In addition, CSCs can escape immune surveillance. They are pivotal in tumor development, drug resistance, and relapse after treatment.[8] The 5-year overall survival (OS) for early-stage OSCC is estimated at 70% to 90%; however, despite advances in its treatment, OS decreases to 50% for late-stage disease because of insufficient screening methods, tumor heterogeneity, and the absence of a definitive panel for diagnosis, prognosis, and management of OSCC patients.[9]

One of the main unique features of CSCs is cell surface markers that can be the target for druggable delivery, identification of the aggressive status of the disease, and giving this opportunity for personalized medicine following the presence of specific CSC subpopulations.[10] The most studied stem cell markers in OSCC patients included a cluster of differentiation 24 (CD24), CD44, octamer-binding transcription factor 4, SRY-box transcription factor 2 (SOX2), NANOG, aldehyde dehydrogenase 1 (ALDH1), phosphorylated signal transducer and activator of transcription 3, CD133, and Musashi-1.[11] The two most frequent CSC biomarkers are CD24 and CD44, which can assess for predictive prognosis of head-and-neck squamous cell carcinoma (HNSCC) patients and are suggested to apply for cancer target therapy.[12] Expression of CD24 and CD44 biomarkers contributes to the beginning, maintenance, and extension of tumor growth and angiogenesis in OSCC.[13]

CD24 is a small cell surface glycoprotein that participates in cell adhesion and metastasis and has been discovered in a wide range of cancer cells. CD44 is a large cell surface hyaluronan receptor protein that plays bimodal edge roles in cell migration and adhesion. Overexpression of CD44 correlated with poor OS following nodal metastasis, invasion, perineural invasion, and local recurrence in OSCC patients.[14] It was demonstrated that $CD24^{+}/CD44^{+}$ cells maintained self-renewal and differentiation stemness features, besides the chemoresistant to gemcitabine and cisplatin. In addition, they showed higher cell invasion *in vitro* and more colonies in collagen gels compared to $CD24^{-}/CD44^{+}$ HNSCC cells. *In vivo,* $CD24^{+}/CD44^{+}$ cells demonstrated larger tumor size in nude mice compared to $CD24^{-}/CD44^{+}$ cell population.[15]

According to our previous study,[16] we followed up with OED and OSCC patients to evaluate the correlation between the expression of CD24 and CD44 markers and clinicopathological indices, including grade, tumor, node, metastasis (TNM), age, sex, recurrence, death, OS, and disease-free survival (DFS).

MATERIALS AND METHODS

This follow-up study was conducted in four centers which include School of Dentisry, Qaim Hospital, Imam Reza Hospital and Omid Hospital of Mashhad in 2022. The study was approved by the local Ethical Committee of the Mashhad University of Medical Sciences (IR.MUMS.DENTSIRY.REC.1400.069). In our previous analytical-cross-sectional study in 2020,[16] the total sample size of 60 cases was determined as 15 cases in the OED group and 45 OSCC cases (20 – low grade and 25 – high grade). We assessed the expression of CD24 and CD44 genes by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). We showed a high expression of CD24 and CD44 markers in OSCC and OED, which correlated to the possibility of malignant transformation, which was more significant in the OSCC group. For following up on patients who participated in the mentioned study, we got access to 37 cases for examining clinicopathological indices, including age, gender, recurrence, type of recurrence, death, expression of CD24 and CD44 markers, OS, DFS, TNM, stage, and grade. If the registered information was incomplete, the necessary information was collected as much as possible by calling the patients or their relatives and then analyzed with appropriate statistical software. In the present study, we focused on the correlation between the expression of CD24 and CD44 biomarkers and clinicopathological indices, while in our previous study, we aimed to evaluate the correlation between mentioned biomarker expression and OSCC patients' grades. In addition, we could access 37 OSCC patients in a determined time; all participants were not primary OSCC, some of them were beginning their therapy, or their diseases were in progress from multiple months to years.

The condition of definite diagnosis based on histological staining, materials, and protocols applied for assessment of CD24 and CD44 biomarker expression by RT-qPCR, including RNA extraction, cDNA synthesis, sequence of primers, and RT-qPCR method besides statistical analysis methods, were mentioned in detail in our previous study.[16] The inclusion criteria were the patients available for follow-up. The exclusion criteria were the unwillingness of patients to continue their cooperation and whose changed number phone or address we could not contact. For the evaluation of OS, the date of disease diagnosis until the death of patients was considered, while some patients were not available or their documents were in a distant city. Data were analyzed using SPSS software (version 26) (IBM, Chicago, IL, USA) and log-rank tests, Fisher's exact test, Chi-square, and one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

In the present study, a total of 37 embedded paraffin-block samples, including 25 OSCC and 12 OED, were taken from archives of the Department of Oral and Maxillofacial Pathology, School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran. The clinicopathological information of the study population is mentioned in Table 1. The mean and standard deviation (SD) of the patient's age was 14.33 ± 59.22, ranging from 31 to 86 years. The mean and SD of the patient's OS were 43.08 ± 54.46, ranging from 9 to 193 months. The mean and SD of the patient's DFS were 8.24 ± 15.34, ranging from 0 to 70 months.

The average minimum and maximum mean age were related to Grades III (56.0) and I (62.8). Based on the results of one-way ANOVA, there was no statistically significant difference in mean age between the OED and OSCC groups ($P = 0.736$) based on the grades. A comparison of DFS between the OSCC group (based on grade) and OED is presented in Table 2.

The minimum and maximum mean DFS were related to the Grade I and II groups, respectively. The OED, Grade I, Grade II, and Grade III groups differed significantly regarding DFS's mean ($P = 0.005$). A pairwise group comparison showed that DFS's mean in Grade I was statistically lower than in Grade II ($P = 0.039$) and OED ($P = 0.002$). However, there was no statistically significant difference in pairwise comparisons between the other study groups ($P > 0.05$).

By Table 2, a comparison of OS between the OSCC group (based on different grades) and OED showed that the minimum and maximum OS mean was related to the Grade III and OED groups, respectively. The study groups differed significantly regarding OS mean ($P = 0.015$). A pairwise group comparison showed that the OS mean in the Grade I ($P = 0.014$) and Grade III ($P = 0.004$) groups

Table 1: Clinicopathological information of the study population

Variant	*n* (%)
OED	12 (32.4)
OSCC	25 (67.5)
Primary tumor (T)	
1.00	2 (5.4)
2.00	13 (35.1)
3.00	7 (18.9)
4.00	3 (8.1)
Lymph node involvement (*n*)	
0.00	10 (27.0)
1.00	6 (16.2)
2.00	7 (18.9)
3.00	2 (5.4)
Metastasis (M)	
Negative	17 (45.9)
Positive	8 (21.6)
Grade	
Grade I	9 (24.3)
Grade II	8 (21.6)
Grade III	8 (21.6)
Stage	
Stage I	2 (5.4)
Stage II	4 (10.8)
Stage III	6 (16.2)
Stage IV	13 (35.1)
Sex	
Men	17 (45.9)
Women	20 (54.1)
Recurrence	
No	22 (59.5)
Yes	15 (40.5)
Type of recurrence	
Local	12 (80.0)
Metastasis	3 (20.0)
Death	
No	17 (45.9)
Yes	20 (54.1)
CD24 expression	
Low	8 (21.6)
High	29 (78.4)
CD44 expression	
Low	12 (32.4)
High	25 (67.6)

OED: Oral epithelial dysplasia; OSCC: Oral squamous cell carcinoma; CD: Cluster of differentiation

was statistically less significant than OED. However, there was no statistically significant difference in pairwise comparisons between the other study groups ($P > 0.05$). We present the chart of survival curve based on grade for DFS [Figure 1a] and OS [Figure 1b] in study patients.

In accordance with Table 3, the comparison between CD24 expression and the study groups ($P = 0.755$), sex ($P = 0.428$), recurrence ($P = 0.690$), type of recurrence ($P = 0.516$), metastasis ($P > 0.99$), and death ($P = 0.246$) showed no statistically significant differences. In addition, the comparison between CD44 expression and the study groups ($P = 0.765$), sex ($P = 0.286$), recurrence ($P = 0.724$), type of recurrence ($P = 0.690$), metastasis ($P > 0.99$), and death ($P > 0.99$) demonstrated no statistically significant differences.

Table 2: Comparison of disease-free survival and overall survival between oral squamous cell carcinoma group (based on grade) and epithelial dysplasia

Group	*n*	DFS (months), mean±SE	OS (months), mean±SE
OED	12	$22.57^{a,*}$±4.41	$154.42^{a,*}$±19.398
Grade I	9	3.50^{b}±1.50	$52.59^{b,c}$±13.274
Grade II	8	28.33^{a}±20.83	$52.875^{a,c}$±11.524
Grade III	8	$18.33^{a,b}$±9.61	$47.000^{b,c}$±23.083
Log-rank test (χ^2, df, *P*)		12.88, 3, 0.005	10.41, 3, 0.015

*The unsimilar minimal letter in the columns showed a statistically significant difference ($P<0.05$). OED: Oral epithelial dysplasia; DFS: Disease-free survival; OS: Overall survival; SE: Standard error

There was no statistically significant difference between the DFS mean and high and low levels of CD24 expression in participants ($\chi^2 = 1.74$, $P = 0.187$), and there was no statistically significant difference between the DFS mean and high and low levels of CD44 expression in participants ($\chi^2 = 2.48$, $P = 0.116$) [Figure 2a and b].

There was no statistically significant difference between the OS mean and the high and low levels of CD24 expression in participants ($\chi^2 = 0.79$, $P = 0.373$), and there was no statistically significant difference between the OS mean and the high and low levels of CD44 expression in participants ($\chi^2 = 0.00$, $P = 0.996$) [Figure 2c and d].

DISCUSSION

In the present study, we were able to follow up 37 patients out of a total of 60 patients who participated in our previous study. These patients included 25 cases with OSCC (grade I: n = 9, grade II: n = 8, grade III: n = 8) and 12 cases with OED[16]. The correlation between clinicopathological indices and the expression of CD24 and CD44 biomarkers in the above patients was evaluated. Although in all the studied groups, more than half of the studied subjects showed high expression of both markers, there was no statistically significant difference in terms of sex, age, grade, stage, presence of recurrence, local

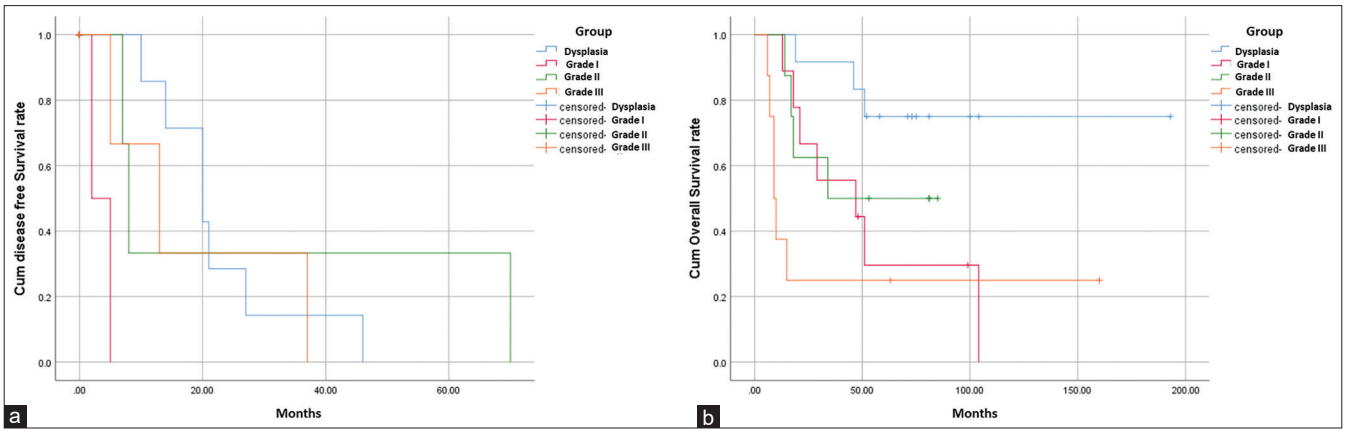

Figure 1: The Kaplan–Meier chart survival curves for disease-free survival (a) and overall survival (b) in study patients.

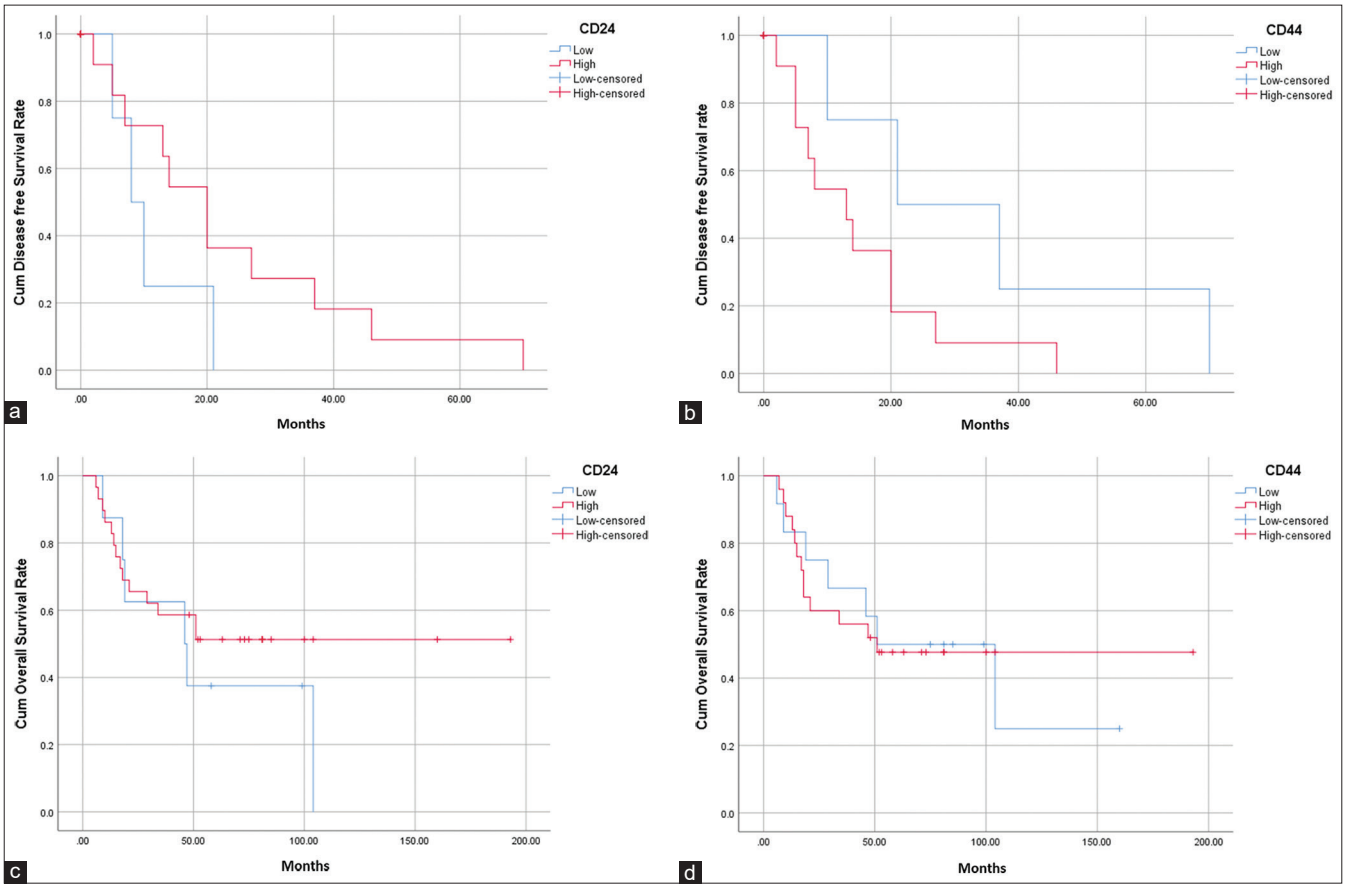

Figure 2: The Kaplan–Meier chart survival curves of disease-free survival for expression of cluster of differentiation 24 (a) and CD44 (b) markers in the study. The Kaplan–Meier chart survival curves of overall survival for expression of cluster of differentiation 24 (c) and CD44 (d) markers in the study.

recurrence, metastasis, death, DFS, and OS. In this study, it was expected that the DFS mean in Grade I was lower than OED. However, one of the study patients was diagnosed with OSCC due to OED, and the premalignant nature of some oral lesions, such as leukoplakia, was confirmed.[17] However, the current criteria do not justify the lower DFS mean compared to Grade II. This challenge may have originated from grading criteria that caused placing some OSCC samples as an intermediate status between Grades I and III, and it needs to improve the grading criteria. According to our previous research,[16] there was no significant difference between the studied groups in the expression of CD24 and CD44. In contrast,

Table 3: Comparison of cluster of differentiation 24 and cluster of differentiation 44 expressions with study groups and clinical indices

Variant	CD24 expression		CD44 expression	
	Low, *n* (%)	High, *n* (%)	Low, *n* (%)	High, *n* (%)
Group				
OED	3 (25)	9 (75)	3 (25)	9 (75)
Grade I	3 (33.3)	6 (66.7)	4 (44.4)	5 (55.6)
Grade II	1 (12.5)	7 (87.5)	2 (25)	6 (75)
Grade III	1 (12.5)	7 (87.5)	3 (37.5)	5 (62.5)
Fisher's exact test (*P*)	0.755		0.765	
Sex				
Women	3 (15)	17 (85)	8 (40)	12 (60)
Men	5 (29.4)	12 (70.6)	4 (23.5)	13 (76.5)
Chi-square test (*P*)	0.428		0.286	
Recurrence				
No	4 (18.2)	18 (81.8)	8 (36.4)	14 (63.6)
Yes	4 (26.7)	11 (73.3)	4 (26.7)	11 (73.3)
Fisher's exact test (*P*)	0.690		0.724	
Type of recurrence				
Regional	4 (33.3)	8 (66.7)	4 (33.3)	8 (66.7)
Metastasis	0	3 (100)	0	3 (100)
Fisher's exact test (*P*)	0.516		0.690	
Metastasis				
No	4 (23.5)	13 (76.5)	6 (35.3)	11 (64.7)
Yes	1 (12.5)	7 (87.5)	3 (37.5)	5 (62.5)
Fisher's exact test (*P*)	>0.99		>0.99	
Death				
No	2 (11.8)	15 (88.2)	5 (29.4)	12 (70.6)
Yes	6 (30)	14 (70)	7 (35)	13 (65)
Fisher's exact test (*P*)	0.246		>0.99	

OED: Oral epithelial dysplasia; CD: Cluster of differentiation

a statistically significant association was reported between the expression of both biomarkers in all three groups. This correlation was more significant in the OSCC groups ($P < 0.001$).

Recent research focused on the potential implications of precisely discovering various stem cell markers, evaluating their frequencies, and determining possible prognostic outcomes. They are involved in specific functions due to tumor progression and metastasis. Application of CD44 and CD24 combination in evaluating OSCC development, metastasis, and OS of patients showed 33.81% of $CD44^{-}/CD24^{+}$ followed by 25.6% of $CD44^{+}/CD24^{+}$, 23.67% of $CD44^{-}/CD24^{-}$, and 16.9% of $CD44^{+}/CD24^{-}$ expressions. Co-expression of both markers has been reported in different malignancies. Double-negative OSCC patients demonstrated satisfying clinical outcomes.[18]

CD44 and CD24 gained considerable interest in oncology, and a combination of these two markers is believed to characterize various tumors, including OSCC.

The association between clinicopathological indices and stage is more substantial than histological grade in OSCC patients.[17] In the current study, the existence of higher Stage III and IV cases with Grades I and II can explain the nonsignificance statistical correlation between the pathological grades or recurrence or other clinicopathological indices as well as the lack of expected progress from Grade I to Grade III. The highest OS mean was in OED, and a statistically significant difference was observed between Grades I and III in comparison to the OED in OS, which was expected since many oral lesions with premalignant features or dysplasia despite treatment not being promoted to the OSCC. The low OS mean in Grade I compared to the other grades and the statistically nonsignificant OS difference between Grade II and OED, besides the stronger correlation between stage rather than grade with clinicopathological indices, can be explained by this issue that more Stage III and IV patients with Grades I and II participated in our study.

In a cohort study, Adnan *et al*. 2022[19] evaluated the expression of CD44, CD133, L1CAM, and SOX2 in OSCC patients ($n = 100$). They found that high expression of CD44 was associated with poor OS but did not affect DFS. The minimum follow-up time for all patients was 5 years, which was longer than in our study. The OS and DFS means were 64 and 52.5 months, higher than our study at 54.46 and 8.24 months, respectively. Compared to the present study, we applied RT-qPCR while they used immunohistochemistry to assess biomarker expression. In addition, our study groups were not significantly different in the expression of biomarkers and all clinical indices, which could be due to the relatively small number of patients available with complete file information for follow-up.[19]

Szafarowski *et al*. in 2020[12] evaluated the levels of CD24, CD44, CD133, and ALDH1A1 expression in HNSCC ($n = 49$) and upper respiratory tract epithelial dysplasia ($n = 11$) patients in comparison to the controls ($n = 12$). Similar to our study, they also evaluated OSCC and OED patients, but their study method was tissue microarray and immunohistochemistry, while we used qRT PCR. In their study, only patients with at least 5 years of follow-up were included, and the follow-up period was between 60 and 104 months, which had a higher minimum follow-up period than ours.[12] In the

present study, we had some limitations; for example, we needed to determine a minimum follow-up point for participants in inclusion criteria. In addition, some information about patients needed to be registered completely, which excluded them from our study. However, in our study, there were patients with a more extended follow-up period, such as an OS of more than 193 months.

Tamatani *et al*. in 2018[20] evaluated the association between expression of CD44, CD44 v9, ABCG2, CD24, Bmi-1, and ALDH1 markers in Stage I and II OSCC (n = 70) and clinicopathological indices. Similar to our study, they assessed CD24 and CD44 markers and clinicopathological factors such as T classification, grade, metastasis, and DFS. Similar to our study findings, they did not find a significant relationship in CD24 expression with tumor size, histological differentiation, lymph node metastasis, or DFS. However, the number of biomarkers, the number of OSCC stages, and the sample size were different. In addition, they applied immunohistochemistry staining while we used RT-qPCR. Their study showed a significant relationship between CD24 expression and invasion.[20] Saghravanian *et al*. in 2017[21] assessed the correlation between CD44 and P63 expression and clinicopathological indices in OSCC patients (n = 45). Similar to our results, there was no statistically significant difference between CD44 expressions, age, sex, local tumor, and OS. In contrast, they found a statistically significant difference between CD44 overexpression and higher grade and stage in OSCC patients. Compared to our study, they evaluated both marker expressions with immunohistochemical staining, while we assessed them using RT-qPCR.

In the current study, the recurrence rate was 40.5%, observed briefly before the initial surgery or therapy, showing the direct correlation between therapeutic approaches and their potential side effects on recurrence that need upgrading and promotion. In addition, the high local recurrence (80%) indicates the need to identify new approaches and apply the ideal implementation of existing methods in the local control of cancer-affected areas. One of the main restrictions due to the following was patients' refusal to refer to the public health center during the COVID-19 pandemic.

Since the CSCs maintain self-renewal and regeneration features during the tumorigenesis process, they can escape from current therapeutic approaches and cause chemo/radiotherapy (RT) resistance, relapse, and metastasis. HNSCC heterogeneity is the main hurdle in response to therapy.[22] Identification of CSC markers that express differently in preneoplastic lesion malignancy, oral cancer progression, and treatment prognosis led us to use them for targeted anti-cancer therapy and improving chemotherapy efficiencies.[23] In a study, CD24 blockade with CD24 mAb promotes anti-tumor immunity in OSCC-related mouse models. This happened because targeting CD24 increased the anti-tumor immune response and inhibited tumor-associated macrophages and increased T-cell numbers that delayed tumor growth *in vivo*.[24] It was reported that circulating tumor stem-like cells expressed CD44 v6 and Nanog markers in OSCC patients were correlated to the different anatomical subsites, locoregional aggressiveness of the disease, and recurrence. This result provided new avenues for better prognostic and therapeutic applications in OSCC patients.[25] Recent studies proposed to assess the circulating tumor cells (CTCs) by liquid biopsy as a noninvasive method for early diagnosis, monitoring tumor process, and prognosis.[26,27] Because CSCs present molecular profile signatures of tumors with specific patterns of biomarker expression that can apply to the diagnosis and prognosis of HNSCC patients.[28] In addition, evaluation of biomarkers' expression before and after therapy can be applied to screening cancer progression and relapse.

Moreover, the remaining CSCs posttreatment through follow-up can be a valuable indicator.[27] However, there are some technical disagreements with appropriate sensitivity and specificity, besides the heterogenicity of HNSCC nature that still needs a definitive biomarker panel for tumor monitoring by biofluid biopsy, which remains the main challenge.[29] Recent studies suggested the application of three-dimensional (3D) organoid for the evaluation of drug sensitivity/resistance response according to the unique genetic profile signature of HNSCC patients, genetic manipulation for cancer modeling by targeting oncogenes/tumor suppressor genes, and single-cell analysis of CTCs for patient monitoring.[30] The establishment of 3D organoid technology, such as patient-derived organoids (PDOs) and organotypic culture, can mimic HNSCC heterogeneity and allow for cancer modeling and manipulation of critical subpopulations, as well as interactions between tumor cells and microenvironment.[31] This technology provides a

tool for biomarker validation and discovery based on personalized medicine for head-and-neck cancer. This Technology can mimic cellular behavior in response to drugs. In this way, it can be applied for therapeutic approaches and prognosis of patients.[32] In future studies, the application of 3D organoid before beginning therapy in HNSCC patients that burden heterogeneity in tumor mass such as CSCs and predict their biomarkers can track patients' response. For future studies, we proposed examining more CSC markers in large-scale populations, more homogenized samples, periodic longer follow-ups, and the utility of a more comprehensive information recording system.

CONCLUSION

Although 78.4% and 67.6% of OSCC and OED patients showed high expression of CD24 and CD44 biomarkers, respectively, no statistically significant difference was found in terms of sex, age, grade, stage, recurrence, regional recurrence, metastasis, OS, and DFS. More studies with larger sample sizes, homogenization of groups, longer period follow-up, more comprehensive information recording systems, and more patient cooperation are needed.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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