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Cyclin D1 and Ki-67 expression and its correlation with histopathological parameters and cervical lymph node metastasis in oral squamous cell carcinoma

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is the most common malignant tumor among oral cancers. Cyclin D1 and Ki-67 have associated with cell division. The aim of this study was to compare the expression of these markers in OSCC with and without cervical lymph node (LN) metastasis.

Materials and Methods: This cross-sectional study was performed on 40 OSCCs with and without cervical LN metastasis (20 in each group) that was recorded in the pathology archive of Ayatollah Kashani Hospital in Isfahan. Clinical information including age, gender, and location was collected. Some histopathological parameters including depth of invasion, lymphovascular invasion (LVI), perineural invasion (PNI), number of LN metastases, histopathological grade, and stage of disease were evaluated. Immunohistochemical staining was performed for cyclin D1 and Ki-67. All data were entered into SPSS24 software and were analyzed by Mann–Whitney, Kruskal–Wallis, Chi-square, Fisher's exact, and *t*-tests. $P < 0.05$ was considered statistically significant.

Results: Based on LVI and stage of disease, a significant correlation was found between the two groups ($P < 0.001$). There was a significant difference between the two groups based on cyclin D1 expression ($P = 0.05$). The expression of the Ki-67 showed a significant difference based on tumor location ($P = 0.026$) and PNI ($P = 0.033$).

Conclusion: The use of markers should be considered in determining the prognosis of OSCC, and the cyclin D1 marker is one of the useful markers for predictors of cervical LN metastasis.

Key Words: Cancer, cyclin D1, immunohistochemistry, Ki-67, oral

INTRODUCTION

Oral cancer is an important tumor and one of the main causes of death in the world. Oral squamous cell carcinoma (OSCC) is the most common malignant tumor among oral cancers.[1] Despite many advances in pathology and oncology, the prognosis of OSCC is still poor. Histopathological examination is still the gold standard method for diagnosis and treatment decisions.[2] Surgical resection, elective neck dissection, radiotherapy, and/or chemotherapy are preferred therapeutic methods for OSCC.[3]

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Tumor stage and lymph node (LN) status are two important factors in determining treatment methods and prognosis of the tumor.[4,5] Delayed detection of cervical LN metastasis may lead to further spread of the tumor.[3] Identifying clinical and histopathological parameters for predicting tumor behavior and the risk of cervical LN metastasis in OSCC patients is necessary. Cyclin D1 is a proto-oncogene that plays an important role in regulating the cell cycle by controlling cell entry from phase G-1 to S by contributing to the inactivation of the retinoblastoma gene product.[6] Increased expression of cyclin D1 is associated with a risk of developing tumors.[7-10] Overexpression of cyclin D1 has been reported in 35% to 40% of cases of head-and-neck SCC.[11] Ki-67 is another nuclear protein and a marker of cell proliferation in the G-2 and M-phases of the cell cycle that is associated with the presence and severity of epithelial dysplasia.[12] Some studies showed that overexpression of cyclin D1 is associated with increased recurrence and decreased survival in patients with OSCC.[12,13] Suresh *et al*. showed that there is a significant relationship between cyclin D1 and Ki-67 expressions with poorly differentiated tumor and cervical LN metastasis.[7] In recent years, many efforts are being made to identify prognostic factors in patients with OSCC including histological indicators and highly specific tissue markers. Hence, cyclin D1 and Ki-67 expressions and their correlation with histopathological parameters and cervical LN metastasis in OSCC were investigated in the present study.

MATERIALS AND METHODS

Specimen selection and clinicopathological study

This retrospective study collected formalin-fixed, paraffin-embedded tumor samples of 40 patients histologically diagnosed as OSCC with (Group 1) and without (Group 2) cervical LN metastasis (20 of each type) sourced from the archival of the Pathology Department of Ayatollah Kashani Hospital affiliated with Isfahan University of Medical Sciences, Iran. All the selected patients in the study had one excisional biopsy of primary tumor and cervical neck dissection between January 2015 and October 2021 and have complete clinicopathological data and available, suitable tissue specimens for review and immunohistochemical staining. The patients with previous treatment such as neoadjuvant chemotherapy or prior radiotherapy at presentation and those with distant metastasis were excluded from the study. The H- and E-stained slides of the cervical LNs of different levels harvested from the radical neck dissection specimens were evaluated for the presence or absence of tumor epithelial cell invasion. Accordingly, the samples with LN metastasis (Group 1) or without LN metastasis (Group 2) were determined. Informed consent was not required because of the retrospective nature of this study. The study was approved by the Institutional Ethics Committee of Isfahan University of Medical Sciences, Iran (IR.MUI.RESEARCH.REC.1399.769).

Demographic data and clinical features including age, gender, primary tumor site, tumor size (largest dimension), TNM stage, and histopathologic grade were collected from pathology reports. H and E stain sections of samples were reviewed by two blinded oral pathologists for confirmation of diagnosis and determination of histopathological parameters such as depth of invasion (DOI), lymphovascular invasion (LVI), perineural invasion (PNI), and the number of LN metastases. Invasion of tumor epithelial cells within or attached to the endothelial cell lining of the arterial, venous, or lymphatic channels was considered LVI. PNI is identified as tumor cell invasion to any layer of the nerve sheath or more than one-third of the nerve circumference. These parameters were identified under ×40, ×100 and confirmed under ×400 of magnification.[3] The distance between the lowest part of the adjacent normal mucosa and the lowest part of the tumor was considered DOI. It was measured by slide caliper and it was divided into D1 (≤5 mm), D2 (>5 mm, ≤10 mm), and D3 (>10 mm).[14] Histopathological grading of SCC was determined according to Bryne's histologic criteria that was grouped into 3 categories: well-differentiated SCC, moderately differentiated, and poorly differentiated SCC carcinoma.[15] TNM staging (Stage I-IVA) was performed according to the American Joint Committee on Cancer 7th edition.[16]

Immunohistochemistry

Immunohistochemistry (IHC) with the biotin–streptavidin method was performed on paraffin-embedded tissue sections of 3–4 μm thickness which were placed on slides for cyclin D1 and Ki-67 IHC staining. For antigen retrieval, tissue sections were deparaffinized and rehydrated with distilled water. To block any endogenous

activity, sections were placed in 3% H2O2 for 3 min and were washed in running tap water. Antigen retrieval was carried out by heating 1500 mL of the recommended retrieval solution (0.01 M citrate buffer, pH 6.0) until boiling in a pressure cooker. After reaching boiling temperature, they were kept at that temperature for 10 min, and the samples were immediately placed in cold water. The sections were washed in Tris-buffered saline (TBS) for 15 min and diluted in normal serum for 10 min. The sections were incubated with primary antibodies at 1:25 dilution (cyclin D1) and 1:50 dilution (Ki-67) overnight at 4°C (Dako EnVision FLEX System; Dako, Glostrup, Denmark) and washed in TBS for 2–5 min and were later incubated in appropriate biotinylated secondary antibody for 1 h at room temperature (Mouse EnVision System HRP, DakoCytomation). Visualization was performed using freshly prepared di-amino-benzidine chromogen for 10 min, and the slides were counter stained with the hematoxylin stain (Merck KGaA, Darmstadt, Germany). Breast tumors and tonsil tissue with intense staining for cyclin D1 and Ki-67, respectively, were considered positive control. For negative controls, the primary antibody was replaced with TBS for both markers.

Assessment of immunohistochemical staining

To analyze immunohistochemical staining, all slides were evaluated by two oral pathologists simultaneously in a blinded manner with light microscopy (Olympus BX41TF, Tokyo, Japan). The nuclear expression of the cyclin D1 and Ki-67 proteins was evaluated semi-quantitatively by a combination of the staining intensity (scored as: 1, weak; 2, moderate; and 3, strong) and the proportion of positively-stained tumor cells in 1000 tumor cells per high-power field (scored as 0, <20%; 1, 20 40%; 2, 41 60%; 3, 61%–80%; and 4, >80%). The sum of the staining intensity and the percentage of positive tumor cell scores were calculated as follows: +, 1–3; ++, 4–5; and +++, 6–7.[9]

Statistical analysis

The data obtained from clinical, histopathological, and immunohistochemical studies were analyzed by the Statistical Package for the Social Sciences, version 24.0 (SPSS Inc., Chicago, IL, USA) to assess statistically significant differences between samples using Mann–Whitney test, Kruskal–Wallis test, Chi-square test, Fisher's exact test, and *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinicopathological findings

A total of 40 cases, 20 each of OSCC with and without cervical LN metastasis were included in the present study. Among Group 1, most patients (70%) have only one LN metastasis. Furthermore, three patients (15%) had 2 LNs involved and three patients (15%) had 4 LNs involved. According to the results, the mean DOI (mean ± SD) was 23.7 ± 1.3 mm in Group 1 and 17.3 ± 1.2 mm in Group 2 which, despite the difference, the difference between the two groups was not significant based on Mann–Whitney test ($P = 0.06$).

According to Chi-square and Mann–Whitney tests, a significant difference was found between the two study groups based on LVI ($P < 0.001$) and stage of disease ($P < 0.001$). There was no significant difference between the groups based on other clinical and histopathological parameters, which is shown in Table 1 and Figure 1a-e.

Immunohistochemical findings

The frequency of the samples based on the sum of the staining intensity and the percentage of positive tumor cell scores for cyclin D1 and its relationship to clinicopathological parameters is presented in Table 2. Cyclin D1 positivity was seen in 32 cases (80.0%) of all samples. Based on Mann–Whitney test, cyclin D1 expression had a significant difference between the two study groups ($P = 0.05$) suggesting an increased rate of LN metastasis in OSCCs with overexpression of cyclin D1. Most OSCCs without LN metastasis have weak expression, and an increase in expression of cyclin D1 has been observed with increasing number of LNs involved. According to Mann–Whitney test, the cyclin D1 expression in OSCCs without LN metastasis compared with the OSCCs with metastasis of two LNs showed a significant difference ($P = 0.002$). Furthermore, there was a significant difference between the samples with one LN involved and two LNs involved in expression levels of cyclin D1 ($P = 0.047$). A statistical difference in cyclin D1 expression was not identified based on other clinicopathological parameters [Figure 1f].

Most samples of the study groups showed strong Ki-67 expression. According to Mann–Whitney test, no significant difference in Ki-67 expression was found between the groups ($P = 0.372$). Therefore, there was no relationship between the cervical

Table 1: Clinicopathological parameters in studied groups

Parameters	Groups		*P* (test)
	Group 1, *n* (%)	Group 2, *n* (%)	
Clinical parameters			
Age, mean±SD	59.45±19.98	59.95±14.2	0.928 (*t*-test)
Gender			
Male	11 (55)	8 (40)	0.342 (χ^2)
Female	9 (45)	12 (60)	
Site			
Tongue	12 (60)	15 (75)	0.132 (Fisher's exact test)
Alveolar mucosa	7 (35)	3 (15)	
Buccal mucosa	0	2 (10)	
Floor of mouth	1 (5)	0	
Size, mean±SD	2.09±1.24	2.02±1.39	0.813 (*t*-test)
Histopathological parameters			
DOI			
D1	6 (30)	9 (45)	0.06 (Mann–Whitney)
D2	4 (20)	8 (40)	
D3	10 (50)	3 (15)	
LVI			
No	1 (5)	18 (90)	<0.001 (χ^2)
Yes	19 (95)	2 (10)	
PNI			
No	17 (85)	13 (65)	0.144 (χ^2)
Yes	3 (15)	7 (35)	
Grade			
WDSCC	12 (60)	13 (65)	0.949 (Mann–Whitney)
MDSCC	8 (40)	5 (25)	
PDSCC	0	2 (10)	
Stage			
Stage I	0	10 (50)	<0.001 (Mann–Whitney)
Stage II	0	8 (40)	
Stage III	17 (75)	2 (10)	
Stage IVA	3 (15)	0	

SD: Standard deviation; DOI: Depth of invasion; LVI: Lymphovascular invasion; PNI: Perineural invasion; WDSCC: Well-differentiated squamous cell carcinoma; MDSCC: Moderately differentiated squamous cell carcinoma; PDSCC: Poorly differentiated squamous cell carcinoma

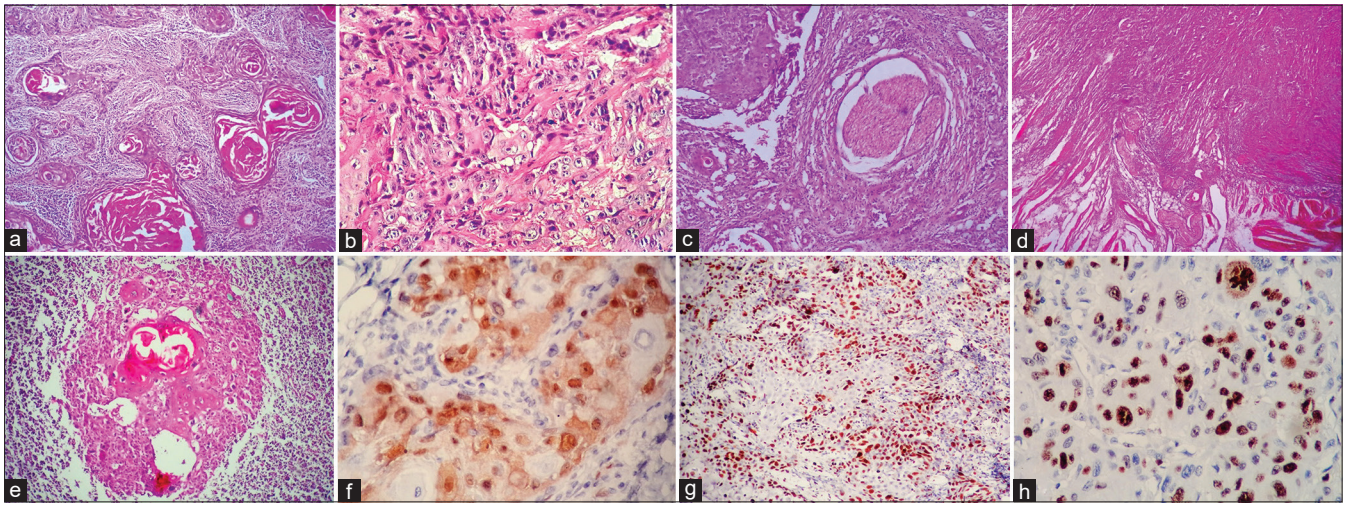

Figure 1: (a) Well-differentiated oral squamous cell carcinoma (OSCC) (H and E, ×100), (b) Poorly differentiated OSCC (H and E, ×400), (c) Perineural invasion (H and E, ×100), (d) Lymphovascular and muscle invasion (H and E, ×40), (e) Lymph node metastasis in OSCC (H and E, ×400), (f) Cyclin D1 expression (IHC, ×400), (g) Ki-67 expression (IHC, ×100), (h) Ki-67 expression (IHC, ×400).

Table 2: Correlation of cyclin D1 expression with clinicopathological parameters

Parameters	Cyclin D1				*P* (test)
	Negative, *n* (%)	+ (weak), *n* (%)	++ (moderate), *n* (%)	+++ (strong), *n* (%)	
Clinical parameters					
Gender					
Male	5 (26.3)	7 (26.8)	2 (10.5)	5 (26.3)	0.75 (Mann–Whitney)
Female	3 (14.3)	10 (47.6)	4 (19)	4 (19)	
Site					
Tongue	7 (25.9)	12 (44.4)	3 (11.1)	5 (18.5)	0.435 (Kruskal–Wallis)
Alveolar mucosa	1 (10)	4 (40)	1 (10)	4 (40)	
Buccal mucosa	0	1 (50)	1 (50)	0	
Floor of mouth	0	0	1 (100)	0	
LNs involved					
0	4 (20)	12 (60)	3 (15)	1 (5)	0.045 (Kruskal–Wallis)
1	3 (21.4)	4 (28.6)	3 (21.4)	4 (28.6)	
2	0	0	0	3 (100)	
4	1 (33.3)	1 (33.3)	0	1 (33.3)	
Histopathological parameters					
DOI					
D1	4 (26.7)	8 (53.3)	2 (13.3)	1 (6.7)	0.203 (Kruskal–Wallis)
D2	1 (8.3)	6 (50)	2 (16.7)	3 (25)	
D3	3 (23.1)	3 (23.1)	2 (15.4)	5 (38.5)	
LVI					
No	3 (15.8)	12 (63.2)	3 (15.8)	1 (5.3)	0.178 (Mann–Whitney)
Yes	5 (23.8)	5 (23.8)	3 (14.3)	8 (38.1)	
PNI					
No	4 (13.3)	13 (43.3)	6 (20)	7 (23.3)	0.148 (Mann–Whitney)
Yes	4 (40)	4 (40)	0	2 (20)	
Grade					
WDSCC	3 (12)	13 (52)	4 (16)	5 (20)	0.807 (Kruskal–Wallis)
MDSCC	5 (38.5)	3 (23.1)	1 (7.7)	4 (30.8)	
PDSCC	0	1 (50)	1 (50)	0	
Stage					
Stage I	1 (10)	7 (70)	1 (10)	1 (10)	0.533 (Kruskal–Wallis)
Stage II	2 (25)	4 (50)	2 (25)	0	
Stage III	4 (21.1)	6 (31.6)	3 (15.8)	6 (31.6)	
Stage IVA	1 (33.3)	0	0	2 (66.7)	
Groups					
Group 1	4 (20)	5 (25)	3 (15)	8 (40)	0.05 (Mann–Whitney)
Group 2	4 (20)	12 (60)	3 (15)	1 (5)	

DOI: Depth of invasion; LVI: Lymphovascular invasion; PNI: Perineural invasion; WDSCC: Well-differentiated squamous cell carcinoma; MDSCC: Moderately differentiated squamous cell carcinoma; PDSCC: Poorly differentiated squamous cell carcinoma; LNs: Lymph nodes

LN status and the Ki-67. There was no significant difference in the expression of this marker based on gender, number of LN metastases, DOI, LVI, grade of tumor, and clinical stage. Statistical analysis showed a significant difference between the Ki-67 expression based on the site of the lesion and PNI. A statistical difference in Ki-67 expression was identified between tongue and alveolar mucosa [$P = 0.005$, Table 3 and Figure 1g and h].

Based on Spearman nonparametric test, tumor size had a direct and significant relationship with the stage of the disease and DOI. Furthermore, DOI had a direct and significant relationship with the stage of disease, grade of tumor, and number of LN metastases. Cyclin D1 expression was also directly and significantly related to Ki-67 expression [Table 4].

DISCUSSION

LN metastasis is one of the important factors influencing the treatment outcomes and prognosis of oral cancer.[17] In the present study, most of the patients with OSCC were in their sixth decade of life, and cervical LN metastasis did not show a significant relationship with the mean age ($P = 0.871$), which

Table 3: Correlation of Ki-67 expression with clinicopathological parameters

Parameters	Ki-67				*P* (test)
	Negative, *n* (%)	+ (weak), *n* (%)	++ (moderate), *n* (%)	+++ (strong), *n* (%)	
Clinical parameters					
Gender					
Male	3 (15.8)	3 (15.8)	5 (26.3)	8 (42.1)	0.64 (Mann–Whitney)
Female	0	7 (33.3)	4 (19)	10 (47.6)	
Site					
Tongue	3 (11.1)	9 (33.3)	7 (25.9)	8 (29.6)	0.026 (Kruskal–Wallis)
Alveolar mucosa	0	1 (10)	0	9 (90)	
Buccal mucosa	0	0	1 (50)	1 (50)	
Floor of mouth	0	0	1 (100)	0	
LNs involved					
0	1 (5)	4 (20)	5 (25)	10 (50)	0.658 (Kruskal–Wallis)
1	2 (14.3)	3 (21.4)	4 (28.6)	5 (35.7)	
2	0	2 (66.7)	0	1 (33.3)	
4	0	1 (33.3)	0	2 (66.7)	
Histopathological parameters					
DOI					
D1	1 (6.7)	3 (20)	5 (33.3)	6 (40)	0.571 (Kruskal–Wallis)
D2	1 (8.3)	2 (16.7)	2 (16.7)	7 (58.3)	
D3	1 (7.7)	5 (38.5)	2 (15.4)	5 (38.5)	
LVI					
No	0	5 (26.3)	5 (26.3)	9 (47.4)	0.486 (Mann–Whitney)
Yes	3 (14.3)	5 (23.8)	4 (19)	9 (42.9)	
PNI					
No	1 (3.3)	6 (20)	7 (23.3)	16 (53.3)	0.033 (Mann–Whitney)
Yes	2 (20)	4 (40)	2 (20)	2 (20)	
Grade					
WDSCC	2 (8)	4 (16)	6 (24)	13 (52)	0.07 (Kruskal–Wallis)
MDSCC	1 (7.7)	6 (46.2)	3 (23.1)	3 (23.1)	
PDSCC	0	0	0	2 (100)	
Stage					
Stage I	0	2 (20)	5 (50)	3 (30)	0.886 (Kruskal–Wallis)
Stage II	1 (12.5)	2 (25)	0	5 (62.5)	
Stage III	2 (10.5)	4 (21.1)	4 (21.1)	9 (47.4)	
Stage IVA	0	2 (66.7)	0	1 (33.3)	
Groups					
Group 1	2 (10)	6 (30)	4 (20)	8 (40)	0.372 (Mann–Whitney)
Group 2	1 (5)	4 (20)	5 (25)	10 (50)	

DOI: Depth of invasion; LVI: Lymphovascular invasion; PNI: Perineural invasion; WDSCC: Well-differentiated squamous cell carcinoma; MDSCC: Moderately differentiated squamous cell carcinoma; PDSCC: Poorly differentiated squamous cell carcinoma; LNs: Lymph nodes

is consistent with the studies of Li *et al.*,[18] Wang *et al.*,[19] and Jardim *et al.*[20] In Nazar *et al.*'s study, the mean age of patients with LN metastasis was higher than patients without LN metastasis.[21] In Kikuchi *et al.*'s study, 59.7% of patients with cervical metastasis and 53.4% of patients without cervical metastasis were more than 70 years old.[22] In Batelja-Vuletic *et al.*'s study, the mean age of patients without cervical LN metastasis was 62.8 years and patients with cervical LN metastasis was 61.9 years, which did not show a statistically significant difference.[23]

In the present study, 60% of patients in Group 2 were females and 55% of Group 1 were males, which can be concluded that the risk of LN metastasis was higher in men, although there was no statistically significant difference (P = 0.342). In most studies, males were more involved than females in both the study groups.[18,19,21,22] However, in Li *et al.*'s[18] and Jardim *et al.*'s[20] studies, no significant difference was reported between patients with and without LN metastasis based on gender, which is similar to the results of the present study. Geographical areas and the importance of lifestyle in the occurrence of OSCC

Table 4: Relation between expression of two markers with each other and other clinicopathological parameters

Parameters	Size	DOI	LNs involved (*n*)	Grade	Stage	Cyclin D1	Ki-67
Age							
Significant	0.314	0.493	0.878	0.284	0.774	0.671	0.277
Correlation coefficients	0.163	0.112	0.025	0.174	0.047	0.069	0.176
Size							
Significant	-	0.007	0.252	0.957	0.003	0.932	0.362
Correlation coefficients	-	0.418	0.185	0.009	0.463	0.014	0.148
DOI							
Significant	-	-	0.027	0.034	0.004	0.101	0.656
Correlation coefficients	-	-	0.349	0.336	0.445	0.263	−0.73
Grade							
Significant	-	-	0.67	-	0.548	0.7	0.345
Correlation coefficients	-	-	0.069	-	0.463	−0.63	−0.153
Stage							
Significant	-	-	<0.001	-	-	0.211	0.76
Correlation coefficients	-	-	0.862	-	-	0.202	−0.5
Cyclin D1							
Significant	-	-	0.321	-	-	-	0.037
Correlation coefficients	-	-	0.205	-	-	-	0.331
Ki-67							
Significant	-	-	0.482	-	-	-	-
Correlation coefficients	-	-	−0.114	-	-	-	-

DOI: Depth of invasion; LNs: Lymph nodes

as well as the sample size can be the reasons for differences in the results.

In the present study, the tongue was the most common site of the tumor, but there was no significant difference between the two groups based on the location (P = 0.132). This result is consistent with the studies of Suresh *et al.*,[7] Li *et al.*,[18] and Kikuchi *et al.*[22] On the other hand, similar to our study, in Li *et al.*'s study, the majority of the samples involved <3 cervical LNs.[18] Limitations in the selection of samples and small sample size compared to other studies can be the reasons for these results.

In this study, there was no significant difference between the groups based on tumor size (P = 0.868). However, the mean size of tumors with LN metastasis (Group 1) was slightly larger which is in line with the Nazar *et al.*'s study.[21] However, in Li *et al.*'s[18] and Hoda *et al.*'s[24] studies, a significant relationship has been reported between the cervical LN status and tumor size. In Woolgar's study, lesions larger than 4 cm were associated with a high rate of cervical LN metastasis.[25]

In this study, the mean DOI was not significantly different between the two groups (P = 0.06). However, the mean DOI in lesions with LN metastasis was higher than in noninvolved specimens, which is similar to the findings of Suresh *et al.*[7] In the study of Li *et al.*,[18] Wang *et al.*,[19] and Sahoo *et al.*,[3] a significant relationship was reported between the DOI and LN status, which differs from the results of the present study.

In our study, a significant relationship was found between the groups based on LVI (P < 0.001), which is contrary to the results of Suresh *et al.*,[7] Li *et al.*,[18] and Chen *et al.*[26] but is consistent with the findings of studies by Wu *et al.*,[27] Adel *et al.*,[28] and Sahoo *et al.*[3] Furthermore, in this study, no significant relationship was found between cervical LN status and PNI (P = 0.144), which contradicts Sahoo *et al.*'s study.[3] Invasion of blood vessels and nerves by tumor cells is an important pathological factor and is one of the early stages of metastasis. PNI alone has been described as one of the pathways of metastasis and tumor spread. However, there are still many contradictions between vascular invasion and neurological invasion with cervical LN metastasis and the prognosis of OSCC.[3,18]

In this study, the cervical LN metastasis was not significantly associated with the grade of tumor (P = 0.255), which is consistent with some studies.[22,24] However, Akhter *et al.*'s,[29] Suresh *et al.*'s,[7] Li *et al.*'s,[18] and Jardim *et al.'s*[20]

studies reported a significant relationship. In most studies, the rate of cervical LN metastasis has increased by decreasing cell differentiation and increasing the grade of tumor. Well-differentiated OSCCs have stronger intercellular connections compared to poorly differentiated tumors and more adhesion molecules such as E-cadherin. Therefore, the rate of metastasis is expected to be higher in poorly differentiated tumors. Thus, cervical neck dissection is recommended for patients with poor differentiation OSCC.[18,24,30,31] Limitations of sample size and medical centers, followed by low sample with poor differentiation, can be the reasons for differences in the results of the present study with other studies.

In this study, a significant relationship was found between cervical LN metastasis and disease stage ($P < 0.001$), which is similar to the results of other studies[18,32] but is in contradiction with the results of Wang *et al*.'s study.[19] Although different rates of cervical LN metastasis have been reported in different studies, the risk of regional and distant metastasis increases with the increasing stage of the disease.[18] However, the clinicopathological factors studied to predict the risk of cervical LN metastasis in various studies have shown contradictory results, so more effective biomarkers with more sensitivity are needed to achieve solutions for predicting regional and distant metastasis.

The cyclin D1 marker plays a role in cell cycle regulation and regulates G1 to S cell cycle transfer. Reduction or overexpression may lead to disruption of normal cell cycle control and tumor formation and has increased overexpression in many malignant tumors.[33-35] The cyclin D1 expression in the two groups of this study showed a significant difference ($P = 0.05$) which resembles other studies.[7,11,21,36] Furthermore, the expression of this marker did not show a significant difference with other clinicopathological factors except the number of cervical LNs involved in our study. In Carlos de Vicente *et al*.'s study, overexpression of cyclin D1 was significantly associated with LN metastasis, stage, and tumor size, but no association was found between the expression of this marker with gender, tumor location, and grade of tumor.[37] In a study by Neves, the expression of this marker increased with increasing grade of tumor, although this difference was not significant.[38] In the study by Huang, overexpression of cyclin D1 was significantly associated with LN metastasis, grade, and stage.[33] Contrary to our study, in this study, the expression of this marker in buccal mucosal lesions was lower than in other sites ($P = 0.007$). However, in line with the present study, there was no significant relationship with patients' age and PNI.[33] Ramasubramanian *et al*.,[39] Angadi and Krishnapillai,[40] and Moharil *et al*.[41] showed that the expression of this marker increased significantly with increasing epithelial dysplasia. In Ohnishi *et al*.'s study, cyclin D1 expression did not show a statistically significant difference based on gender, tumor location, cervical LN status, and grade of tumor, but 90% of metastatic foci had a strong staining for cyclin D1.[9] Zhao also showed that increased cyclin D1 expression was significantly associated with increased tumor size, LN metastasis, grade of tumor, and clinical stage and had a significant negative effect on overall survival.[10] In the study by Mishra, 73.9% of patients under 40 years of age and 50% of patients between 40 and 70 years of age had a positive cyclin D1 marker. 60.8% of males and 68.7% of females had positive staining. 100% of moderately differentiated tumors, 66.6% of well-differentiated samples, 50% of poorly differentiated samples, and 0% of healthy tissue samples were positive for this marker.[2] Patel reported with increasing grade of malignancy, the cyclin D1 expression has increased, although statistically a significant difference has been reported between the well differentiation and poorly differentiated tumors.[12] Suresh *et al*. also showed an association between cervical LN metastasis and the grade of tumor with cyclin D1 expression.[7] Contrary to these studies, in the study of Guimarães *et al*., cyclin D1 expression had no significant relationship with gender, mean age, clinical appearance, LN metastasis, grade, and tumor recurrence.[42] Furthermore, Mahdey *et al*. did not show a relationship between this marker and LN metastasis.[43] Contrary to our study, Saawarn *et al*.[44] and Lam *et al*.[45] reported a significant association between the expression of this marker and grade of tumor. However, similar to our study, they did not report a significant association between stage, age, gender, and location with cyclin D1 expression. While Ramos-García *et al*. showed that overexpression of cyclin D1 has a strong statistical correlation with tumor size, stage, and grade in OSCC.[34] Although different results have been reported in various studies on the expression of cyclin D1, all of these studies have shown overexpression of cyclin D1 along with the rate of

invasion and tumor progression. Therefore, it can be considered a prognostic marker for OSCC.

The Ki-67 protein is a nuclear protein that is expressed in proliferating cells from phase G1 to M of the cell cycle.[26] According to the results of this study, the level of Ki-67 expression in OSCC showed a significant difference only based on the location of the lesion, and this marker was not related to the status of LNs and other clinicopathological parameters. In the study of Carlos de Vicente *et al*., Ki-67 expression was not significantly associated with gender, tumor location, stage, and LN status. However, this marker was significantly associated with the grade of tumor.[37] In the study of Ohnishi *et al.*, Ki-67 expression based on gender, tumor location, stage, and grade did not show a statistically significant difference.[9] In the study of Guimarães *et al*., Ki-67 based on gender, mean age, clinical appearance, LN metastasis, grade, and tumor recurrence was not significantly related.[42] Furthermore, in Nazar *et al*.'s study, the Ki-67 expression did not show a significant difference between samples with and without cervical LN metastasis.[21] Contrary to the results of these studies, in Suresh *et al*.'s study, the Ki-67 expression was significantly associated with cervical LN metastasis in OSCC.[7] In the study of Gadbail *et al*., Ki-67 expression was significantly different based on grade, clinical stage, and LN metastasis.[32] Batelja-Vuletic *et al*. reported that the Ki-67 expression in OSCCs with cervical LN metastasis was higher than without metastasis, which was a significant difference. Furthermore, the expression of this marker had a significant relationship with the disease stage.[23] In Jabbari *et al*.'s study, a significant difference was observed between Ki-67 expression and increased degree of dysplasia.[46] In He *et al*.'s study, Ki-67 protein expression was significantly associated with stage, grade, and cervical LN metastasis.[47] Jing showed that overexpression of Ki-67 was associated with poorly differentiated tumors and samples with cervical LN metastasis.[48,49] In contrast to these studies, Gonzalez-Moles showed that Ki-67 expression was significant only by grade of tumor, but Ki-67 is not a valuable marker for predicting cervical LN metastasis and prognosis.[50] Differences in the sample size can be considered reasons for differences in study results. However, there is still much controversy about the Ki-67 for determining and predicting regional and distant metastasis.

CONCLUSION

Some histopathological features should be given special attention in OSCC. The use of markers should be considered in determining the prognosis and cyclin D1 is one of the useful markers for predictors of cervical LN metastasis.

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Conflicts of interest
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