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Original Article

Human papillomavirus in oral squamous cell carcinoma using p16 and its co-relationship with cervical lymph node metastasis and clinicopathological parameters

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ABSTRACT

Background: Due to the increasing prevalence of oral squamous cell carcinoma (OSCC) in Iran and especially in young people, this study aimed to identify human papillomavirus (HPV) using p16 in OSCC.

Materials and Methods: In this descriptive-analytical cross-sectional study, 40 samples from the archives of the Pathology Department of Kashani Hospital were selected by a definitive diagnosis of OSCC with neck dissection. Demographic information including age, gender, location, and size of the lesion was obtained. Samples were divided into two groups based on lymph node (LN) metastasis. Immunohistochemical staining was performed for p16. Data were entered into SPSS 24 software and statistically analyzed by *t*-test, ANOVA, and Spearman nonparametric test. $P < 0.05$ was statistically significant.

Results: The mean age of patients was 59.7 ± 17.11 which in terms of age and gender there was no significant difference between the two groups including with and without cervical LN metastasis ($P > 0.05$). There was no significant difference between the two groups based on the grade of tumor, perninural invasion, tumor size and location ($P > 0.05$). The only significant difference between the two groups was based on lymphovascular invasion and disease stage ($P < 0.05$). The p16 expression also showed a significant difference between the two groups ($P < 0.05$).

Conclusion: In OSCCs without cervical LN metastasis, a significant increase in p16 expression was observed compared to samples with cervical LNs metastasis. The presence of HPV was higher in samples with less LNs metastasis and possibly a better prognosis.

Key Words: Immunohistochemistry, neoplasms, papillomaviridae

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INTRODUCTION

Squamous cell carcinoma (SCC) is the sixth most common cancer in the world.[1] This tumor accounts for approximately 5% of all malignant tumors in developed countries and also accounts for 90% of all oral malignancies.[2,3] In the last two decades, the prevalence of head and neck cancers including

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oral and pharyngeal SCC has increased.[4] Oral SCC (OSCC) often originates from the squamous epithelium and is morphologically similar to the SCC of other parts of the body such as the cervix, anus, and lungs. The increase in prevalence especially in young people without the common risk factors indicates the presence of other risk factors in the etiology of head-and-neck SCC (HNSCC).[4,5] Lifestyle and environment have important effects on the body's biological system and have a direct and indirect role in causing cancer.[6,7] According to most studies, tobacco use, chewing tobacco, betel quid, and alcohol are major risk factors for OSCC[8,9] but some patients with SCC do have not these risk factors, indicating the presence of other etiological factors such as genetics, diet, oncogenic viruses, and their effect on the physiological mechanism of cell proliferation control.[8]

The human papillomavirus (HPV) is a family of double-stranded DNA viruses with 100 different genotypes that are highly susceptible to skin epithelial cells and mucosal cells.[10,11] The virus usually enters the genome of the host cell and the entry of virus DNA is important in the transformation of malignancy. The carcinogenicity of the HPV is dependent on two proteins encoded by the E6 and E7 genes in the DNA of the virus. The E6 protein binds to P53 and suppresses it. It binds to the PRb tumor suppressor protein. E7 and E6 proteins of HPV inactivate two important tumor suppressor proteins that regulate the cell cycle.[12,13] If precancerous lesions are not detected in the early stages of the infection, they can progress from benign to the malignant lesion. Therefore, early diagnosis and treatment of viral infections can be effective in preventing the progression of lesions.[12] p16 is a protein that regulates the cell cycle. In normal cells, p16 expression is very low in immunohistochemical staining. Due to the change in activity on E7 oncogene, p16 is highly expressed in HPV-positive cells.[13,14]

According to recent studies, HPV-positive HNSCC is most commonly found within the oropharynx lesions.[4,7] However, few studies have been performed on the effects of HPV on clinicopathologic parameters and prognosis of patients with OSCC.[5,6,9] Surgical resection, elective neck dissection, radiotherapy, and/or chemotherapy are preferred therapeutic methods for OSCC.[3] Tumor stage and lymph nodes (LNs) status are two important factors in determining treatment methods and prognosis of the tumor.[5,6] Delayed detection of cervical LNs metastasis may lead to further spread of the tumor.[3] Identifying clinical and histopathological parameters for predicting tumor behavior and the risk of cervical LNs metastasis in OSCC patients are necessary. Due to the high prevalence of OSCC in Iran and its unfavorable prognosis in the most patients and the fact that immunohistochemistry is effective for the detection of viral-specific antigens and has high sensitivity and specificity,[15] the aim of this study was to detection of HPV in OSCC with p16 using immunohistochemical staining and its association with cervical LNs metastasis and other clinicopathological parameters.

MATERIALS AND METHODS

Patient's selection

In this descriptive-analytical cross-sectional study, medical records of patients with OSCC undergoing excisional biopsy and surgical neck dissection treatment between January 2015 and October 2021 from the archival of the Pathology Department of Ayatollah Kashani Hospital of Isfahan University of Medical Sciences, Iran, were retrospectively evaluated. The patients had no history of other cancer and no previous treatment such as neoadjuvant chemotherapy or radiotherapy. The patients had no distant metastasis. Furthermore, there was quality, suitable and sufficient samples for immunohistochemical staining. Finally, formalin-fixed, paraffin-embedded tumor samples of 20 patients with LNs metastasis (Group 1) and 20 patients without cervical LNs metastasis (Group 2) were selected. The study was approved by the Institutional Ethics Committee of Isfahan University of Medical Sciences, Iran (IR.MUI.RESEARCH.REC.1399.802).

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 (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 LNs metastasis. LVI was positive if tumor cells were presented within the lymphovascular channels. PNI is identified as tumor cells 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.[16] 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).[17] Histopathological grading of SCC was determined according to Bryne *et al*. histologic criteria that were grouped into three categories: well-differentiated SCC, moderately differentiated SCC, and poorly differentiated carcinoma.[18] TNM staging (Stage I-IVA) was performed according to the American Joint Committee on Cancer 7th edition.[19]

Immunohistochemical staining

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 p16 IHC staining following deparaffinization done with xylene, rehydration was done with (100%, 80%, and 60%) alcohol and distilled water, and antigen retrieval was carried out using ethylenediaminetetraacetic acid buffer (PH 9.0) for p16. A primary monoclonal mouse anti-human p16 antibody (BioGenex, USA) and a secondary antibody detection kit (Dako Omnis, California, USA) were used in this study. Visualization was performed using freshly prepared di-amino-benzidine chromogen for 10 min and the slides were counterstained with the hematoxylin stain (Merck KGaA, Darmstadt, Germany). Cervix OSCC with intense staining for p16 was considered a positive control. For negative controls, the primary antibody was replaced with Tris-buffered saline.

Assessment of immunohistochemical staining

To analyze immunohistochemical staining, all slides were examined by two oral pathologists blindly and simultaneously with light microscopy (Olympus BX41TF, Tokyo, Japan), and cells were counted at ×400 magnification in 10 randomly selected fields. Tissue samples with cytoplasmic and/or nuclear brown staining of tumor cells were considered positive. The epithelial cells were evaluated using the semi-quantitative scale: 0 (negative: Without immunostained cells), +1 (<25% immunostained), +2 (25%–50%), and +3 (>50%). Furthermore, staining intensity was evaluated on the following scores: 0 (without immunostained cells), +1 (very low staining), +2 (low), +3 (moderate), and +4 (high). The staining intensity distribution (SID) score was calculated by multiplying the distribution by staining intensity.[20] The pattern of cell staining was also divided into focal and diffuse.[21]

Statistical analysis

The clinicopathological data and immunohistochemical staining results were analyzed by the Statistical Package for the Social Sciences, version 24.0 (SPSS Inc., Chicago, IL, USA) to assess statistically significant differences between groups using ANOVA, *t*-test, Spearman nonparametric test. A $P < 0.05$ was considered statistically significant.

RESULTS

In this study, of 40 OSCCs were 4 (10%) negative samples and 36 (90%) positive samples in immunohistochemical staining for p16. Figure 1a shows the positive cells of OSCC for p16. The clinicopathological and IHC findings are summarized in Table 1. p16 expression was slightly higher in females than men and this difference was not significant by *t*-test ($P = 0.774$). The mean age (mean ± standard deviation) of patients in Group 1 was 59.45 ± 19.98 and it was 59/95 ± 14.2 in Group 2 and the mean age of all patients was 59.7 ± 7.11. Based on Spearman nonparametric test, p16 expression had no significant relationship with the mean age of patients ($P = 0.912$, $r = 0.018$).

The highest mean SID index of p16 was related to buccal mucosal lesions and the lowest mean SID index was related to tongue lesions. According to ANOVA, p16 expression was not statistically significant based on tumor location ($P = 0.037$). The samples without cervical LNs metastasis (Group 2) showed the highest mean SID index. The difference in p16 expression was statistically significant based on the number of cervical LNs metastasis ($P < 0.001$). The lesions with dimensions <5 cm had the highest mean SID and with increasing tumor size, the mean SID index decreased. However, this difference in p16 expression was not statistically significant based on tumor size ($P = 0.81$).

In histopathological evaluation, the samples without LVI had higher p16 expression than the lesions with LVI, which was a significant difference based on the ANOVA test ($P < 0.001$). On the other hand, p16 expression in the lesions with PNI was higher than in lesions without it, but there was no statistically significant difference ($P = 0.97$). In addition, with increasing the histopathological grade of tumor, p16

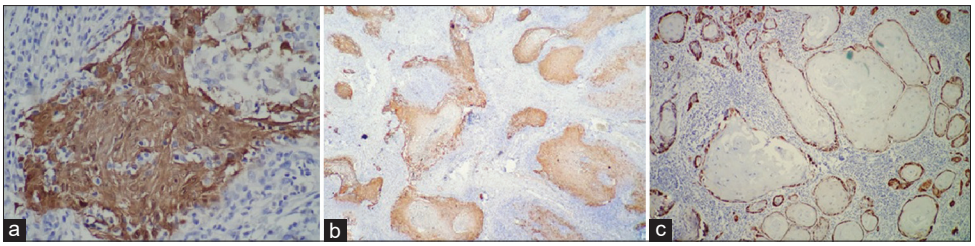

Figure 1: (a) p16 expression in OSCC (IHC × 400), (b) Diffuse pattern of p16 staining (IHC × 100), (c) Focal pattern of p16 expression (IHC × 100). OSCC: Oral squamous cell carcinoma, IHC: Immunohistochemistry.

Table 1: Mean±standard deviation of staining intensity distribution index for p16 expression in oral squamous cell carcinoma

Parameters	p16		*P*
	n (%)	Mean±SD	
Clinical parameters			
Gender			
Male	19 (47.5)	3±2.66	0.774
Female	21 (52.5)	3.33±4.33	
Site			
Tongue	27 (67.5)	2.6±3.12	0.037
Alveolar mucosa	10 (25)	3.2±2.9	
Buccal mucosa	2 (5)	10±8.4	
Floor of mouth	1 (2.5)	4±0	
LNs involved			
0	20 (50)	4.7±4.47	<0.001
1	14 (35)	1.71±1.26	
2	3 (7.5)	1.33±0.57	
4	3 (7.5)	1.33±1.52	
Histopathological parameters			
DOI			
D1	15 (37.5)	4.2±4.6	0.81
D2	12 (30)	3.16±3.71	
D3	13 (32.5)	2±1.15	
LVI			
No	19 (47.5)	1.66±1.15	<0.001
Yes	21 (52.5)	4.84±4.58	
PNI			
No	30 (75)	3.6±3.56	0.97
Yes	10 (25)	3.03±3.66	
Grade			
WDSCC	25 (62.5)	3.56±4.15	0.788
MDSCC	13 (32.5)	2.76±2.52	
PDSCC	2 (5)	1±0	
Stage			
Stage I	10 (25)	6±5.51	<0.001
Stage II	8 (20)	3.12±2.69	
Stage III	19 (47.5)	1.94±1.84	
Stage IVA	3 (7.5)	1.66±1.52	
Groups			
Group 1	20 (50)	1.6±1.18	0.008
Group 2	20 (50)	4.7±4.47	

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

expression has decreased. Furthermore, the difference in p16 expression based on the grade of tumor was not statistically significant (P = 0.788). With the increasing stage of the disease, the mean SID index of p16 was decreased. p16 expression was significant based on the stage of disease according to ANOVA test [P < 0.001, Table 1].

p16 expression in Group 2 was higher than in Group 1 that there was a significant difference between the two groups based on cervical LNs metastasis (P = 0.008). The pattern of tumor cell staining by p16 in the most samples was diffused that there was no significant difference between the two groups based on it (P = 0.574). Figure 1b and c show the diffuse and focal staining pattern of OSCC for p16, respectively.

DISCUSSION

It is now clear that cancer is caused by a number of genetic changes that disrupt the natural control of cell growth and its final differentiation.[22,23] Although many advances have been made in medical science in the field of SCC, the prognosis of OSCC with an overall survival rate of 5 years is about 40%. The most important predictor of prognosis is regional and distant metastasis of these lesions. If there is only one LN involvement, the survival rate is reduced by half.[24,25] Therefore, LNs metastasis is one of the important factors influencing the treatment outcomes and prognosis of oral cancer.[26]

Overexpression of p16 is significantly associated with HPV infection.[27,28] In the present study, most of the patients with OSCC were in their sixth decade of life and cervical LNs metastasis did not show a significant relationship with the mean age of patients, which is consistent with the studies of Li *et al.*,[29] Wang *et al.*,[30] Jardim *et al.*[31] and Rezaei *et al.*[32] While in Nazar *et al.*'s study, the mean age of patients without LNs metastasis was higher than patients with LNs metastasis.[33] In Kikuchi *et al.*'s study, 59.7% of

patients with metastasis and 53.4% of patients without cervical metastasis were more than 70 years old.[34] In Batelja-Vuletic *et al*.'s study, the mean age of patients without cervical LNs metastasis was 62.8 and patients with cervical LNS metastasis was 61.9 years, which did not show a statistically significant difference.[35]

In the present study, the p16 was used to determine the HPV in OSCC. Furthermore, 30% of the OSCC samples had p16-poitive cells, which in studies of Li *et al*.,[29] 12.6%, Zito Marino *et al*.[36] 18.4%, Sabu *et al*.[37] 20.58%, Broccolo *et al*.[38] 9.1%, Wang *et al*.[39] 9.5%, Götz *et al*.[40] 94/5%, Sgaramella *et al*.[41] 33%, Fonmarty *et al*.[42] 7/43%, Patil *et al*.[21] 66/86%, Ramshankar *et al*.[43] 15.38%, Saghravanian *et al*. 13.16%,[44] and 10% in Tokuzen *et al*. study[45] reported positive lesions. These differences may be due to technical sensitivity in the IHC staining method. Another reason is the presence of samples from different parts of the oropharynx. Although the majority of these studies have evaluated oral SCC, some of these studies performed only on lesions on the base of the tongue. Differences in lifestyle and geographical location may also be reasons for this difference.

In our study, p16 expression in OSCC without LNS metastasis was higher than OSCC with LNs metastasis that this difference was statistically significant. Our results are consistent with studies by Li *et al*.,[29] Wang *et al*.,[39,46] Jitani *et al*.[47] and Gillison *et al*.[48] Furthermore, in Mena *et al*.'s study, a significant relationship was found between the incidence of HPV and advanced stages of the disease.[49] In Orosz *et al*.'s study, all HPV-positive samples had LNs metastasis.[50] In contrast to our study, LNs metastasis was significantly higher in the HPV-positive samples than in the negative-HPV lesions in Saghravanian *et al*.'s study.[44] In the study of de Abreu *et al*.,[51] Huang *et al*.,[52] Götz *et al*.,[40] Fonmarty *et al*.[42] and Tokuzen *et al*.,[45] no association was found between the status of cervical LNs and the incidence of HPV. In Sgaramella *et al*.'s study, 72% of the OSCC without LNs metastasis and 52% of the samples with LNs metastasis were negative.[41] Unlike our study, no significant relationship was reported between the with and without LNs metastasis of OSCC based on p16 expression in Rezaei *et al*.'s study.[32]

In the present study, a significant relationship was found between the p16 expression and location of the lesion, LVI, and stage of disease, but there was no significant relationship with other parameters such as age, gender, tumor size, PNI, and tumor grade. In the Orosz´s *et al.* study, no significant difference was reported between the HPV and the location of the lesion and the age of the patients.[50] In Sabu *et al*.'s study, the mean age of p16-positive samples (46 years) was lower than p16 negative samples (53 years). There were also significant HPV-positive samples at the base of the tongue and HPV-negative samples often in the lesions of the soft palate. Although similar to the results of our study, the p16 expression did not show a significant relationship with gender, mean age, and grade of the tumor.[37] In the de Abreu *et al*.'s study, there was a significant relationship between the mean age of patients (HPV-positive patients had a mean age of 61 years and HPV-negative patients had a mean age of 57.5), gender (men were more in both positive and negative HPV), location (tongue is the common location in positive-HPV), stage (all HPV-positive patients were in stages 3 and 4), tumor size and HPV were not found.[51] Consistent with these studies, in the study of Wang *et al*.[39] and Tokuzen *et al*.,[45] the p16 expression in OSCC was not have a significant relationship with gender, age, lesion, tumor grade, and size. In Huang´s *et al*. study, there was no significant relationship between the presence of HPV and location, tumor grade, DOI, LVI, and PNI.[52] Götz *et al.* also found no association between HPV and tumor location.[40] While in Fonmarty´s *et al.* study no significant relationship was found between p16 and age, tumor size, cervical LNs metastasis, and stage, but a significant relationship was found with tumor location and grade.[42] In Gillison *et al*.'s study, HPV was significantly associated with poor tumor differentiation and late-stage disease.[48]

While in our study, p16 expression was higher in more differentiated OSCCs. In Mulder *et al*.'s study, there was a significant difference between well and moderate differentiation tumors and poorly differentiated tumors based on p16 expression.[53] In Saghravanian *et al*.'s study, no relationship was found between age, gender, location and tumor size, tumor grade, and recurrence rate with HPV.[44] In Manjula *et al*.'s study, PNI was identified in 31 (29%) cases of OSCC, which was significantly associated with LNs metastasis.[54] Differences in the sample size, the method of determining the presence of HPV and the location of the lesions can be the reasons for the different results in the studies. We found that there

was no significant relationship between the DOI and LNs status. However, in the Aaboubout´s study, 39% of patients had a DOI more than 4 mm.[55]

Most patients in this study with cervical LNs metastasis had only one LN involved at the time of diagnosis, therefore early detection of OSCC is very important in the prognosis of patients. In the study by Zhang *et al*., thirteen of 1875 patients with OSCC had LNs metastasis. Eight of them (61%) had stage IV and all patients had 3 LNs metastasis.[56] Recent studies have shown that HPV-positive cases respond better to treatment after chemotherapy and have a much better prognosis than others. They have a higher survival rate and a lower risk of progression compared to HPV-negative cases.[57,58] A possible reason for this could be that E6 has deactivated P53 but remains intact. E7 also inactivates PRB in the same way. If E6 and E7 are removed, the apoptotic pathway can be repaired that making the tumor more sensitive to treatment. Conversely, in the absence of HPV, P53, and PRb mutate and leading to their elimination and carcinogenesis, which is commonly seen in tobacco and alcohol-related tumors. These tumors have a worse prognosis.[59]

According to the present study, the pattern staining of tumor cells was mostly diffused, but there was no significant difference between the LNs status and other clinicopathological parameters. Only in Patil *et al*.'s study, the staining pattern was examined, which showed the most samples of well-differentiated OSCC had rare singly dispersed cells, while the patchy pattern was the most samples of moderately differentiation tumors and the most samples of poorly differentiated OSCC had diffuse pattern.[21] Also in the Tokuzen *et al*.'s study, most of the positive samples for p16 had intense and diffuse staining.[45]

CONCLUSION

p16 expression had a significant and inverse relationship with LNs status and the stage of the disease. In other words, with increasing the number of LNs metastasis and the stage of the disease, expression of p16 has been decreased. Therefore, this marker can probably be used to determine the prognosis and appropriate treatment of patients at the time of diagnosis.

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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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