IJMS
Vol 49, No 3, March 2024

Original Article

Human Papillomavirus-Associated Oral Epithelial Dysplasia: A Practical Approach to Make the Diagnosis

Kiarash Parchami[1], DDS; Samira Derakhshan[1], DDS; Hana Saffar[2], MD; Pouyan Aminishakib[1], DDS; Ahmad Reza Shamshiri[3], MD; Samaneh Afshar[2], MSc

[1]Department of Oral and Maxillofacial Pathology, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran;
[2]Department of Pathology, Cancer Institute Hospital, IKHC, Tehran University of Medical Sciences, Tehran, Iran;
[3]Department of Community Oral Health Department, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran

Correspondence:
Kiarash Parchami, DDS;
Department of Oral and Maxillofacial Pathology, School of Dentistry, North Amirabad, Postal code: 14399-5991, Tehran, Iran
Tel: +98 21 88008425
Email: kiarash.parchami@yahoo.com
Received: 20 November 2022
Revised: 31 January 2023
Accepted: 20 March 2023

What's Known

- Human papillomavirus (HPV) plays a significant role in the development of oropharyngeal dysplasia.
- The role of HPV in oral dysplasia has been proven.
- The Chromogenic *in situ* hybridization (CISH) method is standard for determining the presence of HPV in oropharyngeal dysplasia.
- HPV diagnostic method in oral dysplasia needs more studies.

What's New

- HPV has the potential to cause oral dysplasia, as indicated by a prevalence rate of 9.25%.
- HPV has not shown a different incidence in different severity of dysplasia.
- In some cases, polymerase chain reaction (PCR) is necessary in addition to CISH to detect HPV in oral dysplasia.

Abstract

Background: High-risk Human Papillomavirus (HPV) genotypes are found in malignant oral epithelial lesions, and HPV infection is proposed as a risk factor for initiating Squamous cell carcinoma (SCC) in the head and neck region. This study suggests a practical approach to detect HPV in HPV-associated oral epithelial dysplasia (HAOED).

Methods: Fifty-four oral epithelial dysplasia specimens were examined, comprising twenty-seven cases diagnosed with high-grade dysplasia and twenty-seven cases diagnosed with low-grade dysplasia using a binary grading system. To assess the cases for HPV, the specimens were examined for p16 protein using an immunohistochemical (IHC) study, and then, the Chromatin *In Situ* Hybridization (CISH) test was performed for all positive cases. Chromatin Immunoprecipitation-Polymerase Chain Reaction (ChIP-PCR) was performed on CISH-positive specimens to assess the outcome. This cross-sectional study was conducted in 2020 at Tehran University of Medical Science. SPSS software version 22.0 was used to perform the Chi square or Fisher's exact test to examine the relationship between variables (statistically significant level $P<0.05$).

Results: The expression of p16 protein was not associated with the severity of epithelial dysplasia (81.5% in low-grade and 59.2% in high-grade cases) ($P=0.16$). Moreover, according to the CISH test result, 9.25% of all specimens were positive ($P>0.99$), and in the nine cases, undergone the ChIP-PCR study, two cases (22.2%) showed positivity for HPV-16, while one case (11.1%) demonstrated positivity for HPV-51.

Conclusion: Regarding HAOED, here, we proposed a step-by-step combination approach using different diagnostic methods, including IHC for p16 protein, CISH, and ChIP-PCR based on a complementary algorithm.

Please cite this article as: Parchami K, Derakhshan S, Saffar H, Aminishakib P, Shamshiri AR, Afshar S. Human Papillomavirus-Associated Oral Epithelial Dysplasia: A Practical Approach to Make the Diagnosis. Iran J Med Sci. 2024;49(3):186-195. doi: 10.30476/IJMS.2023.96202.2897.

Keywords ● Human papillomavirus virus ● Squamous cell carcinoma of head and neck ● Immunohistochemistry ● *In situ* hybridization ● Polymerase chain reaction

Introduction

Human papillomavirus (HPV) is a well-known etiologic factor in the development of uterine cervical cancers,[1] and oropharyngeal carcinomas.[2] Regarding the distinct clinical behavior of

HPV-associated OPCs, pathologists are committed to clarifying the HPV status of this cancer based on the recently published eighth edition of the American Joint Committee on Cancer (AJCC) staging guideline, that HPV-positive cancers are down-staged from IV to I in comparison with the seventh edition.[3] Additionally, although smoking tobacco and alcohol consumption are the main risk factors for initiation of Oral Squamous Cell Carcinoma (OSCC), the role of high-risk HPV infection is recently associated with the pathogenesis of a subset of this cancer as a cofactor[4] through molecular alteration of epigenetic factors.[5]

Oral Epithelial Dysplasia (OED) is a histopathologically challenging topic for a step of epithelial alteration between normal condition and malignancy, and it is now well-described as an Oral Potentially Malignant Disorder (OPMD).[6] Clinical presentation of OED mostly ranges from leukoplakia to erythroplakia or an irregular mix of these lesions.[7] In the past 50 years, many attempts have been made to provide a reproducible OED grading system and practically link pathologists to clinicians for an effective therapeutic intervention without overtreatment.[8]

Meanwhile, from the primary introduction of "Koilocytic dysplasia",[9] several studies have proposed a distinct uncommon subtype of OED as "HPV-associated Oral Epithelial Dysplasia" (HAOED)[10] and have made the diagnosis of this OPMD more critical and complicated. A recently published meta-analysis showed that 25.3 percent of OEDs are associated with HPV infection; though the method of HPV detection- immunohistochemistry, PCR, or *In Situ* hybridization (ISH)- has a significant impact on the sensitivity of the detection.[11] Although high-risk HPVs- including HPV16- are commonly identified in HAOED similar to oropharyngeal cancers, long-term follow-up is necessary to recognize any clinical difference between the malignant transformation of positive and negative cases.[12]

The present study utilized various HPV detection methods in HAOED cases to identify a feasible, sensitive, and reproducible algorithm to differentiate HAOED from common OED cases. Due to the multifactorial nature of oral dysplasia such as SCC and the effect of various factors on it and also due to the proven role of HPV in dysplasia cases and the onset of SCCs in the head and neck region, aimed to evaluate the presence of HPV in OED cases using several diagnostic methods.

Patients and Methods

Patients and Specimens

Formalin-fixed paraffin-embedded blocks of OED cases were obtained from the Oral and Maxillofacial Pathology Department's laboratory, Tehran University of Medical Sciences (TUMS), Tehran, Iran. The inclusion criteria were the diagnosis of OED in their pathologic reports and the absence of inflammation with lichenoid pattern in the target tissue in histopathological examination. In all stages of this study, samples were previously prepared for diagnostic purposes from patients and were not for this study. For this reason, informed consent was not obtained from the patients. This cross-sectional study was conducted in 2020 at Tehran University of Medical Science and ethically approved by the Ethics Committee of the School of Dentistry, Tehran University of Medical Sciences (No. IR.TUMS.DENTISTRY.REC.1399.096).

Demographic information of all the patients, including age, sex, and anatomical site of the lesion, were extracted from the submitted records.

In accordance with the findings of Angerio and others study,[13] the frequency of high-risk HPV was observed to be 8% and 46% in samples with low and high degrees of hyperplasia, respectively. To scrutinize the variance between the two ratios, amounting to 38%, while maintaining a type 1 error rate of 5% and a statistical power of 80%, at least 17 samples are necessary in each group. As a precautionary measure, if the difference in frequency between the groups is postulated to be 30%, at least 27 samples are required in each group. To estimate the required sample size for evaluating the hypothesis of comparing the prevalence of HPV between samples with low and high degrees of hyperplasia, the following statistical formula was employed to compare two proportions.[14]

$$n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2 (p_1(1-p_1) + p_2(1-p_2))}{(p_1 - p_2)^2}$$

Microscopic Evaluation and Grading

Histopathologic assessment of the specimens was performed on hematoxylin and eosin (H&E) stained 4-µm-thick paraffin sections. Microscopic findings, consisting of acanthosis, hyperkeratosis, papillomatosis, verrucous pattern, and koilocyte-like cells, were included in the histopathologic examination.

All OEDs were graded according to the binary grading system[15, 16] and were divided into two groups of ''low-risk'' or ''high-risk'' cases. The binary system was proposed by Omar Kojan and colleagues and divided dysplastic lesions into two groups: high-grade and low-grade. High-grade dysplasia lesions are determined by having at least four criteria of changes in the

general shape of the epithelium and five criteria of cytological changes of the WHO criteria based on microscopic observation. Low-grade dysplasia is determined based on observing less than four general changes in the epithelium or less than five cytological changes (WHO criteria).[15]

Immunohistochemical Study

An immunohistochemical (IHC) study for p16 was performed on 4-µm-thick paraffin sections. All the sections were deparaffinized and rehydrated. Primary antibody (Mouse anti-human p16INK4A, Monoclonal Antibody, Clone MX007, Master Diagnostica, Spain; dilution 1:40, pH 7.3) incubated for 10 min. Master Polymer Plus Detection System (HRP) (DAB included; Master Diagnostica-000237QK, Spain) was used as the detection system. Finally, the slides were counterstained with hematoxylin and mounted. HPV-positive oropharyngeal carcinoma specimen was used as a positive control. Omitting the antibody and using phosphate-buffered saline were performed as a negative control.

Two experienced oral pathologists (S.D. and P.A.) blindly assessed stained slides for p16 expression. Both nuclear and combined nuclear/cytoplasmic stained specimens were considered positive. The positive cells were scored according to the proportion of stained cells as follows: 0%=0, 1-10%=1, 11-50%=2, 51-80%=3, 81-100%=4. It finally scored as follows:

Score 0: Negative (no positive cells)

Score 1: Focally positive (1-80% of cells show positivity).

Score 2: Diffusely positive (81-100% of cells show positivity).[17]

Chromogenic In Situ Hybridization

A Chromogenic In Situ Hybridization (CISH) study was performed in 37 cases, showing scores of 1 and 2 in the IHC study for p16. Briefly, after dehydration in 100% ethanol for 1 min, 10 µl of ZytoFast CISH Probe (ZytoFast PLUS CISH Implementation Kit AP-Permanent Red, Zytovision, Prod. No. T-1151-40, Germany) for HPV genotypes 16, 18, 31, and 33 (ZytoFast HPV type 16/18 Probe, Prod. No. T-1056-400, Germany and ZytoFast HPV type 31/33 Probe, Prod. No. T-1057-400, Germany) were used, and the slides were assessed using light microscopy.

Chromatin Immunoprecipitation PCR

Among the samples that were subjected to the IHC test for p16 protein and CISH, to finalize and confirm the diagnosis of the presence or absence of HPV, the HPV Direct Flow CHIP test was performed on two groups of samples, including samples whose result was focally positive in the p16 test and equivocal in the CISH test (N=5), and samples whose results were diffusely positive in the p16 test and negative in the CISH test (N=4). We used the HPV Direct-Flow Chip Kit (HS12, PCR Reagents, Master Diagnostia, Granada, Spain). In this protocol, the clinical samples can be amplified directly with no need to extract DNA. Amplification cycling conditions in the peqSTAR XS ThermoCycler followed the manufacturer's instructions. 5 µL of the liquid suspension under the paraffin layer was used as a DNA template. Automated reverse hybridization was performed on hybriSpot 24 (HS24, ref.MAD-003930MU-HS24, Master Diagnostica, Spain), which allows the DNA target molecules to cross the membrane and bind to the complementary probes. NBT-BCIP substrates were added to colorimetric detection by detecting alkaline phosphatase activity and creating insoluble purple precipitates.

Statistical Analysis

The Chi square test (or Fisher's exact test, if appropriate) was performed to investigate the relationship between independent (OED grade and histopathologic factors) and qualitative dependent (IHC for p16 and CISH test for high-risk HPV) variables. The statistically significant level was considered less than 0.05. For quantitative variables, because they did not follow the normal distribution, the median was reported along with the first and third quartiles (Q1 and Q3). Statistical analyses were performed using SPSS software version 22.0 (IBM, Armonk, NY, USA).

Results

A total of fifty-four OED cases were examined, comprising twenty-seven cases diagnosed with high-grade dysplasia and twenty-seven cases diagnosed with low-grade dysplasia. In the low-grade group, 12 females (44.4%) and 15 males (55.6%), and in the high-grade group, 17 females (63.0%) and 10 males (37.0%) were included. The mean age of the patients with low-grade and high-grade dysplasia was 56.93 (ranging from 29 to 78) and 62.89 (ranging from 29 to 78), respectively.

Immunohistochemistry

The percentage of p16 protein expression in both low-grade and high-grade groups indicates a median of 10%. Besides, in the low-grade group, the inter-quarter range was 35% (Q1=5, Q3=40), and in the high-grade group, the inter-quarter range was 50% (Q1=0, Q3=50).

According to the p16 protein expression, in the low-grade group, five out of 27 cases (18.5%) showed a final score of 0, 20 out of 27 cases (74.1%) demonstrated a final score of 1, and two out of 27 cases (7.4 %) were observed with a final score of 2. Furthermore, in the high-grade group, 11 out of 27 specimens (40.7%) showed a final score of 0, 13 out of 27 specimens (48.1%) demonstrated a final score of 1, and three out of 27 specimens (11.1%) were observed with a final score of 2 (figure 1).

There was no significant association between the expression of p16 protein expression and the severity of dysplasia (P=0.16).

Chromogenic In Situ Hybridization

The CISH study for HPV-16 and -18 showed three positive cases (11.1%) in the low-grade group and two positive cases (7.4%) in the high-grade group. There was no significant association between HPV-16 and -18 positivity and the severity of dysplasia (P>0.99).

Moreover, the CISH study for HPV-31 and -33 demonstrated one positive case (3.7%) in the low-grade group and two positive cases (7.4%) in the high-grade group, which showed no significant association between HPV-31 and -33 positivity and the severity of dysplasia (P>0.99).

Furthermore, three specimens showed positive expression for all HPV-16, -18, -31, and -33 using a CISH study (figure 2).

Chromatin Immunoprecipitation PCR

Out of the five samples whose IHC staining result was diffusely positive and the CISH test result was negative, in the ChIP-PCR test, four samples were negative for the presence of HR-HPV, and only one sample reported the presence of HPV-51.

Out of the four samples, the final score of IHC staining was focally positive, and in the CISH tests the results were equivocal. In the ChIP-PCR test for the presence of HR-HPV, HPV-16 was positive in two samples, and in two samples, the presence of the virus was reported as negative.

In total, nine specimens were assessed for high-risk HPVs using ChIP-PCR, and six cases (77.7%) were negative, two cases (22.2%) were positive for HPV-16, and one case (11.1%) was positive for HPV-51.

Finally, three cases from the low-grade group and three cases from the high-grade group showed positivity, and there was no significant association between the presence of high-risk

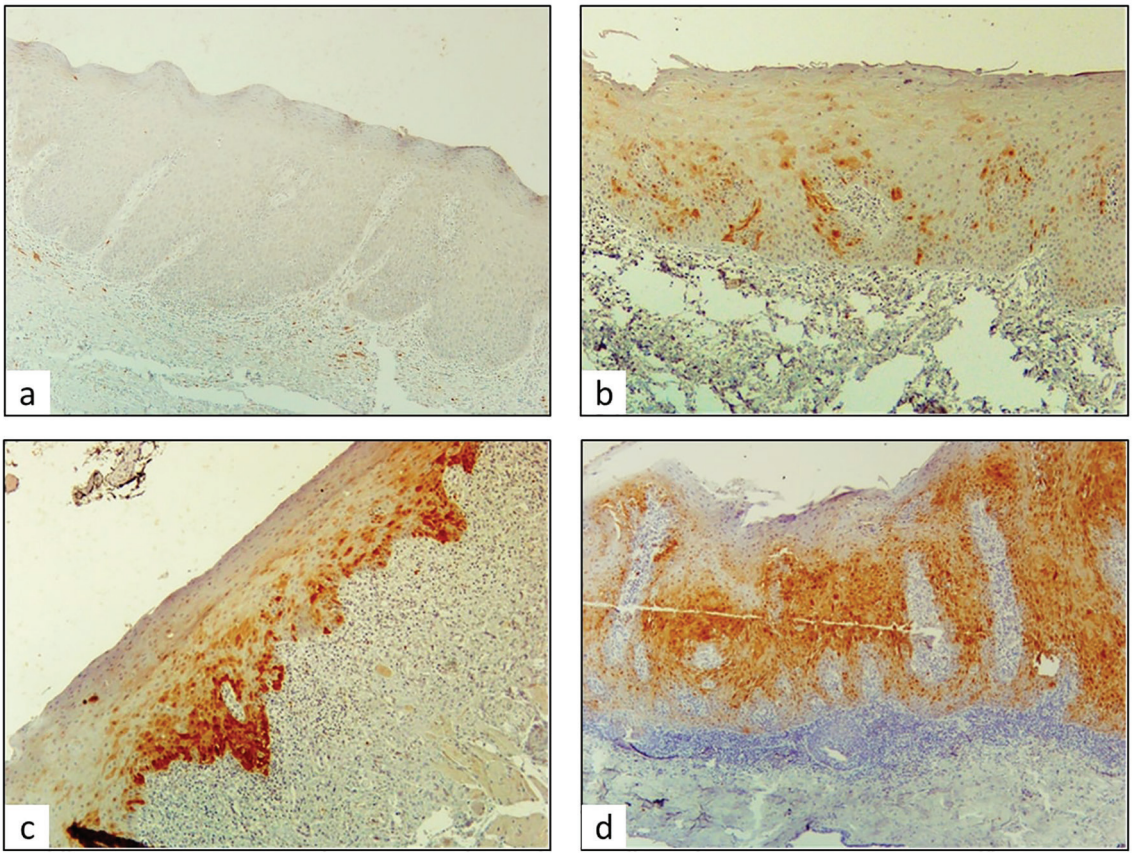

Figure 1: The Immunohistochemistry (IHC) study for p16 was performed for all samples, the brown stained cells indicate p16 expression. a) A specimen in which no p16 protein expression was observed (×40). b) A specimen in which 30% p16 protein expression was observed (×100). c) A specimen in which 60% p16 protein expression was observed (×100). d) A specimen in which 80% p16 protein expression was observed (×40).

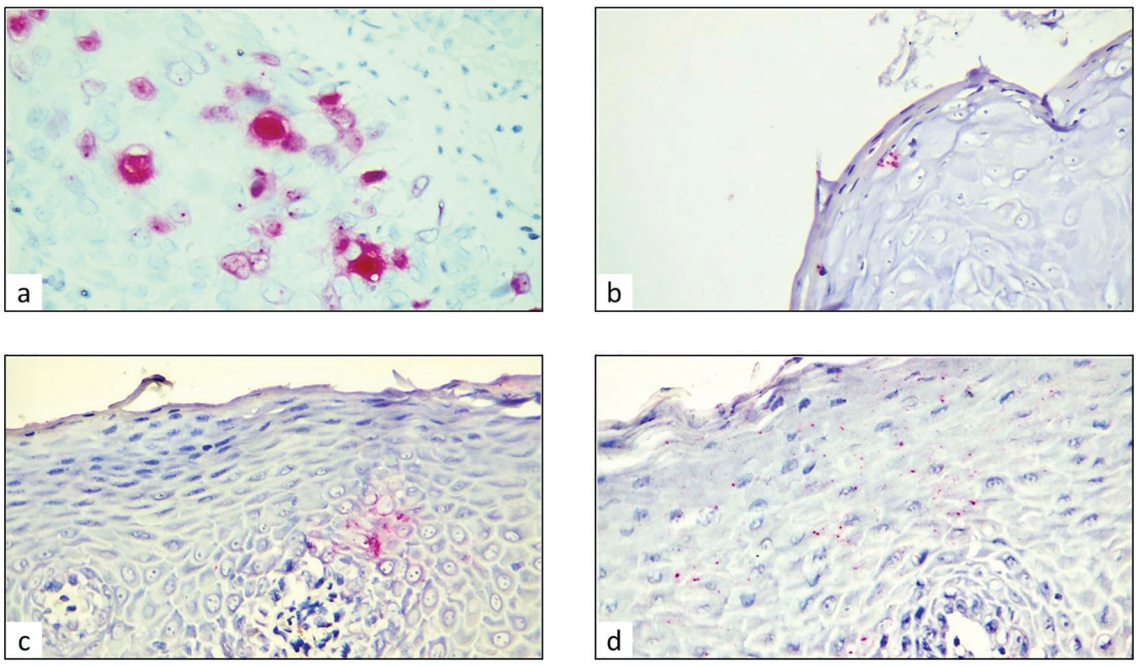

Figure 2: Chromogenic in situ hybridization (CISH) study for human papillomavirus (HPV) was performed for the samples with scores 1 and 2 in the Immunohistochemistry (IHC) study for p16. a) A specimen of uterine cervical tissue, which was performed for a positive control for the CISH study (×400). b, c, d) Specimens that were positive in the CISH study (×400).

Table 1: Microscopic findings in low-grade and high-grade dysplasia cases

Variables		Low-grade dysplasia N=27 N (%)	High-grade dysplasia N=27 N (%)	Odds ratio (95% confidence interval)	P value*
Papillomatosis	Present	8 (29.6%)	13 (48.1%)	2.21 (0.72-6.75)	0.16
	Absent	19 (70.4%)	14 (51.9%)		
Verrucous	Present	3 (11.1%)	2 (7.4%)	0.64 (0.10-4.17)	>0.99
	Absent	24 (88.9%)	25 (92.4%)		
Hyperkeratosis	Present	16 (59.2%)	13 (48.1%)	0.64 (0.22-1.87)	0.41
	Absent	11 (40.8%)	14 (51.9%)		
Acanthosis	Present	26 (96.3%)	26 (96.3%)	1.00 (0.06-16.85)	>0.99
	Absent	1 (3.7%)	1 (3.7%)		
Koilocyte	Present	9 (33.3%)	4 (14.8%)	0.35 (0.09-1.31)	0.11
	Absent	18 (66.7%)	23 (85.2%)		

*A statistical analysis using either the Chi square test or Fisher's exact test, as deemed appropriate, was conducted.

Table 2: Microscopic findings in human papillomavirus-positive and -negative cases

Variables		HPV-Positive N=6 N (%)	HPV-Negative N=48 N (%)	Odds ratio (95% confidence interval)	P value*
Papillomatosis	Positive	3 (50%)	18 (37.5%)	1.67 (0.03-9-16)	0.67
	Negative	3 (50%)	30 (62.5%)		
Verrucous	Positive	2 (33.3%)	3 (6.2%)	7.50 (0.95-59.89)	0.09
	Negative	4 (66.7%)	45 (93.8%)		
Hyperkeratosis	Positive	5 (83.3%)	24 (50%)	5.00 (0.54-46.05)	0.20
	Negative	1 (16.7%)	24 (50%)		
Acanthosis	Positive	6 (100%)	46 (95.8%)	-	>0.99
	Negative	0 (0%)	2 (4.2%)		
Koilocyte	Positive	2 (33.3%)	11 (22.9%)	1.68 (0.27-10.44)	0.62
	Negative	4 (66.7%)	37 (77.1%)		

*A statistical analysis using either the Chi square test or Fisher's exact test, as deemed appropriate, was conducted.

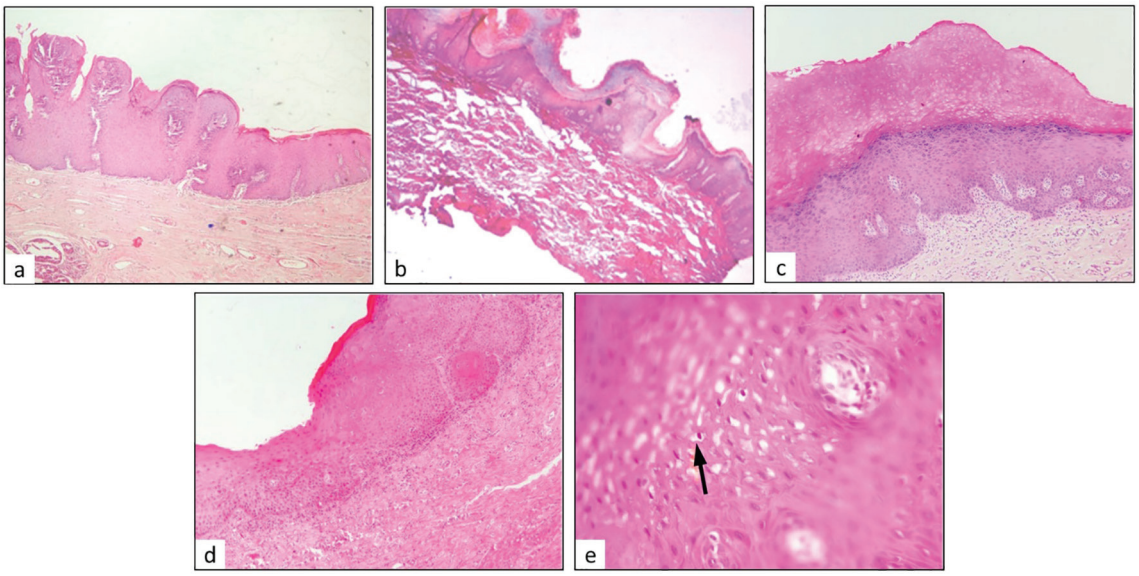

Figure 3: Histopathological features were observed and analyzed in all samples (H&E staining). a) Epithelial tissue with prominent papillomatosis was observed (×40). b) Epithelial tissue with verrucous features was observed (×40). c) Epithelial tissue with hyperkeratosis was observed (×40). d) Epithelial tissue with acanthosis was observed (×100). e) Epithelial tissue with koilocytic cells was observed (×400).

HPVs based on all IHC, CISH, and ChIP-PCR studies and the severity of dysplasia (P>0.99).

Microscopic Evaluation

Microscopic findings including papillomatosis, verrucous, hyperkeratosis, acanthosis, and the presence of koilocyte-like cells are shown in tables 1 and 2 (figure 3).

Discussion

The results of this study showed that the expression of p16 protein was not associated with the severity of epithelial dysplasia (81.5% in low-grade and 59.2% in high-grade cases). Additionally, according to the CISH test result, 9.25% of all specimens were positive, and in the nine cases undergone the ChIP-PCR study, two cases (22.2%) showed positivity for HPV-16, and one case (11.1%) demonstrated positivity for HPV-51.

For clinicians, OED is a dilemma because of the unknown potential transformation risk to OSCC. OED is a range of tissue and cellular

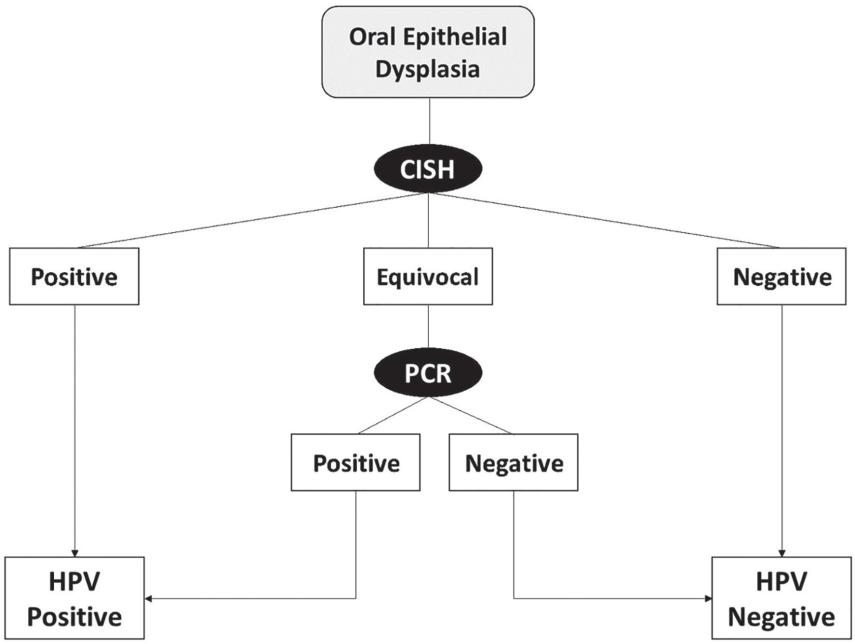

Figure 4: The proposed algorithm to detect human papillomavirus (HPV) in HPV-associated oral epithelial dysplasia (HAOED). (CISH: Chromogenic in situ hybridization, PCR: Polymerase chain reaction, HPV: Human papillomavirus)

changes restricted to the surface epithelial layers without any invasion into the underlying connective tissue.[18]

Although these alterations commonly manifest all the time,[19] HPV has been established as a primary cause of squamous cell carcinoma of the uterine cervix and oropharyngeal region.[20-22] There is no definitive evidence of the carcinogenic role of HPVs in the oral cavity[23] and an extremely variable range of HPV detection, from 0 to 100 percent, is reported in malignant and potentially malignant oral lesions.[24]

One of the most important reasons for this variability is the different methods of HPV detection. Furthermore, some of them are associated with the lesion's anatomical site, based on viral integration into the cellular genome.[25] Another noticeable point that may affect HPV detection is the different grades of dysplasia, especially when different grading systems are used. For example, we analyzed HPV detection in 54 cases of OED and graded them using the binary system in addition to IHC assessment of p16 protein, CISH, and PCR methods. In our study, 70.4% of the specimens demonstrated positive immunoreaction for the p16 antibody.

Although no significant difference between different dysplasia grades was found in our study, some studies demonstrated different expressions of p16 in different grades of dysplasia.[24, 25]

Besides, there are controversial studies about histopathologic features associated with HPV-positive OED.[12] These histopathologic features are named virus-associated dysplasia/bowenoid papulosis.[26]

Woo and others reported specific histopathologic features in 100% of the cases with positive p16 and high-risk HPV-DNA.[27] However, some studies did not confirm the association between some specific histopathologic features and the expression of p16 or HPV-DNA,[25, 28] similar to our study.

A few studies reported an association between HPV and OED, which was confirmed by both positive p16 findings and DNA ISH.[12, 25, 26]

As mentioned, there are various methods for HPV detection, and choosing the best method with the highest sensitivity seems a big dilemma.

Jayaprakash and others, in a meta-analysis on OED, reported that PCR shows significantly higher sensitivity than ISH for the evaluation of HPV-16 and -18.[11]

In another review in 2017, it was reported that although ISH study carries a low sensitivity, PCR has a risk of false positive results in the detection of HPV, especially endemic infections, which are not commonly associated with pathologic tumors.[29]

In our study, we used the CISH method for all p16 positive cases and the PCR method to confirm negative-CISH and diffusely p16 positive cases and equivocal CISH results with focally positive p16 cases.

We showed that the expression of p16 protein could not be a reliable indicator of HPV in OED, regarding all five diffusely-p16 positive cases, which showed negative results using both CISH and ChIP-PCR studies.

The results are consistent with previous studies on HPV DNA integration into the host genome in potentially malignant oral lesions.[24, 25] Although the IHC study for p16 is available and feasible, it is not a sensitive method to detect HPV in OED. However, unlike OED, there is a strong association between HPV and the expression of p16 protein in oropharyngeal squamous cell carcinoma.[29]

In the absence of a universal standardized method to detect HPV in HAOED, we proposed a step-by-step combination approach using different diagnostic methods based on a complementary algorithm (figure 4).

To determine a protocol for checking the presence of high-risk HPV subtypes in oral dysplastic tissues using existing diagnostic methods, the p16 method for HPV detection and subtype determination in oral dysplasia samples is not definitively helpful. It is better to use the CISH diagnostic method from the very beginning, which can identify the high-risk subtypes of HPV. Besides, for samples that have been paraffinized, this diagnostic method has shown high accuracy. In the samples whose CISH test results are reported positive and the staining pattern is similar to the positive control, the presence of HPV in them should be considered positive. In the samples whose result of the CISH test is reported as negative and the staining pattern is similar to the positive control, the presence of HPV in them should be considered definitively positive. In some dyed samples, the percentage of staining may be seen at a deficient level compared to the positive control, which can be used to diagnose these samples by PCR test definitively. In the PCR method, it is possible to distinguish the types of HPV, and good accuracy clearly has been shown for this method in frozen and freshly biopsied tissues in studies. However, for paraffined samples, it is better as an auxiliary method for CISH testing.

All HPV detection methods have their advantages and disadvantages considering cost, sensitivity, DNA or protein detection, and others. Here, we recommended an algorithm to

collect the benefits of all detection methods in a well-designed order. It is clear that using any single method of HPV detection has significant limitations.[30]

Among the limitations of the present study, the following can be mentioned: the difficulty in performing laboratory procedures, the sensitivity of diagnostic tests to the technique of use, the need for a skilled technician to perform these tests, and the small volume of tissues, since some of the initial samples are biopsies.

Conclusion

At the current state of knowledge, HPV detection methods for HAOED still remain a controversial issue. It is very important to have a practical combination of methods to accurately detect HPV in OED lesions. A simple step-by-step algorithm to facilitate the diagnosis of HAOED is suggested here.

Acknowledgment

This study is financially supported by a grant from the School of Dentistry, Tehran University of Medical Sciences (code: 99-02-133-49076). We would like to express our appreciation to Dr Alireza Abdollahi for his kind support. Besides, we are grateful to Mrs Zargar for guiding us on technical issues.

Authors' Contribution

K.P: Specimen collection and experimentation, drafting; S.D: Study design, evaluation of microscopic slides stained with IHC, and drafting; H.S: Analyzing CISH and ChIP-PCR results, reviewing the manuscript; P.A: Study design, evaluation of microscopic slides stained with IHC, and reviewing the manuscript; AR.Sh: statistical data analysis and reviewing the manuscript, S.A: Specimen collection and experimentation, and reviewing the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

References

1 de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE, Lloveras B, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. Lancet Oncol. 2010;11:1048-56. doi: 10.1016/S1470-2045(10)70230-8. PubMed PMID: 20952254.

2 Mazul AL, Taylor JM, Divaris K, Weissler MC, Brennan P, Anantharaman D, et al. Oral health and human papillomavirus-associated head and neck squamous cell carcinoma. Cancer. 2017;123:71-80. doi: 10.1002/cncr.30312. PubMed PMID: 27571516; PubMed Central PMCID: PMCPMC5161679.

3 Amin MB, Greene FL, Edge SB, Compton CC, Gershenwald JE, Brookland RK, et al. The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. CA Cancer J Clin. 2017;67:93-9. doi: 10.3322/caac.21388. PubMed PMID: 28094848.

4 Oliveira MC, Soares RC, Pinto LP, Souza LB, Medeiros SR, Costa Ade L. High-risk human papillomavirus (HPV) is not associated with p53 and bcl-2 expression in oral squamous cell carcinomas. Auris Nasus Larynx. 2009;36:450-6. doi: 10.1016/j.anl.2008.10.011. PubMed PMID: 19124208.

5 O'Regan EM, Toner ME, Finn SP, Fan CY, Ring M, Hagmar B, et al. p16(INK4A) genetic and epigenetic profiles differ in relation to age and site in head and neck squamous cell carcinomas. Hum Pathol. 2008;39:452-8. doi: 10.1016/j.humpath.2007.08.004. PubMed PMID: 18261630.

6 Iocca O, Sollecito TP, Alawi F, Weinstein GS, Newman JG, De Virgilio A, et al. Potentially malignant disorders of the oral cavity and oral dysplasia: A systematic review and meta-analysis of malignant transformation rate by subtype. Head Neck. 2020;42:539-55. doi: 10.1002/hed.26006. PubMed PMID: 31803979.

7 Warnakulasuriya S, Kujan O, Aguirre-Urizar JM, Bagan JV, Gonzalez-Moles MA, Kerr AR, et al. Oral potentially malignant disorders: A consensus report from an international seminar on nomenclature and classification, convened by the WHO Collaborating Centre for Oral Cancer. Oral Dis. 2021;27:1862-80. doi: 10.1111/odi.13704. PubMed PMID: 33128420.

8 Odell E, Kujan O, Warnakulasuriya S, Sloan P. Oral epithelial dysplasia: Recognition, grading and clinical significance. Oral Dis. 2021;27:1947-76. doi: 10.1111/odi.13993. PubMed PMID: 34418233.

9 Nalli G, Mastrotta P, Garcia MG, Tatti S, Verdu S. Detection of Oral Human Papillomavirus (HPV) and its Clinical Importance. J

Dent (Shiraz). 2022;23:51-7. doi: 10.30476/DENTJODS.2021.88338.1326. PubMed PMID: 35291686; PubMed Central PMCID: PMCPMC8918636.
10 Woo SB, Cashman EC, Lerman MA. Human papillomavirus-associated oral intraepithelial neoplasia. Mod Pathol. 2013;26:1288-97. doi: 10.1038/modpathol.2013.70. PubMed PMID: 23599160.
11 Jayaprakash V, Reid M, Hatton E, Merzianu M, Rigual N, Marshall J, et al. Human papillomavirus types 16 and 18 in epithelial dysplasia of oral cavity and oropharynx: a meta-analysis, 1985-2010. Oral Oncol. 2011;47:1048-54. doi: 10.1016/j.oraloncology.2011.07.009. PubMed PMID: 21816661; PubMed Central PMCID: PMCPMC3640331.
12 Chen X, Zhao Y. Human papillomavirus infection in oral potentially malignant disorders and cancer. Arch Oral Biol. 2017;83:334-9. doi: 10.1016/j.archoralbio.2017.08.011. PubMed PMID: 28886585.
13 Angiero F, Gatta LB, Seramondi R, Berenzi A, Benetti A, Magistro S, et al. Frequency and role of HPV in the progression of epithelial dysplasia to oral cancer. Anticancer Res. 2010;30:3435-40. PubMed PMID: 20944119.
14 Armitage P, Berry G, Matthews J. Analysing means and proportions. Statistical methods in medical research. 2002;83:146. doi: 10.1002/9780470773666.ch4.
15 Kujan O, Oliver RJ, Khattab A, Roberts SA, Thakker N, Sloan P. Evaluation of a new binary system of grading oral epithelial dysplasia for prediction of malignant transformation. Oral Oncol. 2006;42:987-93. doi: 10.1016/j.oraloncology.2005.12.014. PubMed PMID: 16731030.
16 Thompson L. World Health Organization classification of tumours: pathology and genetics of head and neck tumours. Ear Nose Throat J. 2006;85:74. PubMed PMID: 16579185.
17 Koo CL, Kok LF, Lee MY, Wu TS, Cheng YW, Hsu JD, et al. Scoring mechanisms of p16INK4a immunohistochemistry based on either independent nucleic stain or mixed cytoplasmic with nucleic expression can significantly signal to distinguish between endocervical and endometrial adenocarcinomas in a tissue microarray study. J Transl Med. 2009;7:25. doi: 10.1186/1479-5876-7-25. PubMed PMID: 19366452; PubMed Central PMCID: PMCPMC2672079.
18 Dost F, Le Cao K, Ford PJ, Ades C, Farah CS. Malignant transformation of oral epithelial dysplasia: a real-world evaluation of histopathologic grading. Oral Surg Oral Med Oral Pathol Oral Radiol. 2014;117:343-52. doi: 10.1016/j.oooo.2013.09.017. PubMed PMID: 24388536.
19 Fonseca-Silva T, Farias LC, Cardoso CM, Souza LR, Carvalho Fraga CA, Oliveira MV, et al. Analysis of p16(CDKN2A) methylation and HPV-16 infection in oral mucosal dysplasia. Pathobiology. 2012;79:94-100. doi: 10.1159/000334926. PubMed PMID: 22285991.
20 Antonsson A, Neale RE, Boros S, Lampe G, Coman WB, Pryor DI, et al. Human papillomavirus status and p16(INK4A) expression in patients with mucosal squamous cell carcinoma of the head and neck in Queensland, Australia. Cancer Epidemiol. 2015;39:174-81. doi: 10.1016/j.canep.2015.01.010. PubMed PMID: 25677091.
21 Szalmas A, Konya J. Epigenetic alterations in cervical carcinogenesis. Semin Cancer Biol. 2009;19:144-52. doi: 10.1016/j.semcancer.2009.02.011. PubMed PMID: 19429477.
22 Rahrotaban S, Mahdavi N, Abdollahi A, Yazdani F, Kaghazloo A, Derakhshan S. Carcinoma-associated Fibroblasts are a Common Finding in the Microenvironment of HPV-positive Oropharyngeal Squamous Cell Carcinoma. Appl Immunohistochem Mol Morphol. 2019;27:683-8. doi: 10.1097/PAI.0000000000000687. PubMed PMID: 31593979.
23 Dalakoti P, Ramaswamy B, Bhandarkar AM, Nayak DR, Sabeena S, Arunkumar G. Prevalence of HPV in Oral Squamous Cell Carcinoma in South West India. Indian J Otolaryngol Head Neck Surg. 2019;71:657-64. doi: 10.1007/s12070-018-1470-9. PubMed PMID: 31742038; PubMed Central PMCID: PMCPMC6848302.
24 Thankappan P, Ramadoss MN, Joseph TI, Augustine PI, Shaga IB, Thilak J. Human Papilloma Virus and Cancer Stem Cell markers in Oral Epithelial Dysplasia-An Immunohistochemical Study. Rambam Maimonides Med J. 2021;12. doi: 10.5041/RMMJ.10451. PubMed PMID: 34709167; PubMed Central PMCID: PMCPMC8549839
25 Nemes JA, Deli L, Nemes Z, Marton IJ. Expression of p16(INK4A), p53, and Rb proteins are independent from the presence of human papillomavirus genes in oral squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006;102:344-52. doi: 10.1016/j.tripleo.2005.10.069. PubMed PMID: 16920543.
26 Zhang L, Lewis JS, Jr., El-Mofty SK, Gandhi M, Chernock RD. Nonkeratinizing Squamous Cell Carcinoma In Situ of the Upper

Aerodigestive Tract: An HPV-Related Entity. Head Neck Pathol. 2017;11:152-61. doi: 10.1007/s12105-016-0749-y. PubMed PMID: 27492445; PubMed Central PMCID: PMCPMC5429272.

27 Venkatesh A, Elengkumaran S, Ravindran C, Malathi N. Association of Human Papilloma Virus in Oral Squamous Cell Carcinoma: An Alarming Need for Human Papillomavirus 16 Screening in Cancer Patients. J Pharm Bioallied Sci. 2021;13:S1224-S7. doi: 10.4103/jpbs.jpbs_370_21. PubMed PMID: 35017960; PubMed Central PMCID: PMCPMC8686889.

28 Cunningham LL, Jr., Pagano GM, Li M, Tandon R, Holm SW, White DK, et al. Overexpression of p16INK4 is a reliable marker of human papillomavirus-induced oral high-grade squamous dysplasia. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006;102:77-81. doi: 10.1016/j.tripleo.2005.11.028. PubMed PMID: 16831676.

29 Husain N, Neyaz A. Human papillomavirus associated head and neck squamous cell carcinoma: Controversies and new concepts. J Oral Biol Craniofac Res. 2017;7:198-205. doi: 10.1016/j.jobcr.2017.08.003. PubMed PMID: 29124000; PubMed Central PMCID: PMCPMC5670302.

30 Fauzi FH, Hamzan NI, Rahman NA, Suraiya S, Mohamad S. Detection of human papillomavirus in oropharyngeal squamous cell carcinoma. J Zhejiang Univ Sci B. 2020;21:961-76. doi: 10.1631/jzus.B2000161. PubMed PMID: 33843162; PubMed Central PMCID: PMCPMC7759454.