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HPV-16 in a distinct subset of oral epithelial dysplasia

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Human papillomavirus (HPV) 16 is the most common high-risk HPV type identified in oropharyngeal and cervical neoplasia. Recently, HPV-associated oral epithelial dysplasia with specific histopathologic features and demographics similar to HPV-oropharyngeal carcinoma has been identified. The objective of this study was to evaluate histopathologically all cases of HPV-oral epithelial dysplasia seen in one center and identify HPV types in a subset of cases. Cases with specific histopathology for HPV-oral epithelial dysplasia that were positive both by immunohistochemical studies for p16 and by *in situ* hybridization for high-risk types of HPV were further analyzed using QIAamp DNA Tissue Kits (Qiagen, Hilden, Germany). DNA was extracted, amplified, and digested with restriction enzymes and run on a polyacrylamide gel. Digestion patterns were visually compared with a database of known HPV digestion patterns for identification. There were 53 specimens included in the analysis. There were 47 males and six females (7.8:1), with a median age of 55 years (range 41–81). The most common site of involvement was the tongue/floor of mouth (77% of cases). Of the 53 cases, 94% exhibited parakeratosis and/or hyperkeratosis. All the cases featured karyorrhexis, apoptosis, and characteristics of conventional carcinoma *in situ*. The quantity of DNA extracted was sufficient for analysis in 22 cases. HPV-16 was identified in 20/22 (91%) cases. One case was associated with HPV-33 and one with HPV-58 (5% each). Eight of the 53 cases (15%) were associated with invasive squamous cell carcinomas.
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Human papillomavirus (HPV) infection has been well established as a cause of anal and genital tract cancers and, more recently, oropharyngeal cancers.[1–6] HPV-16 is the most common high-risk HPV type identifed, accounting for at least 50% of cervical squamous cell carcinomas.[2,7] HPV-16 has been identified in over 90% of oropharyngeal squamous cell carcinomas, with HPV types HPV-18, -33, and -52 less frequently identified in oral and oropharyngeal squamous cell carcinomas.[8–13] Studies have shown an improved prognosis for HPV-associated head and neck squamous cell carcinomas compared with HPV-negative cancers.[14,15] The prevalence of HPV in oral cavity squamous cell carcinomas is 4–6% and there have been very few studies that evaluated the prevalence of HPV in oral epithelial dysplasia.[8,16–20]

In 2013, we reported high-grade oral epithelial dysplasia that exhibited distinctive histopathologic features of dysplasia that predicted positivity both for p16 and for high-risk types of HPV by DNA *in situ* hybridization in 100% of cases.[21] The objective of the current study is to identify specific HPV types associated with this distinctive form of HPV-oral epithelial dysplasia.

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Materials and methods

Cases of HPV-oral epithelial dysplasia were identified from September 2008 to August 2016 from the

archives of StrataDx, a surgical pathology laboratory in Lexington, MA, USA affiliated with the Harvard School of Dental Medicine. All the cases were reviewed by two oral and maxillofacial pathologists (SBW and MAL). Only cases that demonstrated the specific histopathologic features for HPV-oral epithelial dysplasia that had been described in a previous study and with immunohistochemical studies for p16 that were positive in a contiuous band through the full thickness of the epithelium were considered for inclusion.[21]

Regions of interest were macro-dissected from 5-micron sections of formalin-fixed paraffin-embedded tissue and DNA was isolated using QIAamp Mini kit (Qiagen, Hilken, Germany) following manufacturer's instructions. HPV genotyping was done by a polymerase chain reaction-restriction fragment length polymorphism (PCR-restriction fragment length polymorphism) method. Briefly, a PCR reaction was first performed using HPV consensus primers designed to amplify a conserved 332–470 bp fragment of the HPV L1 gene, and the PCR products were analyzed on a 5% polyacrylamide gel stained with ethidium bromide. If a visible product was present, the PCR product was digested with three different restriction enzymes, Pst I, Rsa I, and Hae III (New England Biolabs), following the manufacturer's directions. The digested products were visualized on a 5% polyacrylamide gel stained with ethidium bromide and the patterns of the digested products compared with a database of known patterns to determine the genotype (Figure 1). A control PCR reaction with primers to a 500 bp fragment of the human beta-globin was also performed to assess the quality of the DNA.

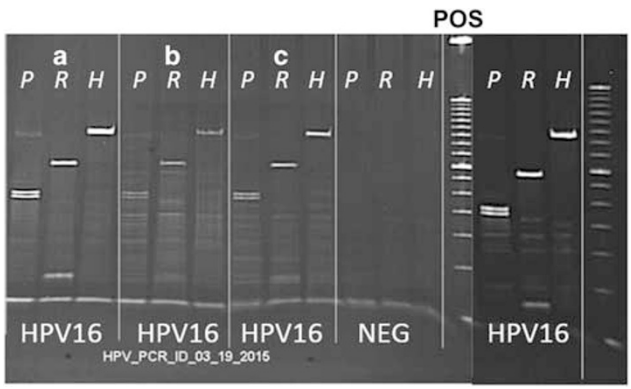

Figure 1 Molecular diagnostic test results for HPV. Each sample (labeled 1, 2, 3, NEG: negative control, POS: positive control for HPV-16) is amplified by PCR with primers to a portion of the HPV L1 major capsid protein gene, digested with one of three restriction enzymes (Pst I, Rsa I, HaeI), and separated by polyacrylamide (5%) gel electrophoresis. The pattern of fragments is matched to a library of fragments derived from different HPV genotypes to arrive at a designation for each sample. The patterns for specimens, a, b, and c each match to HPV-16. H: Hael; P: Pst l; R: Rsa I.

Results

There were 53 cases included in the analysis, of which the histopathology for 19 had been previously reported (Table 1).[21] The median age was 55 years (range 41–81) and there were 47 males (M:F ratio = 7.8:1). Of the 45 lesions accompanied by a clinical description, 28 (62%) were reported as white,

Table 1 Clinical data and HPV type

Case	*Age*	*Gender*	*Location*	*HPV type*
1	51	M	Tongue/floor of mouth	NAD
2	60	M	Lateral tongue	16
3	43	M	Tongue	NAD
4	45	F	Ventral tongue	NAD
5[a]	70	M	Ventral tongue	NAD
6	41	M	Ventral tongue	NAD
7	56	M	Buccal mucosa	16
8	69	M	Floor of mouth	16
9	62	M	Buccal mucosa	NAD
10	48	M	Ventral tongue	NAD
11	56	M	Ventral tongue	16
12	46	M	Anterior tongue	16
13[a]	62	M	Tongue	NAD
14	55	F	Gingiva	16
15[a]	55	F	Gingiva	16
16	71	M	Ventral tongue	16
17	54	M	Floor of mouth	16
18	61	M	Floor of mouth	16
19	67	M	Ventral tongue	16
20[a]	72	M	Ventral tongue	33
21	61	M	Ventral tongue	58
22	51	M	Ventral tongue	16
23	49	F	Ventral tongue	16
24	55	F	Buccal mucosa	16
25	57	M	Ventral tongue/floor of mouth	NAD
26	46	M	Retromolar pad	NAD
27	59	M	Ventral tongue	16
28	51	M	Lateral tongue	16
29	57	M	Floor of mouth	16
30	57	M	Floor of mouth	16
31	49	M	Ventral tongue	16
32	47	M	Buccal mucosa	16
33	55	M	Labial mucosa	NAD
34	64	M	Lateral tongue	NAD
35	52	M	Lip	NAD
36	54	M	Floor of mouth	NAD
37	67	M	Lateral tongue	NAD
38[a]	66	F	Tongue	NAD
39[b]	61	M	Tongue	NAD
40[a,b]	50	M	Buccal mucosa	NAD
41[a]	49	M	Floor of mouth	NAD
42[a]	58	M	Soft palate	NAD
43	62	M	Ventral tongue	NAD
44	64	M	Lingual frenum	NAD
45	55	M	Soft palate	NAD
46	60	M	Floor of mouth	NAD
47	81	M	Ventral tongue	NAD
48	50	M	Ventral tongue	NAD
49[b]	60	M	Ventral tongue/floor of mouth	NAD
50[b]	52	M	Lateral tongue	NAD
51	50	M	Floor of mouth	NAD
52[b]	46	M	Ventral tongue	NAD
53	43	M	Ventral tongue	NAD

Abbreviation: NAD, no amplification of DNA.
[a]Dysplasia associated with invasive squamous cell carcinoma.
[b]*In situ* hybridization studies negative or equivocal for high-risk human papillomavirus.

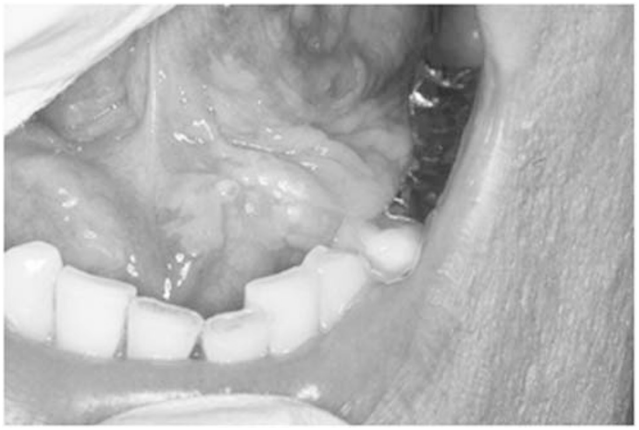

Figure 2 Demarcated leukoplakia of the left floor of mouth.

leukoplakia, or hyperkeratosis, three as red or erythroplakia (7%), and three (7%) as both red and white or erythro-leukoplakia (Figure 2). Of the 53 lesions, 28 (53%) were located on the tongue, 9 (17%) on the floor of mouth, and 3 (6%) involved both sites; one case was on the lingual frenum. As such, 41 cases (77%) involved the tongue and/or floor of mouth and lingual frenum (Table 1).

Fifty (94%) exhibited either parakeratosis and/or hyperkeratosis. Of the 53 cases, 68% were parakeratinized only, exhibiting brightly eosinophilic parakeratin (Figure 3a). Nine cases (17%) demonstrated hyperkeratosis only, and five cases (9%) featured both parakeratosis and hyperkeratosis.

All cases showed karyorrhexis and apoptosis throughout the hyperplastic stratified squamous epithelium with a continuum of changes (Figures 3a, Figures 4a and b). There were cells with karyorrhexis and dense chromatin resembling mitotic figures, similar to the characteristic cells of Heck disease.[22] Other cells were apoptotic and contained pyknotic nuclei with brightly eosinophilic cytoplasm and a halo from loss of attachment to the adjacent keratinocytes, and finally, nuclei are lost altogether (Figures 3b and 4c). These characteristic findings distinguish these cases from conventional oral epithelial dysplasia. The surrounding cells are generally hyperchromatic and basaloid with increased nucleus:cytoplasm ratio involving the full thickness of the epithelium in all the cases.

All the cases showed strong p16 positivity in a continuous band through the full thickness of the epithelium, excluding the keratin layer (Figures 3c and 4d). *In situ* hybridization was positive for high-risk types of HPV in 48/53 cases (91%; Figure 3d). Eight of 53 cases (15%) were associated with invasive squamous cell carcinomas and these carcinomas developed on the tongue/floor of mouth (5 cases), gingiva, buccal mucosa, and soft palate. Five of the eight carcinomas (63%) were non-keratinizing/basaloid and three were minimally keratinizing (38%; Figure 5).

The quantity of DNA extracted was sufficient for analysis in 22 cases. The following results pertain to this subgroup of patients and there was no statistically significant difference between this subset and the group that was not analyzed in terms of gender, age, or anatomic site (Table 2). The most common site of involvement (12) was the tongue; 5 cases (23%) presented on the floor of mouth, 3 cases (14%) presented on the buccal mucosa, and 2 cases (9%) were present on the gingiva. HPV-16 was identified in 20/22 (91%) cases. Of these, half were from the tongue, 25% from the floor of mouth, 15% from the buccal mucosa, and 10% from the gingiva. One case each was associated with HPV-33 and and HPV-58 (5% each); these two patients were both male, aged 72 and 61, respectively, and both specimens were from the ventral tongue. Of the cases associated with invasive squamous cell carcinomas, two underwent DNA analysis; one each harbored HPV-16 and HPV-33, similar to the overlying oral epithelial dysplasia.

Discussion

The prevalence of HPV within the oral cavity ranges from 0.9 to 12.0%,[12,23,24] with the prevalence of high-risk types reported to be between 1 and 3%.[17,25] HPV has also been detected in the oral cavity of children younger than age 20 with a prevalence of 1.9 to 6.0%.[23,26] However, most incident and prevalent HPV infections in the mouth are cleared within 1 to 2 years and high viral load predicts persistence of infection.[27–29]

In the United States, the prevalence of HPV in oropharyngeal squamous cell carcinoma has increased significantly over the last several decades. The prevalence was reported at 16.3% in the 1980s and the prevalence has increased to 72.2% by 2009.[30,31] HPV is the cause of squamous cell carcinoma in 70% of oropharyngeal carcinomas and approximately 85.0% of the base of tongue and tonsillar squamous cell carcinomas.[30,32,33] In 2012, Isayeva *et al*[34] reported that the prevalence of HPV detected by PCR in 4195 squamous cell carcinomas of the oral cavity was 20.2%, with the most common type being HPV 16. However, in contrast to the oropharynx, E6/E7 mRNA expression is limited and less than 5% of oral cavity squamous cell carcinomas appear related to HPV.[35,36] In a meta-analysis performed in 2011, Jayaprakash *et al*[16] reported that HPV is associated with oral epithelial dysplasia in 25.3% of cases; however, it should be noted that diverse methods of HPV detection were used in the studies included and sensitivity therefore may have varied with PCR showing the significantly higher sensitivity than *in situ* hybridization. HPV has been detected in squamous cell carcinoma in only 4.0 to 6.0% of cases by identification of high-risk HPV E6/E7.[18,19] Most oropharyngeal squamous cell carcinomas (>85%) are caused by HPV-16, with other HPV

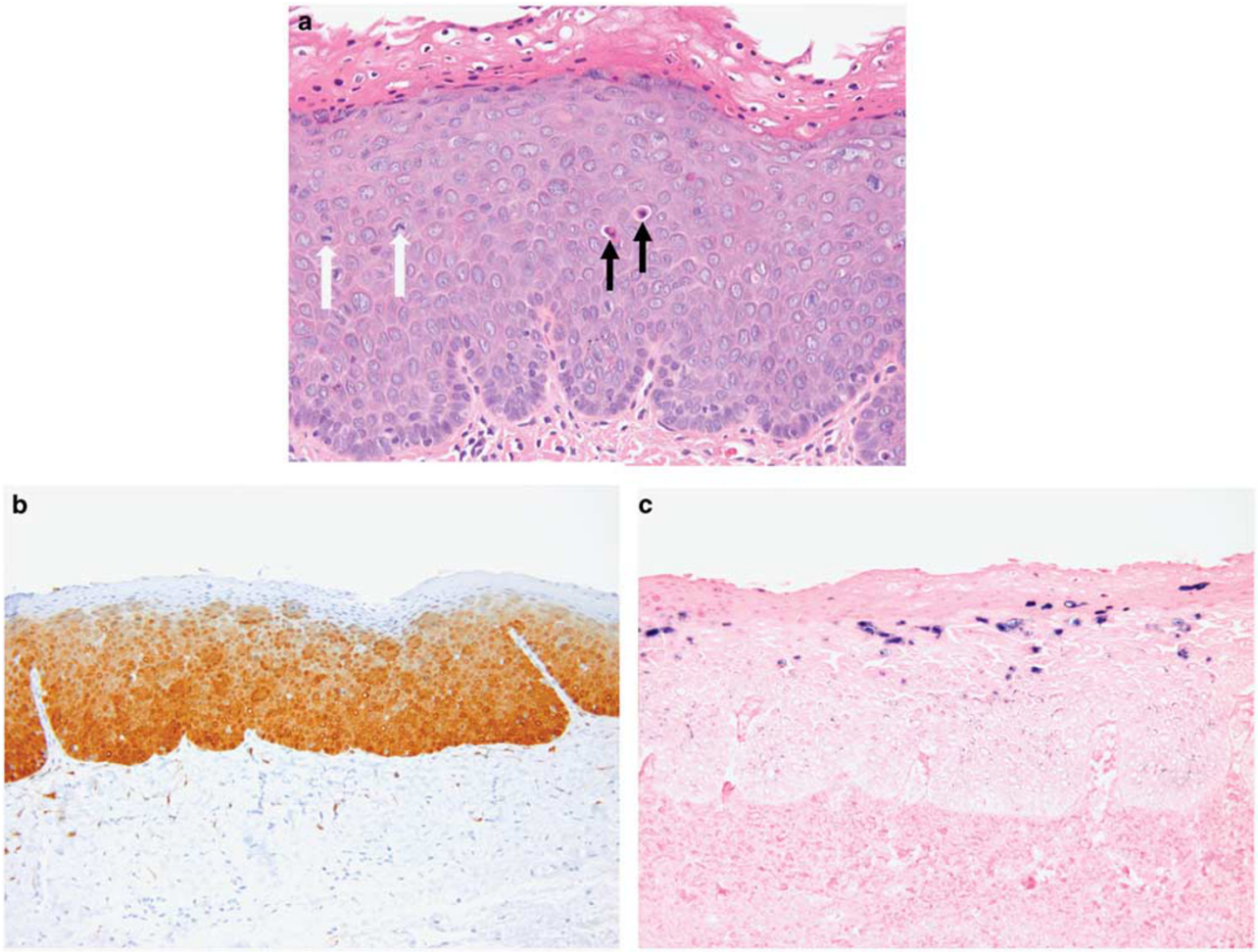

Figure 3 (**a**) Karyorrhectic cells (white arrows) and apoptotic cells (black arrows) noted throughout the lesion (hematoxylin and eosin, maginification × 400). (**b**) Study for p16 exhibits a continuous band of positivity within the cytoplasm and nuclei of the epithelium, short of the keratin layer (immunohistochemical study for p16, magnification × 200). (**c**) Corresponding study exhibits nuclear positivity for high-risk human papillomavirus (*in situ* hybridization study for high-risk HPV, magnification × 200).

types such as HPV-18, -33, and -52 identified less frequently.[13,30] In the cervix, HPV-18, -33, -45, and -58 are the most common HPV types implicated in cervical cancer after HPV-16.[5,37] In this study of oral epithelial dysplasia, HPV-16 was detected by restriction fragment length polymorphism in 91% of cases while HPV-33 and -58 were each detected in one case each.

There have been a few investigations that evaluated the relationship between HPV and oral epithelial dysplasia confirmed by both p16 and DNA *in situ* hybridization studies. Two studies reported that 14.6%[38] and 35.7%[39] of cases of oral epithelial dysplasia were positive for p16 and high-risk HPV by PCR, and 17.5% of cases were positive for high-risk HPV by DNA *in situ* hybridization.[40] Those studies did not specify whether the oral epithelial dysplasias that were positive for HPV had different histopathologic features from those that were not positive. However, a recent study showed that oral epithelial dysplasia that had specific histopathologic features (namely, high numbers of apoptotic and karyorrhectic cells) were positive for p16 and high-risk HPV by DNA *in situ* hybridization in 100% of cases.[21] Similar histopathologic features have been reported previously in oral warts/condylomas without dysplasia and have been referred to as oral bowenoid lesions or virus-associated dysplasia/bowenoid papulosis.[41,42] Although five cases (9%) in the current series were negative or equivocal for HPV by DNA *in situ* hybridization, this test is not highly sensitive and these results may represent false negatives.

In oropharyngeal cancer, the precursor dysplastic mucosal lesion has not been identified although it is assumed that one is present. In the tonsil, dysplastic precursor lesions within the tonsillar crypt epithelium are difficult to identify because of the complex, invaginated, and tortuous nature of tonsillar crypt epithelium. It is likely that invasive squamous cell carcinoma overgrows the precursor lesion in most cases. On the other hand, the oral cavity is amenable to direct visual examination and most oral squamous cell carcinomas arise from dysplastic precursor

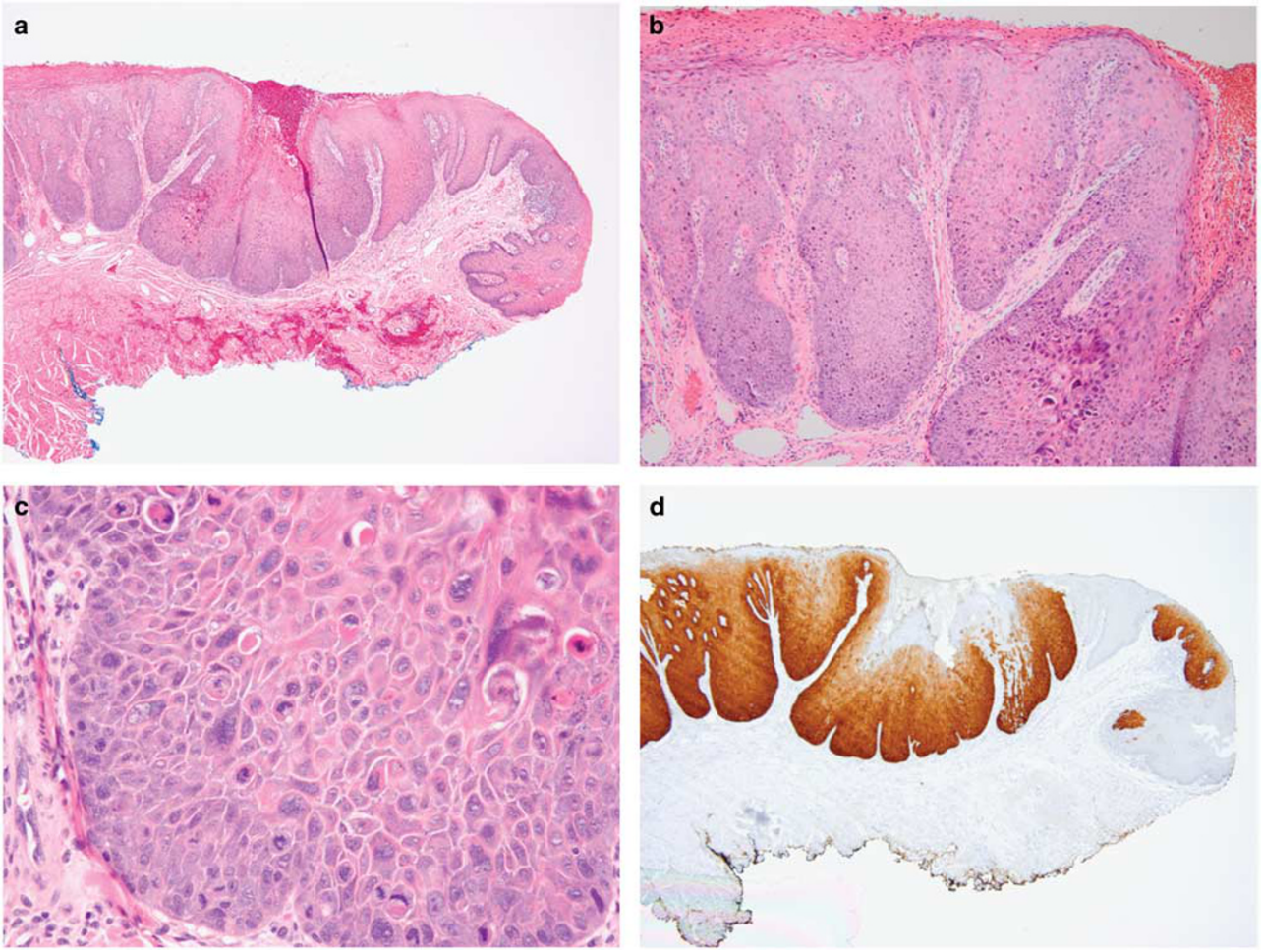

Figure 4 (**a**) Dysplastic tissue exhibiting parakeratosis and epithelial hyperplasia (hematoxylin and eosin, maginification ×40). (**b**) Numerous karyorrhectic cells and features of conventional epithelial dysplasia throughout the tissue (hematoxylin and eosin, magnification ×100). (**c**) Karyorrhectic cells and apoptotic cells within the bulbous rete ridge of a dysplastic lesion (hematoxylin and eosin, magnification ×400). (**d**) Corresponding study for p16 exhibits a continuous band of positivity within the 'skip' lesions of the dysplastic epithelium (immunohistochemical study for p16, magnification ×40).

lesions, the most common of which are leukoplakias and variants such as erythro-leukoplakia.[43] HPV-oral epithelial dysplasia is no different and presents as leukoplakia occurring at oral sites associated with conventional smoking-related leukoplakia and squamous cell carcinoma, such as tongue and floor of mouth, which constituted 77% of the cases in this series (Figure 2). Eight out of 53 cases (15%) in this series showed invasive squamous cell carcinoma and it is therefore likely that such HPV-oral epithelial dysplasias represent the precursor lesions of HPV-associated oral squamous cell carcinoma. There was a stronger male predilection (7.8:1) compared with the 4:1 ratio of HPV-associated oropharyngeal squamous cell carcinoma, and there was a median age of 55 years, younger than patients with smoking-related cancers, but comparable to the median age of 54 for patients with HPV+ oropharyngeal squamous cell carcinoma.[29,44,45]

One case excluded from this study that exhibited the characteristic features of HPV-oral epithelial dysplasia with high-risk HPV identified by DNA *in situ* hybridization, but was negative for p16 (Figure 6). Oropharyngeal carcinomas have been described previously that have been positive for HPV but are p16-negative (HPV+p16 –).[46,47] It has been suggested that such tumors, which have high recurrence rates and molecular profiles closer to HPV-negative carcinomas than to HPV+p16+ carcinomas, may represent HPV opportunistic infection of pre-existing neoplasms.[48] At this time, too few HPV+p16 – carcinomas have been identified in the oral cavity to determine their significance.

Many studies have demonstrated improved survival associated with HPV-positive oropharyngeal carcinomas compared with non-HPV-positive tumors.[49–52] One recent study showed that HPV-16 is associated with lower disease-free survival than other high-risk HPV types.[53] At this time, there are too few cases of HPV-associated oral cavity squamous cell carcinomas to determine whether

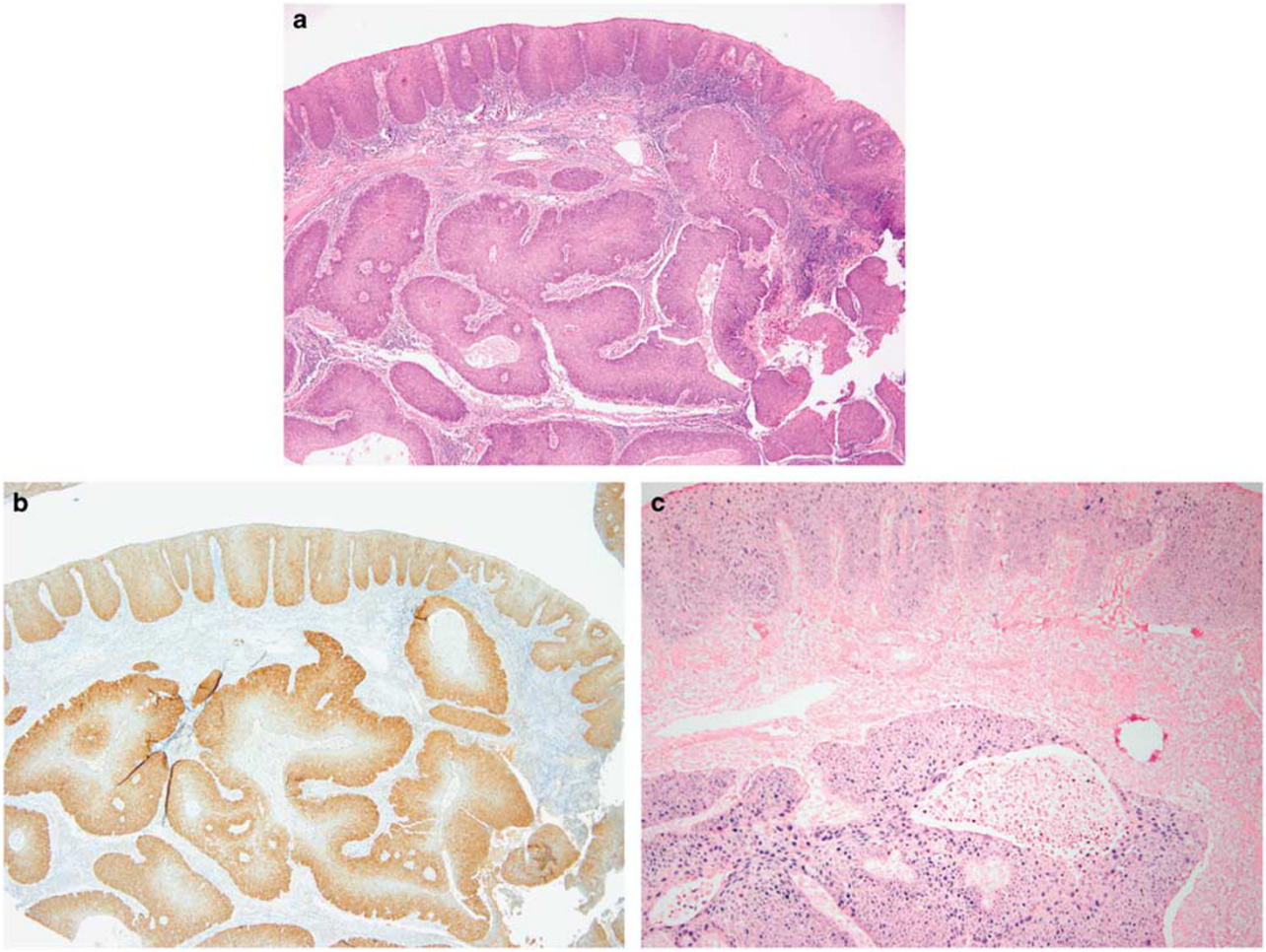

Figure 5 (a) HPV-oral epithelial dysplasia overlying invasive squamous cell carcinoma (hematoxylin and eosin, maginification ×40). (**b**) Corresponding study for p16 exhibits a continuous band of positivity within the invasive tumor islands and within the surface epithelium (immunohistochemical study for p16, magnification ×40). (**c**) Corresponding study exhibits nuclear positivity for high-risk human papillomavirus (*in situ* hybridization study for high-risk HPV, magnification ×100).

Table 2 Comparison of patients with oral epithelial dysplasia with and without HPV genotyping

	Patients with no HPV typing (n = 31) (%)	*Patients with HPV typing (n = 22) (%)*	P-*value*
Median age	55.0	56.0	0.39
Gender			
Males	29 (94)	18 (82)	0.18
Females	2 (7)	4 (18)	
Anatomic site			
Tongue and/or floor of mouth	24 (77)	17 (77)	0.36
Buccal mucosa	2 (7)	3 (14)	
Soft palate	2 (7)	0 (0)	
Gingiva	1 (3)	2 (9)	
Lip/labial mucosa	2 (7)	0 (0)	

they too behave in a less aggressive manner than conventional squamous cell carcinomas. Although a recent retrospective study did not show a statistically significant difference in the survival of patients whose non-oropharyngeal tumors were p16-positive compared with those who were p16-negative, another recent review suggested that patients with p16-positive tumors did have a better prognosis.[54,55]

Conclusion

HPV-associated oral epithelial dysplasias present as leukoplakias in the oral cavity, similar to non-HPV leukoplakias associated with smoking. However, HPV-associated oral epithelial dysplasias have distinct histopathologic features namely prominent karyorrhexis and apoptosis, and all show strong positivity for p16, similar to oropharyngeal HPV-associated squamous cell carcinomas. As with other cancers, HPV-16 was the most common type identified (91% of cases) followed by HPV-33 and HPV-58 (4% each). In this series of cases, HPV-oral epithelial dysplasia was associated with invasive squamous cell carcinoma in 8/53 (15%) cases and it is unclear whether the prognosis of these squamous cell carcinomas will be different from

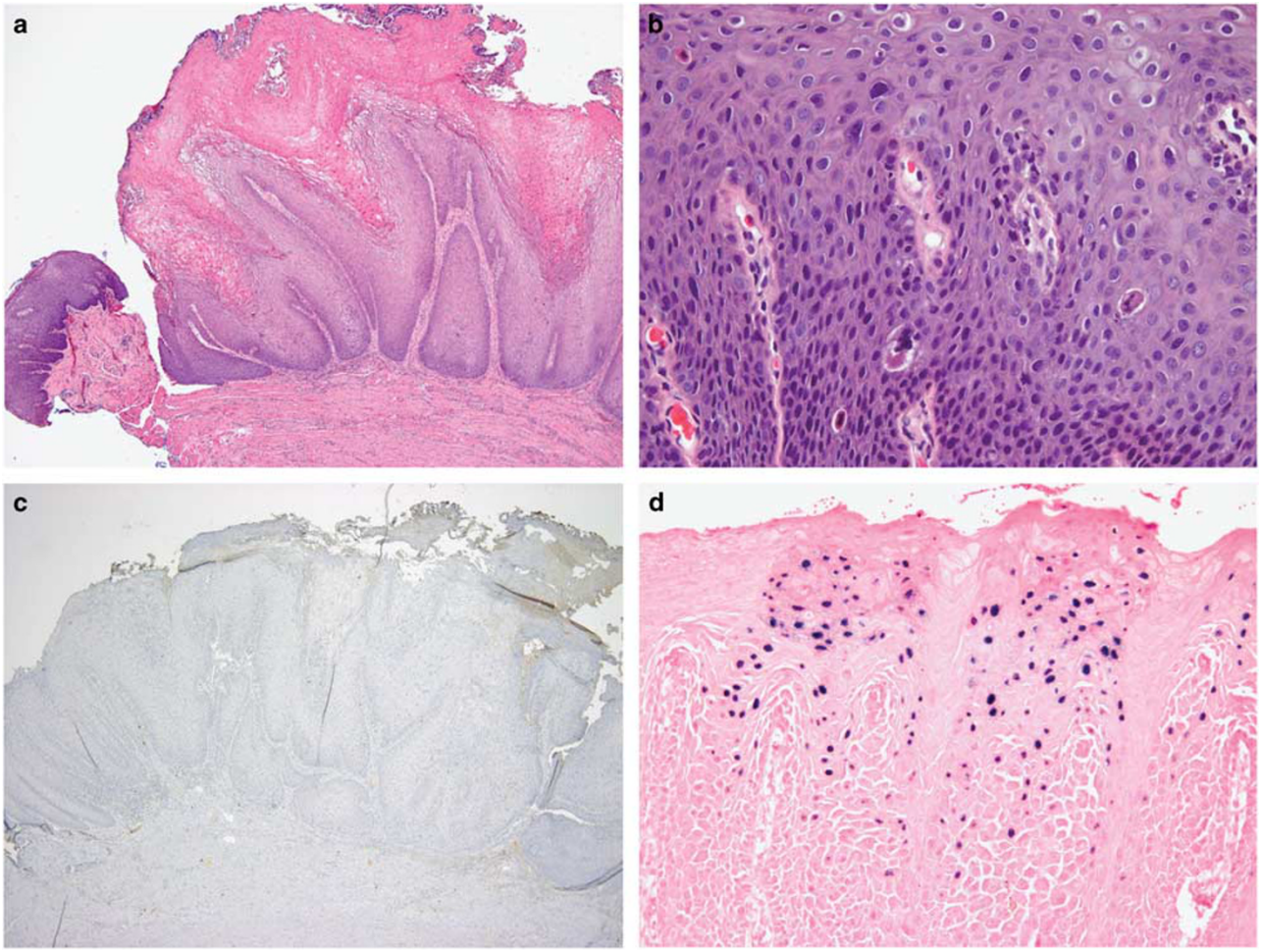

Figure 6 (**a**) Thick layer of parakeratin overlying dysplastic surface epithelium (hematoxylin and eosin, maginification ×40) (**b**) Characteristic features of HPV-oral epithelial dysplasia, including karyorrhectic cells and apoptotic cells (hematoxylin and eosin, magnification ×400). (**c**) Immunohistochemical studies for p16 are negative (immunohistochemical study for p16, magnification ×40). (**d**) Corresponding study exhibits nuclear positivity for high-risk human papillomavirus (*in situ* hybridization study for high-risk HPV, magnification ×200).

non-HPV-associated squamous cell carcinomas. Further studies may offer additional information on this entity in the future.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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