Comparison Between p16^{INK4A} Immunohistochemistry and Human Papillomavirus Polymerase Chain Reaction Assay in Oral Papillary Squamous Cell Carcinoma

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Purpose: Oral papillary squamous cell carcinoma (OPSCC) is a histologic variant of SCC with a growth pattern suggesting human papillomavirus (HPV) infection. The aim of this study was to investigate the presence of HPV genotypes in OPSCC.

Materials and Methods: All cases with a histologic diagnosis of OPSCC from 1993 through 2008 were retrieved and confirmed. Immunohistochemical evaluation for the surrogate marker p16^{INK4A} and HPV polymerase chain reaction were performed in tissue and DNA derived from formalin-fixed paraffin-embedded tissue.

Results: Forty-four patients with confirmed OPSCC (mean age, 71.96 yr; female-to-male ratio, 1.75:1) comprised the study population. The most common site of involvement was the gingiva followed by the palate and buccal mucosa. Forty cases exhibited an invasive component, 1 was noninvasive, and in 3 cases invasion could not be confirmed owing to suboptimal thickness of the biopsy. Paraffin tissue blocks were available in 41 cases. Twenty-three cases (56.1%) exhibited positive p16^{INK4A} staining, which was primarily weak to moderate with a generally focal pattern. Polymerase chain reaction assays were negative for HPV DNA in all cases.

Conclusions: In this study, there was a clinical predilection of OPSCC in older women, with most cases occurring in the masticatory mucosa. Weak to moderate and focal p16^{INK4A} staining was appreciated in contrast to reported staining properties in genital and oropharyngeal PSCC. Failure of the polymerase chain reaction assay to exhibit transcriptionally active HPV genotypes suggests that HPV is not associated with OPSCC tumorigenesis.

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Papillary squamous cell carcinoma (PSCC) constitutes a distinct histopathologic variant of SCC affecting predominantly men in the sixth and seventh decades of life.[1] Clinically, PSCC manifests as an exophytic proliferation with a papillary or warty surface involving, in order of descending frequency, the larynx, hypopharynx, oropharynx, and nasopharynx.[1-3] Risk factors for oropharyngeal PSCC include smoking and alcohol.[1]

Although frequently encountered in the upper aerodigestive tract, involvement of the oral cavity (OPSCC) is considered uncommon, with limited cases reported.[4,5] Intraoral locations include mainly the gingiva, tongue, and palate.[3,6] Rarely, OPSCC develops in lesions of proliferative verrucous leukoplakia.[7]

Microscopically, OPSCC is characterized by a dysplastic epithelial papillary growth pattern of variable thickness supported by a fibrovascular core. The neoplastic epithelium is composed of predominantly immature basaloid-type cells with prominent nuclear atypia and cellular pleomorphism.[1,8] Keratinization can vary from minimal to moderate.[1,9] However, most PSCCs exhibit absent or limited keratosis.[1,7,10] In addition, koilocytic changes in the upper epithelial layers may be present.[7] Invasion is characterized by single or multiple nests of neoplastic cells usually localized in the superficial aspect of the connective tissue.[8] If no stromal invasion is detected, the term *atypical papillary hyperplasia* or *PSCC in situ* is used.[9] Secondary microscopic features of PSCC include chronic lymphoplasmacytic inflammation, necrosis, and hemorrhage.[1,8] The limited invasion encountered in PSCCs has been associated with better prognosis and less aggressive biologic behavior of this histologic subtype of SCC, underlining the need for more conservative treatment compared with conventional SCC.[1,9]

Based on its histologic characteristics and scientific data supporting the involvement of human papillomavirus (HPV) in head and neck cancer,[11,12] an association between PSCC and viral infection has been speculated.[8,10] The important role of HPV infection in cervical and anogenital carcinogenesis is well established.[11,13] Specifically, it has been documented that high-risk HPV tumorigenesis is mediated through the inactivation of 2 major cell cycle regulators, p53 and retinoblastoma protein (pRb), by viral gene products E6 and E7.[14,15] E6-expressing epithelial cells are incapable of activating the p53-mediated DNA damage response pathway, whereas inactivation of pRb by E7 causes the overexpression of $p16^{INK4A}$, which results in the initiation of S phase, cell cycle dysregulation, proliferation, and then malignant transformation.[11,16,17]

Concerning the oral epithelium, a thorough investigation has been performed to elucidate the possible correlation of certain HPV genotypes, especially those with known high oncogenic potential such as HPVs 16 and 18, with dysplastic and malignant intraoral lesions.[18] Despite the increasing volume of information, results regarding reported HPV detection rates remain inconclusive because of various differences principally concerning the demographics and sample sizes and the sensitivity of the applied molecular techniques.[8]

OPSCC has not received significant attention in the oral pathology and surgery literature. Further, investigation of HPV infection as a possible pathogenetic mechanism for PSCC of the oral cavity proper is limited. In the present report, the authors review the clinicopathologic features of 44 cases of OPSCC and evaluate their possible correlation with HPV genotypes. The latter was attempted through immunohistochemical expression of the surrogate marker $p16^{INK4A}$ and polymerase chain reaction (PCR). The results of these methods were compared with their efficacy for HPV detection in OPSCC.

Materials and Methods

The study was approved by the University of Minnesota institutional review board. All cases of OPSCC accessioned in the Division of Oral and Maxillofacial Pathology, School of Dentistry, University of Minnesota (Minneapolis, MN) from 1993 through 2008 were retrieved. The diagnosis of OPSCC was verified by 1 oral pathologist (I.G.K.) and 1 surgical pathologist (S.E.P.) using the 2005 World Health Organization criteria, and available clinical data on patient age, gender, and race and cancer location were tabulated.

Immunohistochemical staining was performed using a specific mouse antihuman monoclonal antibody against $p16^{INK4A}$ (clone E6H4; MTM Laboratories AG, Heidelberg, Germany) on the Ventana Nexes Automated Immunostaining Platform (Ventana Medical Systems, Tucson, AZ) according to the manufacturers' instructions. Appropriate positive and negative controls were included. Grading of immunohistochemical staining was performed by the 2 pathologists (I.G.K. and S.E.P). Positive $p16^{INK4A}$ immunostaining was assessed using a double evaluating system calculated by multiplying the intensity of staining (0, negative; 1, weak; 2, moderate; 3, strong) with the percentage of stained neoplastic cells in a low-power field (1, 1% to 10%; 2, 11% to 50%; 3, 51% to 75%; 4, >75%). The immunohistochemical staining pattern (focal vs diffuse [bandlike]) was recorded.

For additional detection of HPV genotypes, PCR was performed on total DNA extracted from formalin-fixed paraffin-embedded tissues. All paraffin blocks used contained more than 30% lesional tissue. Ten to 20 paraffin ribbons per case were obtained to extract DNA. For PCR, the E6 (L1) highly conserved region was amplified using the QIAxcel system (Qiagen, Valencia, CA) and L1 primers (MY11, L1

cons-S: 5′-GCMCAGGGWCATAAYAATGG-3′; MY9, L1 cons-A: 5′-CGTCCMARRGGAWACTGATC-3′). An HPV-16–infected cell line was used as a positive control. PCR was performed in an iCycler IQ thermocycler (Bio-Rad Laboratories, Hercules, CA) and PCR products were analyzed on the QIAxcel instrument. Negative or positive results were those with an absence or presence of a band in the positive HPV range of 431 to 486 bp, respectively.

Results

Of 834 cases of oral SCC diagnosed from 1993 through 2008, 44 (5.3%) were subclassified as OPSCC, with 28 (63.6%) affecting women and 16 (36.4%) affecting men. With regard to racial distribution, 41 patients (93.2%) were Caucasian, 1 (2.3%) was Native American, and no information was recorded for 2 (4.5%). The mean age was 71.96 years (standard deviation, 15.96 yr; median, 76 yr). The demographic characteristics of the 44 cases of OPSCC are presented in Table 1. Unfortunately, no information was provided concerning the smoking habits of these patients. Regarding the location of the primary intraoral tumors, 18 (41%) occurred on the gingiva, 13 (72.2%) involving the mandible and 5 (27.8%) the maxilla; 7 (16%) on the palate; 6 (14%) on the buccal mucosa; 5 (11%) on the tongue (Fig 1); 5 (11%) on the floor of the mouth; and 1 each (2%) on the lip and retromolar mucosa (Fig 2). In 1 case, the location was not specified. Based on these observations, it is evident that more than half (25 cases; 56.8%) involved the masticatory mucosa (gingiva and palate). The histologic material of this study consisted of diagnostic biopsies procured from the database of the Division of Oral and Maxillofacial Pathology, School of Dentistry, University of Minnesota. Hence, outcome data, including the incidence of cervical metastasis and survival rate, were not available for inclusion in this study.

Table 1. DEMOGRAPHIC CHARACTERISTICS OF 44 CASES OF ORAL PAPILLARY SQUAMOUS CELL CARCINOMA

Gender, n (%)	
Female	28 (63.6)
Male	16 (36.4)
Race, n (%)	
Caucasian	41 (93.2)
Native American	1 (2.3)
No available data	2 (4.5)
Age (yr)	
Average ± SD	71.96 ± 15.96
Median	76

Abbreviation: SD, standard deviation.

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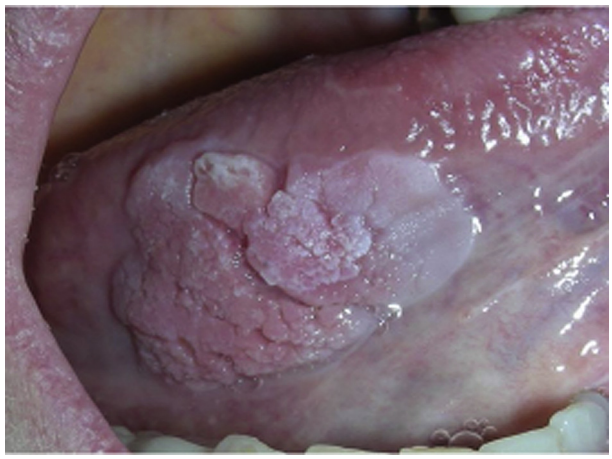

FIGURE 1. Oral papillary squamous cell carcinoma manifesting as an exophytic verrucoid lesion of the right lateral border of the tongue.

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Histologically, 40 cases (91%) exhibited invasion of the connective tissue by epithelial neoplastic cells (Figs 3, 4), 1 (2%) was noninvasive OPSCC (OPSCC in situ), and in 3 (7%) invasion could not be determined owing to the limited thickness of the incisional biopsy (Table 2). Forty-one blocks were available for immunohistochemical staining and PCR evaluation. Against p16^{INK4A}, 23 cases (56.1%) showed positive staining, with a score from 1 to 9 (mean, 4.52;

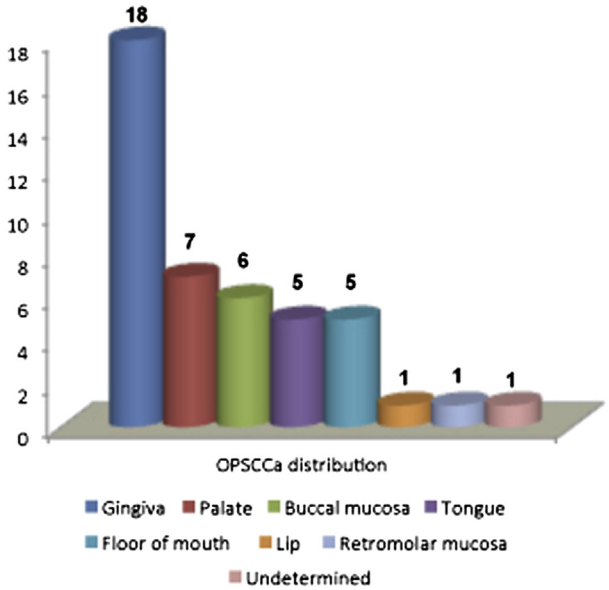

FIGURE 2. Intraoral distribution of 44 cases of oral papillary squamous cell carcinoma (OPSCC).

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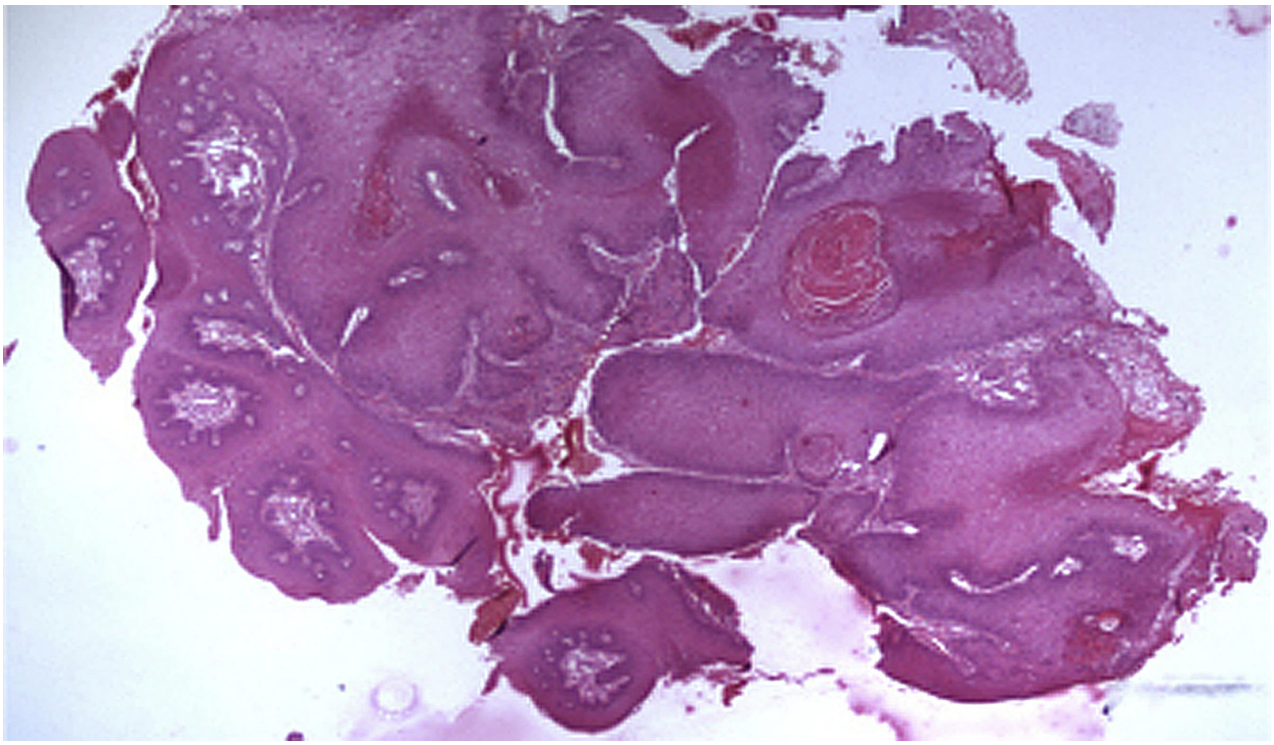

FIGURE 3. Low-power microphotograph depicting a pronounced broad-based papillary architectural pattern (hematoxylin and eosin stain; magnification, ×100).

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standard deviation, 2.15; median, 4; Fig 5). In 18 cases (43.9%), the score was 0, indicating negative immunohistochemical expression of the molecule p16^{INK4A} by neoplastic epithelium. Table 3 presents the results of immunohistochemical evaluation with p16^{INK4A}. Positive p16^{INK4A} tumors exhibited a predominantly focal immunostaining pattern (20 of 23; 87%). Only in 3 of 23 cases (13%) of OPSCC did neoplastic cells show diffuse immunopositivity. HPV DNA testing by PCR was negative in all 41 tissue samples.

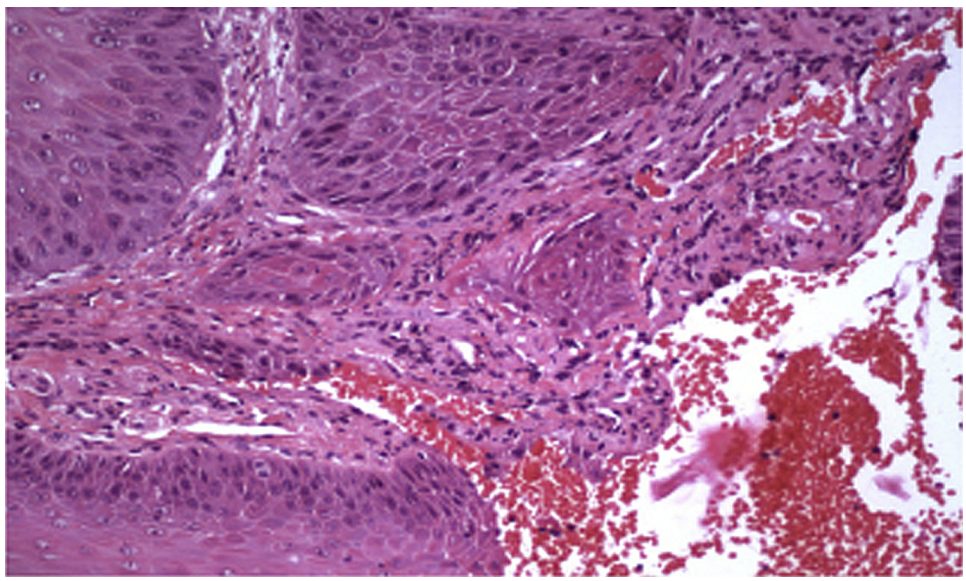

FIGURE 4. High-power microphotograph depicting invasion of subjacent fibrous connective tissue by epithelial neoplastic nests. Cytologic and histologic variations, including cellular atypia and pleomorphism with loss of polarity, hyperchromatism, increased nuclear-to-cytoplasmic ratio, and atypical mitoses, are visible (hematoxylin and eosin stain; magnification, ×300).

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Table 2. HISTOPATHOLOGIC FEATURES OF 44 CASES OF OPSCC

Invasive OPSCC	40 (91%)
Noninvasive OPSCC	1 (2%)
Invasion could not be evaluated	3 (7%)

Abbreviation: OPSCC, oral papillary squamous cell carcinoma.

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Table 3. ASSESSMENT OF IMMUNOHISTOCHEMICAL EXPRESSION OF P16INK4A IN ORAL PAPILLARY SQUAMOUS CELL CARCINOMA

Score, 1-9 (mean, 4.52; SD, 2.15; median, 4)	23 (56.1%)
Score, 0	18 (43.9%)
Could not be evaluated	3

Abbreviation: SD, standard deviation.

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Discussion

PSCC represents a rare histopathologic variant of SCC, with an estimated incidence in the head and neck region of approximately 0.1%.[7] The upper aerodigestive tract, including the oropharynx, hypopharynx, larynx, and sinonasal tract, is the most common site of involvement.[7,8,10] Intraoral occurrence is, in the authors' opinion, under-reported because in their data OPSCCs comprised 5.3% of all oral SCCs diagnosed in a 15-year period. There are few relevant publications in the English-language literature.[19]

Clinically, OPSCC reportedly affects older patients compared with typical oral SCC. In a clinicopathologic study of 12 cases of OPSCC, Ding et al[19] reported a mean age of 72.9 years at the time of initial diagnosis, which is in agreement with the average age of the patients in the present study (71.96 yr). Regarding gender distribution, oropharyngeal PSCC exhibits a male predilection.[1] However, the present study indicated a female predominance, with a female-to-male ratio of 1.75:1. A female predilection (female-to-male ratio, 1.4:1) also was observed by Ding et al.[19] In the present study, the most frequent location of OPSCC was the gingiva and particularly the mandibular gingiva, followed by the palate and buccal mucosa. Ding et al[19] found that the most common locations were the cheek and gingiva.

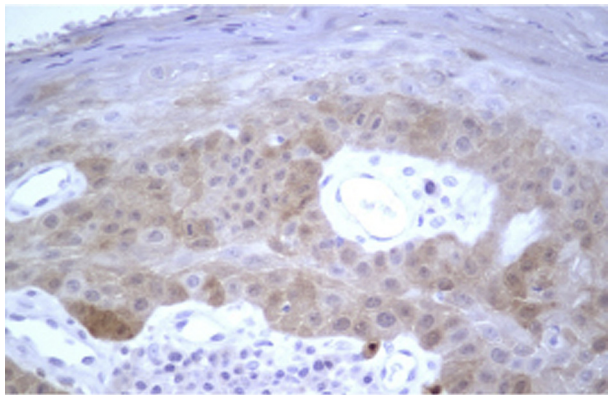

FIGURE 5. Immunohistochemical findings against p16INK4A. Mild to moderate and focally intense staining of the basal and suprabasal cell layers is observed (avidin-biotin complex stain; hematoxylin counterstain; original magnification, ×400).

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Histopathologically, 2 different architectural patterns, papillary and exophytic, were recognized, although their significance is questionable.[9] The papillary pattern consists mainly of multiple, thin, delicate, fingerlike papillary projections supported by fibrovascular cores, whereas the exophytic pattern is characterized by broad-based bulbous to "cauliflowerlike" exophytic proliferations with multiple blunt, rounded projections.[9,19] Owing to similarities in their clinicopathologic features, cases of OPSCC have been diagnosed as exophytic SCC or lumped together with ordinary SCC in various studies.

Invasion of subjacent connective tissue occurs in the form of single or multiple nests of neoplastic epithelial cells infiltrating, in most cases, the superficial aspect of the connective tissue.[8] In the present study, invasion was observed in 40 cases (91%), and 1 case (2%) represented a noninvasive pattern. Interestingly, in tissue specimens derived from incisional biopsies, locating invasive foci can be challenging or even impossible, even after deeper sections are evaluated.[10] In 3 cases (7%) of OPSCC included in the present study, invasion could not be confirmed owing to the suboptimal thickness of the incisional biopsy. Thus, to confirm invasion, an adequate depth of incisional biopsy is recommended for clinical lesions suspicious for OPSCC.

Considering the growth pattern of PSCC, Batsakis et al[7] suggested an HPV etiology for at least some cases. HPV has been confirmed as the etiologic factor of various epithelial malignancies, especially of the anogenital area, in men and women.[20-22] In addition to HPV-related anogenital carcinogenesis, recent molecular findings have associated oropharyngeal carcinoma with HPV, with a reported positivity of 0% to 64%, or even higher.[10,23,24]

The oncogenic effect of HPV is regulated through the expression of viral oncoproteins E6 and E7 in low- and high-risk subtypes.[25] E6 and E7 in high-risk

HPV genotypes, such as 16 and 18, bind their targeted tumor suppressor proteins with stronger affinity, thus acquiring an increased tumorigenic and oncogenic potential[12,26] that leads to cell immortalization and carcinogenesis.[27]

Biologically, the E6 protein induces degradation of tumor suppressor protein p53 through ubiquitin-mediated proteolysis. This phenomenon leads to substantial loss of p53 function, such as cell cycle arrest (G1 phase) and apoptosis. In consequence, infected epithelial cells with pronounced expression of E6 are incapable of initiating p53-regulated DNA damage repair mechanisms and, inevitably, are susceptible to an accumulation of genetic alterations.[12,17] Furthermore, E7 protein is responsible for inactivation of the Rb tumor suppressor gene product pRb, resulting in malfunction of the cell cycle and proliferation.[17] After inactivation of pRb, a feedback overexpression of $p16^{INK4A}$ is observed.[11,28] The p16 protein is encoded by the *CDKN2A* (*INK4A*) gene located on chromosome 9q21 and functions as a decelerator of the cell cycle by inactivation of the cdk4 and cdk6 cyclin D complexes.[11]

Immunohistochemical assessment of $p16^{INK4A}$ overexpression by neoplastic cells has been proposed as a reliable biomarker for the identification of HPV genotypes[11,24,29] and is easier than other options, such as DNA-based PCR,[30] DNA-based in situ hybridization (ISH),[31] and RNA-based reverse transcriptase PCR.[32] Nevertheless, these methods present different degrees of sensitivity and specificity against HPV genotypes, with p16 immunohistochemistry and PCR-based assays yielding high sensitivity and ISH yielding high specificity.[12]

In addition to being a trustworthy diagnostic marker for HPV infection, immunohistochemical expression of $p16^{INK4A}$ is considered an important prognostic factor because $p16^{INK4A}$-positive findings for oropharyngeal SCC are associated with a more favorable prognosis regardless of tumor HPV status as identified with ISH or PCR.[33]

The present study concerns the authors' experience regarding OPSCC and their investigation of a possible correlation with HPV through the immunohistochemical expression of the surrogate marker $p16^{INK4A}$ and PCR. With respect to immunohistochemical staining against $p16^{INK4A}$, 56.1% of OPSCCs included in the present study exhibited positive staining of predominantly weak to moderate intensity, whereas in 43.9% of specimens the score was 0, indicating negative immunohistochemical expression of the molecule $p16^{INK4A}$. Moreover, positivity of $p16^{INK4A}$ lesions was characterized by predominantly focal immunostaining (87%). The significance of the observed staining pattern for $p16^{INK4A}$ is of great interest because a strong and diffuse positive staining pattern in HPV-related lesions has been observed in the genital and upper aerodigestive tracts,[32] whereas the positive staining pattern was mostly weak to moderate and focal in the present study. Similar results have been observed in previous studies.[33-35] Lewis et al[32] proposed the following criteria as a marker for accepted transcriptionally active HPV in SCC: *1)* more than 75% of neoplastic cells positive for p16 or *2)* more than 50% of neoplastic cells positive for p16 with the simultaneous presence of confluent areas.

In the present study, the PCR assay failed to identify transcriptionally active HPV genotypes in $p16^{INK4A}$-positive OPSCCs. Various explanations can be proposed for this observation, mainly associated with molecular events during the multistage process of carcinogenesis. Epigenetic mutation of Rb or disruption of the Rb pathway leading to inactivation of the suppressor effect of pRb is a common phenomenon in SCC,[36] thus causing a potential reactive overexpression of the $p16^{INK4A}$ molecule that is not attributed to HPV infection. Another scenario, although less probable, is the accumulation of nonspecific immunohistochemical staining that is misevaluated as HPV-related $p16^{INK4A}$ positivity. In this study, the authors focused on the role of HPV in the pathogenesis of OPSCC. OPSCCs have been studied with PSCCs of the upper aerodigestive tract. Molecular data from studies focusing on PSCC of the head and neck region have reported a 36% to 68% presence of transcriptionally active HPV subtypes, with low-risk (6 and 11) and high-risk (16 and 18) genotypes identified.[8,10] The observed discrepancies are attributed to sample sizes, demographic variations, and sensitivity of the molecular method applied for HPV detection.

In summary, of 44 cases of OPSCC, 56.1% exhibited $p16^{INK4A}$-positive immunohistochemical expression, but without confirmation of transcriptionally active HPV genotypes by PCR. Further studies should be pursued to elucidate the molecular events that cause the phenotypic features of OPSCC and the extent of HPV participation in the etiopathogenesis of this rare type of neoplasm. Combined diagnostic assays, including $p16^{INK4A}$ immunohistochemistry with ISH or PCR, will better clarify this phenomenon, providing the basis for modified anticancer treatment.

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