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Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas

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SUMMARY

Background: Human papillomavirus (HPV) is a cause of oropharyngeal cancer, but a role for HPV in the etiology of oral cavity squamous cell carcinomas (OCSCC) remains uncertain.
Methods: We sought to estimate the etiologic fraction for HPV among consecutive, incident OCSCC diagnosed from 2005 to 2011 at four North American hospitals. DNA and RNA purified from paraffin-embedded tumors were considered evaluable if positive for DNA and mRNA control genes by quantitative PCR. Fifteen high-risk (HR) HPV types were detected in tumors by consensus PCR followed by type-specific HR-HPV E6/7 oncogene expression by quantitative reverse-transcriptase PCR. P16 expression was evaluated by immunohistochemistry (IHC). A study of 400 cases allowed for precision to estimate an etiologic fraction of as low as 0% (97.5% confidence interval, 0–0.92%).
Results: Of 409 evaluable OCSCC, 24 (5.9%, 95%CI 3.6–8.2) were HR-HPV E6/7 expression positive; 3.7% (95%CI 1.8–5.5) for HPV16 and 2.2% (95%CI 0.8–3.6) for other HR-HPV types. HPV-positive tumors arose from throughout the oral cavity (floor of mouth [$n = 9$], anterior tongue [6], alveolar process [4], hard palate [3], gingiva [1] and lip [1]) and were significantly associated with male gender, small tumor stage, poor tumor differentiation, and basaloid histopathology. P16 IHC had very good-to-excellent sensitivity (79.2%, 95%CI 57.9–92.9), specificity (93.0%, 95%CI 90.0–95.3), and negative-predictive value (98.6%, 95%CI 96.8–99.6), but poor positive-predictive value (41.3%, 95%CI 27.0–56.8) for HR-HPV E6/7 expression in OCSCC.
Conclusion: The etiologic fraction for HR-HPV in OCSCC was 5.9%. p16 IHC had poor positive predictive value for detection of HPV in these cancers.

Introduction

Human papillomavirus (HPV) infection is the principal cause of a subset of oropharyngeal squamous cell carcinomas (OPSCC). Epidemiological associations with sexual behavior and HPV exposure are strong and consistent for OPSCC, but less so for oral cavity squamous cell carcinoma (OCSCC).[1] A role for HPV in the pathogenesis of OCSCC therefore remains somewhat controversial.

In a systematic review by Kreimer and colleagues, HPV DNA was detected in 24% of OCSCC worldwide.[2] However, the presence of HPV DNA alone is insufficient evidence for a causal association from a molecular perspective. Expression of HPV oncogenes E6 and E7 remains a gold standard for classification of an HPV-caused cancer and is necessary for tumor initiation[3] and maintenance of the malignant phenotype[4,5] in model systems of oral cancer. While case reports have provided compelling evidence of HPV E6/E7 expression in some cases of OCSCC[5,6], comprehensive analyses of large series have not been reported.

A recent analysis of OPSCC collected as part of the Surveillance, Epidemiology and End Results (SEERs) program in the United States (US) estimated that the proportion of OPSCC attributable to HPV infection increased from 16% to 72% between 1988 and 2004.[7] An analogous fourfold increase in the HPV-attributable fraction for OCSCC could elevate even a negligible fraction to a significant fraction at the population level. We therefore evaluated a large series of consecutive cases of OCSCC diagnosed in North America from 2005 to 2011 for high-risk (HR)-HPV E6/E7 oncogene expression.

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Methods

Study population and design

A four-institution, retrospective case-series was designed to estimate the etiologic fraction for HPV in OCSCC and was powered to detect a prevalence of as low as 0% with precision. Zero positives among 400 cases would provide a one-sided 97.5%CI of 0–0.92%. Adjusted for an estimated 7.5% in-evaluable samples, a total of 430 cases were included in the analysis.

Eligible tumor specimens included consecutive, newly diagnosed cases of formalin-fixed, paraffin-embedded, pathologically-confirmed, in situ or invasive squamous cell carcinoma of the oral cavity (inclusive of lip, ventibule of mouth, gingiva, alveolar process, tongue, buccal mucosa, hard palate, floor of mouth and retromolar trigone) diagnosed at four academic medical centers in North America, including: The Ohio State University, Columbus, OH; Princess Margaret Hospital, Toronto, CA; University of Chicago, Chicago, IL; and The University of California, San Francisco (UCSF), CA. Consecutive cases were identified from pathology archives retrospective from a diagnosis on December 31, 2011 until a total of 430 were obtained. Anatomic site of tumor origin was determined by the operating physician and confirmed by the pathologist. Subject age, gender and AJCC TNM stage were extracted from pathology reports, but stage was not available for some cases from a biopsy referral service at UCSF. Institutional Review Board approval was obtained from all participating sites.

Histopathological analysis

Histopathological interpretation was performed by pathologists (AS, ML) masked to laboratory analysis. Hematoxylin and eosin stained slides were used to confirm presence and estimate the proportion of in situ or invasive squamous cell carcinoma in the specimen as well as to classify histopathological features, including differentiation status (well, moderate, poor) and histopathological variants of squamous cell carcinoma. Tumors were categorized into variants of squamous cell carcinoma based upon the presence of specific histopathological features as previously described for acantholytic[8,9], adenosquamous[9], basaloid[8,10], carcinoma cuniculatum[11,12], verrucous carcinoma[8,13], papillary[8,9], spindle cell[8,10,12], and lymphoepithelial-like variants[8,9].

All tumors were evaluated for expression of a surrogate biomarker of HPV E7 oncoprotein function, the cdk inhibitor p16, by means of an immunohistochemical analysis with a mouse monoclonal antibody (MTM Laboratories, City State) visualized with use of an autostainer and a cone-view secondary detection kit[14]. Positive p16 expression was defined as an H score of 60 or greater as previously described[15], where the H score was derived from the cross product of intensity of staining (0, 1, 2, 3+) and percent of tumor staining at maximum intensity.

The specificity of HPV to tumor cell nuclei was evaluated for all tumors positive for HPV DNA by use of the in situ hybridization-catalyzed signal amplification method for biotinylated probe (Genpoint, Dako, Carpinteria, CA.)[16] with either a biotinylated DNA probe that was specific for HPV16 (code Y1407, Dako) or a wide spectrum probe for detection of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 (code Y1443, Dako). Tumors with punctuate or diffuse staining specific to tumor cell nuclei were considered positive.

Laboratory analysis

A study-specific standard operating procedure was used by all sites for serial sectioning of paraffin embedded tumor blocks. New blades were used for each tumor sample. Sectioning included: hematoxylin and eosin verification of tumor in the specimen; 10 μm section paraffin curls × two for DNA and RNA isolation; and 4 μm sections × 10 mounted on adherent slides.

DNA was isolated from paraffin curls by use of proteinase K digestion, phenol–chloroform extraction and ethanol precipitation.[17]

Total RNA was extracted using High Pure RNA Paraffin Kits (Roche, Mannheim, Germany) per the manufacturer's protocol. DNA and RNA quantity and purity (calculated by use of the ratio of the absorbance at 260 nm to that at 280 nm [260/280 ratio]) were measured with the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc, Wilmington, DE).

After DNase treatment, 0.3 μg of total RNA was reverse transcribed to cDNA by use of High Capacity RNA-to-cDNA Master Mix per the manufacturer's protocol (Applied Biosystems, Carlsbad, California). Controls with no reverse transcriptase were performed in parallel for each sample.

Specimens were classified as evaluable or in-evaluable for DNA analysis by use of a real-time Taq-Man PCR assay that amplified a 58 bp region of a control gene (human endogenous retrovirus-3, ERV-3) as previously described.[18] Briefly, 2 μL of purified tumor tissue DNA was analyzed. A standard curve was generated in duplicate from a fivefold dilution series (from 150,000 to 1.92 cells) of a diploid human cell line, CCD-18LU (ATCC, Manassas, VA). Samples with ERV-3 values above the lower limit of reproducibility of the assays (>3 copies) were considered evaluable.

Specimens were classified as evaluable for RNA analysis (after reverse transcription to cDNA) by use of a real-time quantitative Taq-Man reverse transcriptase (qRT)-PCR assay designed to amplify a 73 bp region of a housekeeping gene, human ribosomal protein large P0 (RPLPO) as previously described.[18] Samples with RPLPO values above the lower limit of reproducibility of the assays (>3 copies) were considered evaluable.

Purified tumor DNA was evaluated for the presence of DNA of 15 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), three potentially high-risk types (26, 53, 66) and seven low-risk types (6, 11, 40, 43, 44, 54, 69, 71, 70, 74) by consensus primer PCR amplification by use of the SPF_{10} primer system designed to amplify a 65 bp fragment of the conserved L1 region of the genome, followed by reverse line blot hybridization for HPV type specification (The Inno-LiPA assay, Innogenetics, Gent, Belgium). Samples positive for H(human)DNA control were considered evaluable as indicated by the manufacturer.

HPV type-specific TaqMan quantitative real-time PCR assays designed to amplify a 60–136 bp fragment of the E6 or E7 region (depending on type) of the 15 HPV types classified as high-risk as per Munoz and colleagues[19] noted above were used: (1) to analyze all tumors for HPV16 E6 DNA; (2) to confirm HPV type-specific detection in samples positive by the Inno-LiPA assay; and (3) to analyze samples in-evaluable by Inno-LiPA (human DNA control negative) but evaluable by ERV3 for 15 HR-HPV DNA types as previously described.[7,15,18] Primer and probe sequences as well as reaction conditions are shown in Supplementary Table 1. Samples above the lower limit of reproducibility of the assays (for all, $\geqslant 3$ copies) were considered positive. HPV viral load in tumors was estimated from the quotient of viral load and ERV-3, adjusted to the percent tumor present in the sample.

Purified tumor RNA was evaluated for HR-HPV E6/7 mRNA expression after reverse transcription to cDNA by use of HPV type-specific quantitative real-time TaqMan PCR assays noted above. All tumors were evaluated for HPV16 E6/7 expression. Additionally, all tumors positive for HPV DNA were evaluated for HPV E6/7 expression by qRT-PCR for the corresponding HPV type(s) detected. Results were reported as HPV E6/7 mRNA expression level

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Table 1
Demographic and clinical characteristics of 409 cases of oral cavity squamous cell carcinoma, stratified by HPV E6/7 oncogene expression.

Factor	*N* (%) (*n* = 409)	Type-specific		χ^2 p[a]
		Gold standard HPV		
		Neg. (%) (*n* = 385)	Pos. (%) (*n* = 24)	
Gender				
Female	173 (42.3)	170 (44.2)	3 (12.5)	
Male	236 (57.7)	215 (55.8)	21 (87.5)	0.004
Site[b]				
OSU	130 (31.8)	120 (31.2)	10 (41.7)	
PMH	94 (23.0)	90 (23.4)	4 (16.7)	
UC	95 (23.2)	90 (23.4)	5 (20.8)	
UCSF	90 (22.0)	85 (22.1)	5 (20.8)	0.558
Year of collection				
2005–2007	102 (24.9)	98 (25.5)	4 (16.7)	
2008–2009	168 (41.1)	155 (40.3)	13 (54.2)	
2010–2011	139 (34.0)	132 (34.3)	7 (29.2)	0.694
Anatomic subsite				
Alveolar process	39 (9.5)	35 (9.1)	4 (16.7)	
Buccal mucosa	34 (8.3)	34 (8.8)	0 (0)	
Floor of mouth	83 (20.3)	74 (19.2)	9 (37.5)	
Gingiva	31 (7.6)	30 (7.8)	1 (4.2)	
Hard palate	25 (6.1)	22 (5.7)	3 (12.5)	
Lip	21 (5.1)	20 (5.2)	1 (4.2)	
Retromolar trigone	7 (1.7)	7 (1.8)	0 (0)	
Tongue	162 (39.6)	156 (40.5)	6 (25.0)	
Vestibule of mouth	7 (1.7)	7 (1.8)	0 (0)	0.383
Staging type				
Clinical	122 (29.8)	117 (30.4)	5 (20.8)	
Pathological	269 (65.8)	252 (65.5)	17 (70.8)	0.481[c]
Biopsy service	13 (3.2)	13 (3.4)	0 (0)	
Missing	5 (1.3)	3 (0.8)	2 (8.3)	
AJCC tumor stage				
Tis	4 (1.0)	2 (0.5)	2 (8.3)	
T1	132 (32.3)	122 (31.7)	10 (41.7)	
T2	126 (30.8)	121 (31.4)	5 (20.8)	
T3	44 (10.8)	44 (11.4)	0 (0)	
T4/a/b/NOS	86 (21.0)	80 (20.8)	6 (25.0)	0.012[c]
Biopsy service	13 (3.2)	13 (3.4)	0 (0)	
Missing	4 (1.0)	3 (0.8)	1 (4.2)	
AJCC nodal stage				
N0	185 (45.2)	172 (44.7)	13 (54.2)	
N1	43 (10.5)	42 (10.9)	1 (4.2)	
N2/a/b/c	80 (19.6)	77 (20.0)	3 (12.5)	
N3	4 (1.0)	3 (0.8)	1 (4.2)	
Nx	81 (19.8)	76 (19.7)	5 (20.8)	0.316[c]
Biopsy service	13 (3.2)	13 (3.4)	0 (0)	
Missing	3 (0.7)	2 (0.5)	1 (4.2)	
AJCC metastasis stage				
M0	103 (25.2)	99 (25.7)	4 (16.7)	
M1	3 (0.7)	3 (0.8)	0 (0)	
Mx	266 (65.0)	247 (64.2)	19 (79.2)	0.454[c]
Biopsy service	13 (3.2)	13 (3.4)	0 (0)	
Missing	24 (5.9)	23 (6.0)	1 (4.2)	
Tumor differentiation				
Grade 1	119 (29.1)	112 (29.1)	7 (29.2)	
Grade 2	220 (53.8)	214 (55.6)	6 (25.0)	
Grade 3	70 (17.1)	59 (15.3)	11 (45.8)	0.001
Histologic variant				
Acantholytic squamous cell carcinoma	21 (5.1)	20 (5.2)	1 (4.2)	
Basaloid squamous cell carcinoma	14 (3.4)	5 (1.3)	9 (37.5)	
Lymphoepithelial carcinoma (non-nasopharyngeal)	1 (0.2)	1 (0.3)	0 (0)	
Papillary squamous cell carcinoma	2 (0.5)	2 (0.5)	0 (0)	
Spindle cell squamous carcinoma	3 (0.7)	3 (0.8)	0 (0)	
Squamous cell carcinoma, conventional	365 (89.2)	352 (91.4)	13 (54.2)	
Verrucous carcinoma	3 (0.7)	2 (0.5)	1 (4.2)	<0.001

[a] Pearson χ^2 or Fisher's exact test.
[b] Dates of collection: 2007–2010, OSU; 2007–2010, PMH; 2005–2011, UC; 2007–2011, UCSF.
[c] Excluding missing, biopsy service.

normalized to 1000 copies of RPLP0 mRNA as evaluated by qRT-PCR.

All p16-positive tumors that were HPV-negative by the above analysis were further screened for HPV DNA sequences from HPV

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types other than the high-risk and low-risk types noted above by consensus primer PCR by use of GP5+/6+ primer sets[20] followed by gel electrophoresis and DNA sequencing.

Statistical considerations

Differences between evaluable and in-evaluable cases as well as type-specific gold standard HPV-positive and -negative samples in demographic and clinical characteristics were analyzed using contingency table chi-square tests or Fisher's exact test. Nonparametric Mann–Whitney or Kruskal–Wallis tests were used to determine equality of medians in age at diagnosis and laboratory testing values. Contingency tables were used to determine sensitivity, specificity, and positive and negative predictive values (and 95% confidence intervals [CI]) for p16 classification compared to HR-HPV E6/7 expression. All reported p values were two-sided. Statistical analyses were performed by use of Stata 10.1 software (StataCorp, College Station, TX).

Results

A flow diagram of the laboratory analysis performed is shown in Fig. 1. A total of 430 OCSCC were obtained for analysis, and 21 (4.7%) tumors were deemed in-evaluable due to inability to amplify a control gene (ERV-3) by PCR or absence of detectable expression of a control gene (RPLPO) by qRT-PCR. The demographic and clinical characteristics of the 409 cases of OCSCC included in the final analysis are shown in Table 1. When evaluable and in-evaluable OCSCC were compared with regard to these factors, evaluable tumors were significantly more frequent among men and were more likely to be tongue cancers (Supplementary Tables 2 and 3).

The total yield and purity of DNA (median 1.95 ugs [interquartile range [IQR], 1.05–5.98], 260/280 ratio 1.63 [IQR 1.56–1.69]) extracted from paraffin-embedded tumors was very good, with a median yield of 1,068 (IQR 562–1860) human diploid genome equivalents per microliter of sample (total 50 μL), as estimated by real-time PCR for a control gene (ERV-3). The RNA was of similarly high quantity and quality (median 2.70 ugs [IQR 1.38–6.36], 260/280 ratio 1.95 [IQR1.83–2.0]), with a median of 371 (IQR 167–878) RPLPO copies per microliter of sample, as estimated by qRT-PCR. The distribution of values for ERV3 and RPLPO among evaluable cases is shown in Fig. 2, stratified by calendar period of diagnosis. Although values declined significantly with calendar time, yield among older samples remained sufficient for analysis.

In our analysis, HR-HPV DNA was detected in 40 of 409 (9.8%, 95%CI 6.9–12.7) OCSCC. Neither possibly high-risk (26, 53, 66) nor low-risk (6, 11, 40, 43, 44, 54, 69, 71, 70, 74) HPV types were detected in any of the tumors. When evaluated by use of the Inno-LiPA assay, 31 of 409 OCSCC were negative for the human DNA control. However, all of these samples had high human DNA content when evaluated for a control gene (ERV-3) by quantitative real-time PCR (median 7327, IQR [3343, 12678]), consistent with the presence of an inhibitor of the Inno-LiPA assay. Therefore, these 31 samples were also evaluated for HPV DNA and mRNA by use of 15 different HR-HPV type-specific real-time PCR assays, and none were positive. Forty of 378 evaluable samples were positive in the Inno-LiPA assay [HPV16 ($n = 27$), HPV 18 (2), HPV31 (2), HPV33 (2), HPV35 (1), HPV39 (2), HPV45 (1), HPV51 (2), HPV 52 (1)]. HPV16 DNA and mRNA were not detected in any additional cases when all 409 tumors were evaluated by real-time PCR and qRT-PCR.

Of the 40 samples positive for HR-HPV DNA by the Inno-LiPA assay, 27 were confirmed positive by type-specific real-time PCR for the corresponding HPV type. When evaluated for HR-HPV E6/7 expression, 24 cases (5.9%, 95%CI 3.6–8.2) were positive [HPV16 ($n = 15$), HPV 18 (2), HPV31 (2), HPV33 (2), HPV35 (1), HPV39 (1), HPV45 (1)]. The laboratory analysis for the 16 OCSCC that were HPV positive by Inno-LiPA but negative for HPV E6/7 expression analysis is summarized in Supplementary Table 4. Of note, the HPV viral load among cases that were positive for HPV DNA but negative for E6/7 expression was significantly lower than for cases positive for E6/7 expression (0 [IQR 0.0–0.002] versus 9.8 [IQR 2.2–152.0] copies per cell, $p < 0.001$). The specificity of HPV DNA to tu-

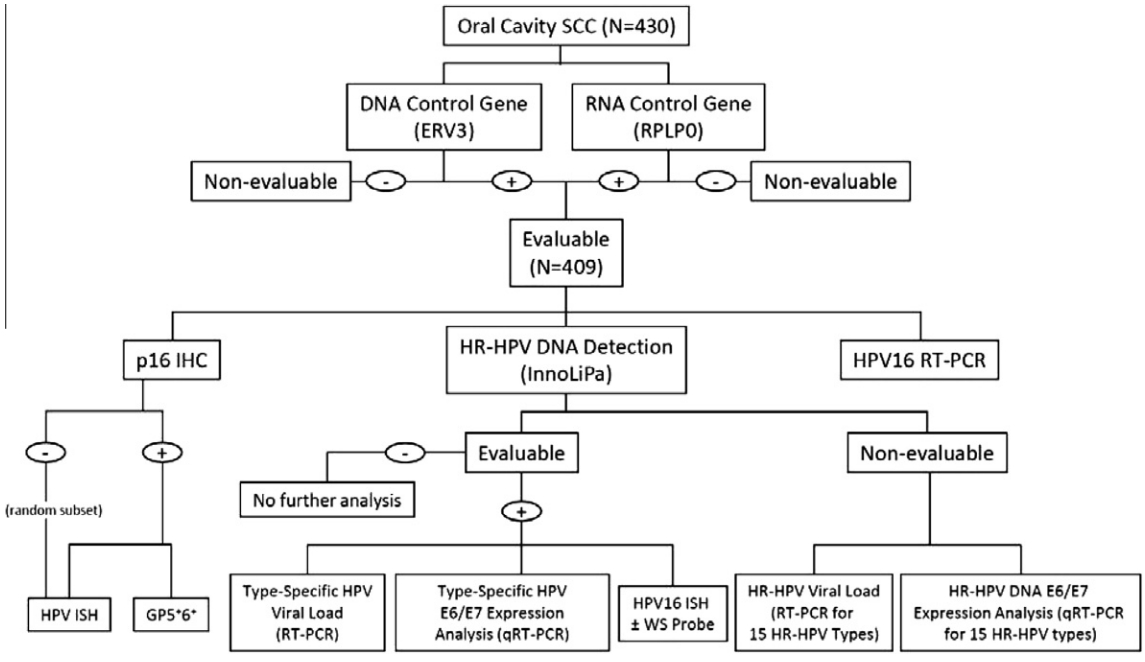

Figure 1 Flow diagram of laboratory analysis of 430 oral cavity squamous cell carcinomas. See text for detailed methods. Abbreviations: SCC, squamous cell carcinoma; ERV3, endogenous retrovirus 3; RPLPO, human ribosomal protein large; IHC, immunohistochemistry; HR-HPV, high-risk human papillomavirus; RT-PCR, real-time polymerase chain reaction; ISH, in situ hybridization; qRT-PRC, quantitative reverse transcriptase polymerase chain reaction; WS, wide spectrum.

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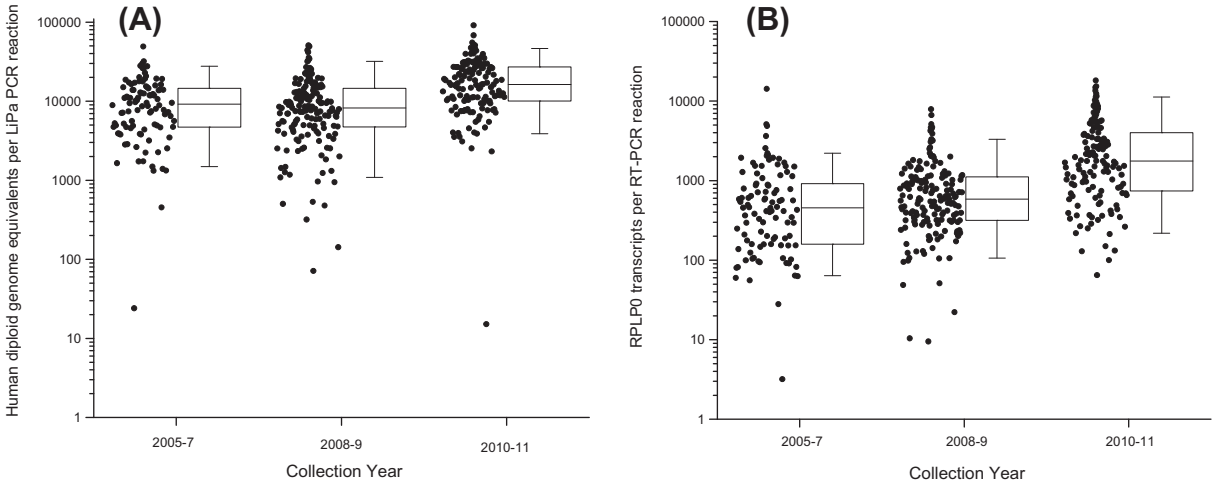

Figure 2 The distribution of values for controls for PCR amplification of human DNA (ERV-3) and human mRNA reverse transcribed to cDNA (RPLPO) observed in the 409 oral cavity squamous cell carcinomas scored as evaluable, stratified by year of specimen collection. (A) The number of human diploid genome equivalents (e.g. cell number) per sample that were evaluated for the presence of HPV DNA by means of Inno-LiPA PCR analysis are shown as estimated by quantitative real-time PCR for a single copy human gene, ERV-3. (B) The number of transcripts for a control gene (RPLPO) per sample, corresponding to that evaluated for the presence of HPV E6/7 mRNA transcripts by qRT-PCR, is shown as estimated by qRT-PCR. The box plot to the right of the scatter plot indicates the median and the 25th and 75th percentiles; whiskers extend to the 5th and 95th percentiles. Kruskal–Wallis test for equality of medians, $p = 0.0001$ for both factors.

mor cell nuclei was confirmed by HPV in situ hybridization for 20 of 24 cases positive for HR-HPV E6/7 expression. Representative cases are shown in Fig. 3.

The characteristics of HR-HPV E6/7 expression positive and negative oral cavity cases are compared in Table 1. Median age of HPV-positive cases was similar to HPV negative cases (61 versus 64 years, $p = 0.35$). A majority of HPV-positive cases were floor of mouth or tongue cancers, but the anatomic-site distribution did not differ from HPV-negative cases. HPV-positive cases were significantly more likely than HPV-negative cases to be diagnosed among men, to be of early tumor stage, to be poorly differentiated and to have basaloid histopathology (Table 1).

When tumors were evaluated for p16 expression, 46 (11.2%, 95%CI 8.2–14.3) of 409 were positive by H score criteria, including 19 of 24 HR-HPV E6/7 positive tumors and 27 of 385 negative tumors. The sensitivity of p16 for HR-HPV E6/7 expression was 79.2% (95%CI 57.9–92.9), specificity 93.0% (95%CI 90.0–95.3), positive-predictive value 41.3% (95%CI 27.0–56.8) and negative-predictive value 98.6% (95%CI 96.8–99.6). When evaluated for the presence of additional HPV types by use of GP5+/6+ consensus primer PCR, no HPV DNA sequences were detected in the 27 p16-positive/HPV-negative cases.

A summary of the clinical characteristics and HPV testing results for all 24 OCSCC positive for HR-HPV E6/7 expression is shown in Table 2.

Discussion

The incidence of HPV-positive oropharynx cancer increased by 225% from 1988 to 2004 in the United States, in contrast to a 50% decline in incidence of HPV-negative cancers.[7] Presumably, the population-level changes over calendar time in tobacco smoking and sexual behaviors that are hypothesized to underlie this etiologic shift would similarly affect OCSCC. Given the consistently high estimates ($\geqslant$70%) of the HPV-etiologic fraction in oropharyngeal cancers diagnosed in North America after 2000[7,15,21], we specifically chose to investigate the etiologic fraction for HPV among a series of contemporaneously diagnosed cases of OCSCC from North America. To our knowledge, this is the largest and most comprehensive analysis of HR-HPV E6/7 expression specific to oral cavity cancers. We observed HR-HPV E6/7 expression in approximately 6% of OSCC. Analogous to oropharynx cancers, HPV-positive cancers were significantly associated with male gender, early tumor stage, and poorly differentiated, basaloid histopathology.

In contrast to the anatomic site specificity for the lingual and palatine tonsils in the oropharynx, HPV-positive and negative OCSCC had a similar anatomic site distribution. In this study, the small tumor size and the anatomic discontinuity of the HPV-positive OCSCC from the oropharynx indicate they are unlikely to represent misclassified oropharynx cancers. The oral region therefore appears analogous to the genital tract with regard to field effects of HPV infection, wherein the overwhelming majority of cancers arise from distinct anatomic sites with apparent increased susceptibility to HPV infection and/or transformation (e.g. the tonsil crypt and cervical transformation zone), with a minority sporadically arising from other anatomic sites (e.g. the oral cavity and the vagina, vulva).

Several recently published studies have identified a high proportion (35–55%) of OCSCC with detectable HPV DNA by sensitive PCR methods.[22,23] Our data indicate that caution should be used in inferring HPV-causality based on DNA detection alone, as approximately half of HPV DNA-positive cases were confirmed as positive for HR-HPV E6/7 expression. Additionally, the HPV viral load was markedly lower among HR-HPV-E6/7 expression-negative tumors, possibly consistent with the presence of a pathophysiologically unrelated oral HPV infection. In a recent population-based study in the US, gender, age and intensity of current tobacco smoking were strongly associated with prevalent oral HPV infection.[24] In that study, oral HPV infection was detected in ~10% of men, ~11% of individuals aged 55–64 years and among 20% of current smokers of one or more packs per day. Given the gender and age distribution of our study population, approximately 10–20% would be expected to have an oral HPV infection. In a recent meta-analysis of case-control studies, oral HPV infection was associated with a fourfold increase in odds of oral cavity carcinoma (OR 3.98, 95%CI 2.62–6.02).[25] However, the authors noted that their analysis did not account for the possible effects of tobacco smoking. Therefore, there is considerable potential for residual confounding of the association between oral HPV infection and OCSCC by tobacco smoking. Nevertheless, relatively strong associations have been observed between high-risk oral HPV infections overall and OCSCC

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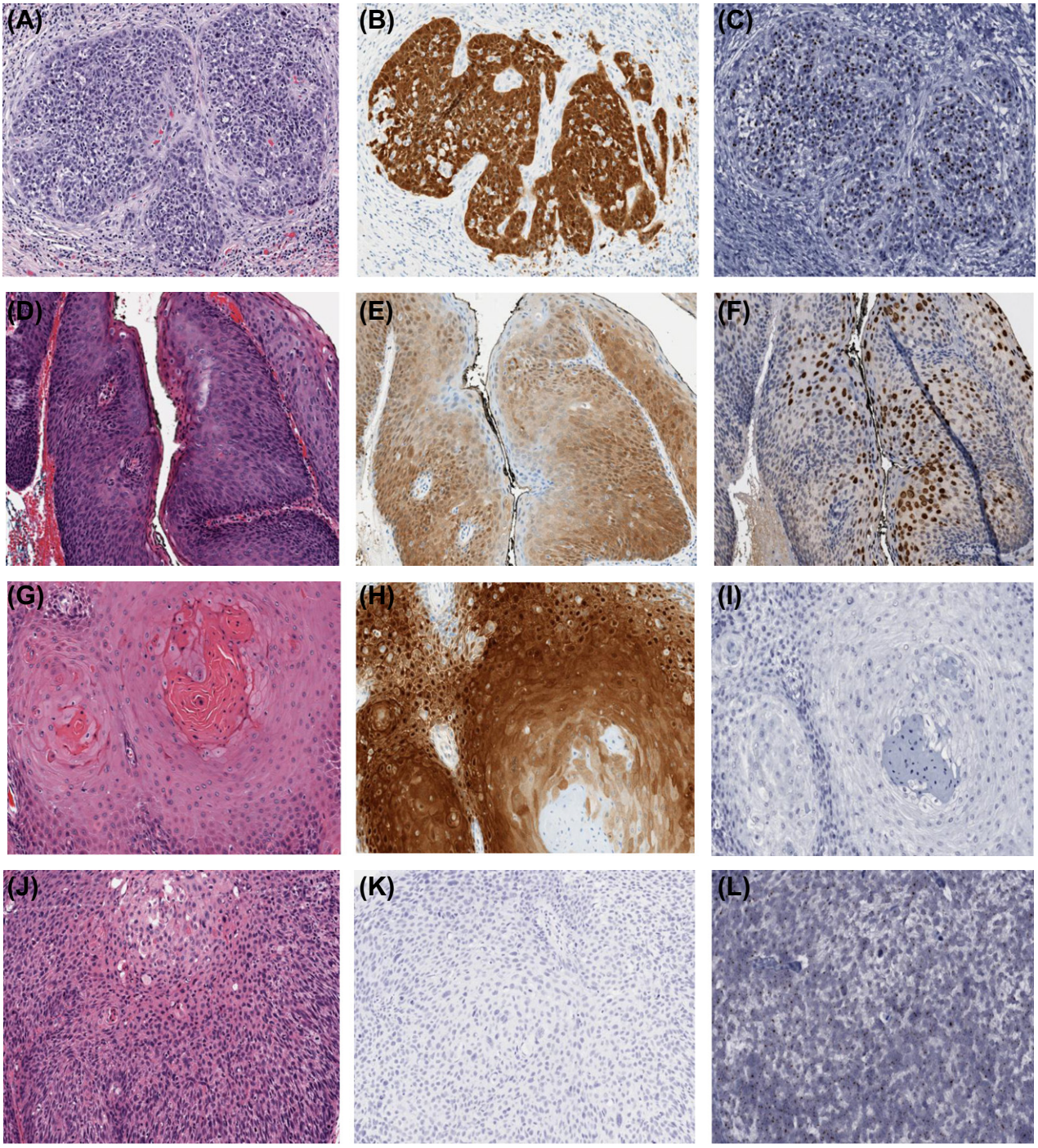

Figure 3 Representative histopathology for oral cavity squamous cell carcinomas that were positive for HR-HPV E6/7 expression. Cases evaluated by hematoxylin and eosin staining (A, D, G, J), for expression of p16 by IHC (B, E, H, K), and for HPV presence by ISH (C, F, I, L). Panel A–C is an example of a poorly differentiated, basaloid squamous cell carcinoma of the alveolar process that was p16-positive (B) and HPV16 ISH-positive (C). The tumor was positive for HPV16 E6/7 expression. Note the hyperchromatic cells with scant cytoplasm, marked nuclear atypia, and high mitotic activity characteristic of basaloid carcinoma. Panel D–F is an example of a well differentiated squamous cell carcinoma in situ of the floor of mouth that was p16-positive (E) and HPV31 ISH-positive (F). The tumor was positive for HPV31 E6/7 expression. Panel G-I is an example of a well differentiated squamous cell carcinoma of the hard palate that was p16-positive (H) and HPV16 ISH-negative (I). The tumor was negative for HPV DNA and mRNA. Panel J–L is an example of a poorly differentiated, basaloid squamous cell carcinoma of the floor of mouth that was p16-negative (K) and HPV16 ISH-positive (L). The tumor was positive for HPV16 E6/7 expression.

in case-control studies after adjustment for tobacco exposure,[26,27] in contrast to weak associations observed for serologic measures of HPV16 exposure.[28–32] The HPV type distribution we observed in our series among OCSCC was significantly more diverse than for the corresponding series of oropharyngeal cancers (94.9% HPV16, 5.1% non-16),[15] with approximately 38% of positive cases attributable to HR-HPV types other than 16. Studies that focus exclusively on HPV16 may therefore underestimate associations.

p16 IHC is an accepted surrogate diagnostic biomarker for HPV status for OPSCC, and in our recent analysis had a positive predictive value of ~93% for HR-HPV E6/7 expression.[15] However, the positive-predictive value of p16 for HPV in OCSCC is very poor

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Table 2
Clinical and pathologic characteristics of 24 oral cavity squamous cell carcinomas with HPV E6/7 expression.

Sample	Anatomic subsite	Histologic variant	Tumor differentiation	Staging type	Tumor stage	Nodal stage	HPV type	Viral copies per single ERV3 (adj)	E6/7 mRNA copies per 1000 RPLP0	p16 IHC status	HPV ISH status
1	Alveolar process	SCC, conventional	Well	Clinical	T1	N0	16	0.9	127.3	Pos	Pos
2	Floor of mouth	Basaloid SCC	Poor	Pathological	T2	N0	16	8.4	94.6	Neg	Pos
3	Floor of mouth	Basaloid SCC	Poor	Pathological	T1	N0	16	3.9	10.5	Pos	Pos
4	Tongue	SCC, conventional	Moderate	Pathological	T4a	N2b	16	153.7	144.5	Pos	Pos
5	Alveolar process	Basaloid SCC	Poor	Pathological	T2	N2c	16	11.2	509.0	Pos	Pos
6	Tongue	Verrucous carcinoma	Well	Pathological	T1	N0	31	6.2	1121.5	Pos	Pos
7	Floor of mouth	SCC, conventional	Well	–	Tis	Nx	18	7205.4	686.2	Pos	Pos
8	Gingiva	Basaloid SCC	Poor	Pathological	T4a	N0	35	573.6	5179.0	Neg	Neg
9	Tongue	SCC, conventional	Moderate	Pathological	T2	N0	18	3.3	170.7	Pos	Pos
10	Hard palate	SCC, conventional	Poor	Clinical	T1	N0	16	162.0	11.7	Neg	Pos
11	Floor of mouth	Basaloid SCC	Poor	Clinical	Tis	Nx	16	0.1	3.1	Neg	Pos
12	Tongue	SCC, conventional	Moderate	Pathological	T1	N0	31	28.3	583.1	Pos	Neg
13	Tongue	SCC, conventional	Well	Pathological	T1	N0	33	0.1	32.2	Pos	Pos
14	Floor of mouth	Acantholytic SCC	Poor	Pathological	T2	N1	16	0.02	37.5	Neg	Neg
15	Hard palate	SCC, conventional	Moderate	Clinical	T2	Nx	16	43.6	5.2	Pos	Pos
16	Alveolar process	Basaloid SCC	Poor	Pathological	T1	N0	16	85.9	536.3	Pos	Pos
17	Tongue	SCC, conventional	Moderate	Pathological	T1	Nx	16	2.0	168.6	Pos	Pos
18	Alveolar process	SCC, conventional	Well	Pathological	T1	N0	33	149.9	536.4	Pos	Pos
19	Floor of mouth	SCC, conventional	Moderate	–	–	–	16	2.5	456.5	Pos	Pos
20	Floor of mouth	SCC, conventional	Well	Clinical	T1	N0	16	286.5	122.1	Pos	Pos
21	Hard palate	Basaloid SCC	Poor	Pathological	T4a	Nx	45	0.8	544.4	Pos	Neg
22	Floor of mouth	Basaloid SCC	Poor	Pathological	T4a	N2b	16	46.2	138.0	Pos	Pos
23	Floor of mouth	Basaloid SCC	Poor	Pathological	T4b	N3	16	6.7	1565045.0	Pos	Pos
24	Lip	SCC, conventional	Well	Pathological	T4a	N0	39	309.2	3373.1	Pos	Pos

(~41%), with the majority of p16-positive cancers being negative for HR-HPV E6/7 expression. Given the lower overall prevalence of HPV in OCSCC relative to OPSCC, this is not unexpected. Although constituting a low percentage of OCSCC overall, the p16-positive HPV-negative proportion (7.0%) exceeded that for HR-HPV E6/7 expression-positive OCSCC. Our data therefore indicate that p16 IHC should not be used as a surrogate for HPV testing in OCSCC. Whether or not p16 expression is predictive of tumor HPV status in tumors arising from other anatomic sites such as the larynx will need to be evaluated.

Our study has several potential limitations. Although our estimate of the HPV-etiologic fraction was precise, it may not reflect the population-level HPV-attributable fraction in North America. Our study was not population based. We note, however, that the HPV-etiologic fraction of 68% for oropharynx cancers in our series of patients diagnosed after 2000[15] was quite similar to the 72% we previously observed in a US population-based analysis with identical laboratory methods.[7] A US population based analysis of HPV in OCSCC currently being conducted by the US Centers for Disease Control and Prevention will address this issue. DNA and RNA degradation in paraffin may decrease sensitivity of HPV detection, but would result in an underestimate of the etiologic fraction. Additionally, our PCR assays are all specifically designed to yield small amplicons, as appropriate for degraded DNA. Although we observed a significant decline in DNA and RNA yield with increasing time since diagnosis, the quality and quantity remained far above that necessary for the specimen to be evaluable.

It is now clear that HPV-positive OPSCC constitutes a unique epidemiological and clinical entity. Risk factor profiles[17] and prognosis[21] are different for HPV-positive and HPV-negative OPSCC. Due to absence of relevant data, we could not investigate associations between HPV status and cofactors (e.g. tobacco and alcohol use) or survival outcomes for OCSCC, and therefore this will require further study. Of particular interest will be the association of HPV and p16 status in combination with survival of OCSCC and whether or not the small subset of HR-HPV E6/7 positive OCSCC could be treated with organ preservation therapy in lieu of surgical resection.

Despite these limitations, our data may have important implications for the primary prevention of oral cavity carcinoma. The International Agency for Research on Cancer (IARC) recently estimated the HPV-attributable fraction for OPSCC to be 26% world-

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wide, accounting for approximately 22,500 incident cases of OPSCC each year.[33] If as high as 5% of the 263,000 annual cases of OCSCC[34] are also due to HPV infection, a total of 35,650 oral cancer cases worldwide might be preventable through HPV vaccination (assuming efficacy equivalent to that for genital infection). In the US for 2012, the corresponding number would be ~10,500 cases.[7] The broader HPV type distribution for OCSCC than OPSCC might also have implications for potential benefits from newer generation HPV vaccines that may protect against a broader range of HPV types.

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Conflict of interest statement

None declared.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2012.07.002.

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