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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