Real-Time Quantitative PCR Demonstrates Low Prevalence of Human Papillomavirus Type 16 in Premalignant and Malignant Lesions of the Oral Cavity

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ABSTRACT

Purpose: Human papillomavirus (HPV) type-16 has been associated with invasive squamous cell carcinoma of the head and neck. This study examines the role of HPV-16 in the progression of oral head and neck cancer by determining the quantity of HPV-16 DNA in premalignant and malignant lesions, using real-time quantitative PCR, to more accurately determine the role of HPV-16 in oral head and neck squamous cell carcinogenesis.

Experimental Design: We examined 102 microdissected premalignant head and neck lesions (85 from the oral cavity), 34 invasive oral cavity squamous cell carcinomas, as well as 18 invasive tumors known to be HPV positive by traditional molecular technology for the presence of HPV-16 DNA using real-time quantitative PCR.

Results: HPV DNA was detected in 1 of 102 premalignant lesions (0.98%), 1 of 34 (2.9%) invasive oral cavity carcinomas, and 14 of 18 (78%) known HPV-positive tumors.

Conclusions: HPV-16 infection and integration is seldom found in oral premalignant lesions and invasive carcinoma, and therefore rarely contributes to malignant progression in the oral cavity. Furthermore, quantitative PCR is a useful technique that reliably excludes contaminated samples and those with minimal HPV DNA content that is unlikely to be significant in carcinogenesis.

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INTRODUCTION

HPV has been implicated in human tumor progression based largely on data gathered from cervical cancer patients. High-risk HPV types (16, 18, 31, 33) have been known to affect epithelial cells by expressing proteins that alter tumor suppressor pathways by inactivating p53 and Rb (1, 2). Recently, HPV type 16 has been identified in 90% of HPV-associated head and neck tumors (3) and has been found in 50% of oropharyngeal head and neck squamous cell carcinoma (3–5). However, markedly varied estimates of HPV prevalence in premalignant head and neck lesions (0–100%; Refs. 6–13) have made it difficult to clarify the timing and nature of the contribution of HPV to head and neck carcinogenesis.

Head and neck premalignant lesions have been studied extensively for genetic alterations. A genetic progression model for the predictable loss of heterozygosity that occurs within premalignant epithelium has been established (14). Determining the timing of viral infection in these lesions would additionally elucidate the role of HPV in carcinogenesis and could help guide future strategies for the prevention and early detection of head and neck squamous cell carcinoma. As such, many studies have been performed to detect the presence of HPV in head and neck epithelia by use of a variety of laboratory methods.

Several investigators have attempted to determine the prevalence of HPV infection in normal individuals. The prevalence estimates have ranged from 0 to 60% (9, 15, 16), depending on the patient population as well as the sampling and assay methods used. There is likely some background rate of HPV infection in clinically normal individuals, suggesting that infection does not always promote progression to dysplasia.

Studies examining invasive squamous cell carcinomas of the head and neck have also reported many estimates (0–100%; Refs. 9, 17–20). Many different techniques have been used for detection, including PCR, Southern blot, dot blotting, and in situ hybridization. In a meta-analysis of the studies looking at these lesions, Miller and White (21) noted that there was a significantly higher rate of HPV positivity reported by those studies using PCR techniques for either consensus or type-specific HPV sequences. This raises the possibility that nonquantitative PCR may allow for increased sensitivity as opposed to other techniques but may also indicate that nonquantitative PCR has a higher occurrence of false-positive results. More recent studies have used a combination of techniques and have established a higher prevalence of HPV detection in oropharyngeal tumors, noting an inverse relationship between p53 mutations and HPV positivity (3).

In an effort to determine the significance of HPV infection...
in cancer progression, several studies have examined the prevalence of HPV in premalignant lesions of the head and neck (9, 11, 20, 22–27). Again, these studies demonstrated many prevalence estimates, using methods of variable sensitivity and specificity. Furthermore, the different clinical and histopathological classifications used to describe these lesions have made it difficult to compare the results of the different studies.

The goal of our study was to use a novel technique for quantifying levels of HPV-16 DNA present in premalignant and malignant lesions of the head and neck to provide insight into conflicting reports of HPV prevalence in these lesions. Our hypothesis was that this study would provide a more accurate estimation of the true prevalence of HPV in oral cavity lesions because of the ability to quantitate the amount of HPV DNA. The technique was compared with traditional molecular techniques on invasive tumors found previously to contain HPV DNA, and was noted to be more sensitive than Southern blot and either comparable with or more specific than consensus PCR to the L1 MY09/MY11 region of HPV. To our knowledge, this is one of the largest series looking at well-characterized premalignant and malignant lesions of the head and neck, and the first series using real time quantitative PCR (TaqMan; Perkin-Elmer Corp., Foster City, CA) for this purpose. Quantitative PCR minimizes the risk of contamination by eliminating postamplification processing of samples. Additionally, the significance of the HPV detected in the samples can be evaluated by comparing HPV genome copy number to somatic host cell genome copy number, making it the ideal assay for HPV DNA detection.

MATERIALS AND METHODS

Tissue. Samples from 102 patients were collected from archived, paraffin-embedded, head and neck premalignant specimens from the Johns Hopkins Hospital Department of Pathology between 1982 and 2000. The majority of lesions were isolated leukoplakias, and no lesions were associated with a prior or synchronous invasive squamous cell carcinoma. The average age of the patients was 59 (range 21–99), and they were predominantly male (85 of 102). Ten of 102 patients subsequently progressed to invasive carcinoma after initial excisional biopsy of a premalignant lesion. H&E-stained slides were reviewed by a pathologist (W. H. W.) and evaluated for the presence and degree of squamous epithelial dysplasia. The histological diagnoses in increasing order of severity were as follows: (a) hyperplasia without atypia; (b) mild dysplasia; (c) moderate dysplasia; (d) severe dysplasia; and (e) carcinoma in situ. Of note, hyperplastic lesions without atypia were included, because >30% of these lesions have been shown to demonstrate the similar genetic alterations found in their fully malignant counterparts (14, 28). In addition, 34 oral cavity invasive carcinomas were obtained. The average age of these patients was 44 (range 20–81), with 17 males and 17 females. Twenty-three of these tumors were fresh, frozen samples, and 11 were obtained from paraffin-embedded tissue blocks.

DNA Extraction. Fifteen 10-μm sections were cut from paraffin blocks or fresh tissue and microdissected using a microsurgical forcep. Uninvolved nonmucosal tissue was microdissected as a representative normal. DNA was extracted using techniques described previously (14). Paraffin-embedded samples were placed in xylene for 12 h and centrifuged at 13,500 rpm. The pellet was then digested in SDS/proteinase K over the next 48 h at 45°C. The product was extracted with phenol/chloroform, ethanol precipitated, and stored at −20°C. The samples were diluted 20-fold and then analyzed by quantitative PCR. Fresh tissue was treated in a similar fashion, although these samples did not require xylene treatment.

Quantitative PCR. Specific primers and probes have been designed to amplify the E6 and E7 regions of HPV type 16 (29). Using this combination and the protocol described by Capone et al. (29), the normal and affected samples from the 102 patients were run in duplicate. Primers and probes to a housekeeping gene (β-globin) were run in parallel to standardize the input DNA. By using serial dilutions, standard curves were developed for the HPV viral copy number using CaSkI (American Type Culture Collection, Manassas, VA) cell line genomic DNA, known to have 600 copies/genome equivalent (6.6 pg of DNA/genome).

Validation. DNA was obtained from 18 invasive squamous cell tumors from the head and neck that were found previously to contain HPV DNA by traditional molecular techniques (consensus PCR to the L1 MY09/MY11 region, type-specific PCR to the HPV-16 E7 region, direct sequencing of HPV-16 E6, Southern blot for HPV-16, and in situ hybridization for HPV-16). These samples were derived from fresh frozen tissue and microdissected. Normal tissue DNA was also obtained for 14 of these 18 samples. Quantitative PCR was performed on these samples as described above.

Data Analysis. Standard curves were developed for both HPV E6 and E7, using serial dilutions of DNA extracted from CaSkI cells with 50 ng, 5 ng, 0.5 ng, 0.05 ng, and 0.005 ng of DNA (see Fig. 1A). Standard curves were developed as well for the β-globin housekeeping gene (2 copies/genome), using the same serial dilutions of the CaSkI genomic DNA. This additional step allowed for relative quantification of the input DNA level and the expression of the final quantity as the number of viral copies/genome/cell (30). HPV quantities derived that were >0.1 (>1 HPV genome copy/10 cells) were scored as positive. This threshold was based on the assumption that the presence of <1 HPV genome copy/10 cellular genomes indicates that HPV is only present in a minority of the dominant clonal population in a premalignant lesion. Although this threshold may seem low, this reflected the intention to err on the side of overestimation of HPV-16 positivity in these lesions. Lesions were also excluded if there was detection of HPV DNA in the normal control samples, indicative of contamination. Spearman’s rank correlation was used to evaluate a linear relationship between viral copy number in tumors as determined by use of E6 and E7 primers.

RESULTS

Overall, 1 of 102 (0.98%) of all premalignant samples had evidence of significant HPV-16 involvement using the criteria defined above. Six lesions were excluded because of contamination of the normal control (range between 0.00013 and 5 HPV copies/genome). Two samples were judged as negative for HPV-16 because they did not meet the minimum criteria of at
least 1 copy/10-cell genome’s worth of DNA, although no HPV was detectable in the normal control DNA. The remainder of the samples had no evidence of HPV in the lesions or corresponding normal control DNA, with positive \(/H9252\)-globin controls in both sample and normal (indicating sufficient amounts and quality of DNA present for analysis).

HPV was detected in 1 of 24 (4.2%) moderate dysplasias (see Table 1). The copy number of HPV E6 DNA was 19.3
copies/genome, and E7 DNA was 11.6 copies/genome. HPV was not detected in any of the 34 hyperplasias without atypia, 10 mild dysplasias, 18 severe dysplasias, or 16 carcinoma in situ lesions.

One of 34 (2.9%) invasive oral cavity cancer samples had evidence of significant HPV 16 infection, with 28.4 copies/ genome of HPV E7, and 12.4 copies/genome of HPV E6. Six other tumor samples fell below the threshold of significant infection but were detected consistently with both E6 and E7 markers.

Fig. 1 demonstrates sample data produced using the Primer Express software (Perkin-Elmer Corp, Foster City, CA). Fig. 1A depicts a representative standard curve of 10-fold serial dilutions of the CaSki cell line DNA in duplicate. Fig. 1B illustrates a representative negative run, with a positive β-globin curve indicating an adequate DNA sample and a negative curve for detection of HPV. Fig. 1C shows the oral cavity sample positive for the E7 region of HPV-16 DNA along with a positive curve for the β-globin internal control.

The biopsy site of the HPV-positive lesion within the pre-malignant subset was located in the oral cavity (1 of 85). There were no HPV-positive lesions in the oropharynx (0 of 5 patients) or larynx (0 of 12 patients), although these were of a small sample size. Of note, of the oropharyngeal lesions, only 1 was taken from the tonsil, with the remaining 4 lesions located in the soft palate.

The use of quantitative PCR for HPV detection was evaluated by performing the assay on 18 head and neck squamous cell carcinoma samples found previously to be HPV type-16 positive based on standard traditional techniques (Ref. 3; See Table 2). Fourteen of 18 of these samples (77.8%) were positive by quantitative PCR for both the E6 and E7 sequences. The median number of HPV-16 viral copies per genome in positive tumor samples was 34.6 for E6 (range 0.046–322.4 copies/ genome) and 14.3 for E7 (range 0.016–270.0 copies/genome). The number of viral copies per genome calculated by use of E6 primers was strongly correlated with the number of viral copies in the tumor by use of E7 primers (r = 0.75; P < 0.01). The 4 samples found to be negative or below threshold by quantitative PCR were originally defined as HPV-positive by only standard PCR techniques (Southern blot and in situ hybridization were not performed secondary to insufficient quantities of DNA). Corresponding normal DNA samples for 14 of these tumors were analyzed using quantitative PCR, and 12 of 14 (85.7%) were negative for HPV DNA. Two of 14 (14.3%) of the normal samples demonstrated very low levels of contamination, only for the presence of E6 viral DNA (see Fig. 2).

### DISCUSSION

The body of literature implicating HPV as a major carcinogen in humans continues to grow. The role of HPV-16 and other high-risk HPV types in the progression of pre-malignant cervical lesions to malignancy has been well established, such that HPV infection is necessary for cervical cancer development. However, in the case of head and neck pre-malignant lesions, the variability inherent in detection and sampling methods has made it difficult to estimate the prevalence of HPV in normal mucosa, pre-malignant lesions, and invasive tumors.

Despite this variability, an association of HPV-16 with a subset of head and neck cancers has been established. High-risk HPV-16 has clearly been shown to be the dominant type in head and neck cancers (3–5). Recently, exposure to HPV-16, as measured by seroreactivity to viral L1 capsid protein, was associated with a 14-fold increase in risk of oropharyngeal cancer (31). Furthermore, HPV-16 exposure clearly preceded the development of disease in these patients.

In our study, only 1 of 102 pre-malignant samples and 1 of 34 invasive cancers demonstrated clinically significant amounts of HPV-16 DNA. Whereas these numbers may seem low, it may be explained by the predominance of our samples taken from the oral cavity, which have not been as well demonstrated to harbor HPV-16 DNA as oropharyngeal lesions. Our study suggests that, unlike oropharyngeal lesions, HPV-16 infection in oral cavity carcinomas is not a frequent event. It is also possible, although unlikely, that DNA extraction from paraffin-embedded tissue may decrease the likelihood of adequate detection. To account for the differences in DNA extraction methods and yield, we used a β-globin internal control, which amplified without difficulty, thereby confirming the quality of the DNA. Furthermore, 23 of 34 oral cavity cancer cases were taken from fresh tissue and still exhibited a low detection rate of HPV DNA, implying that the tissue processing technique did not affect the results.

Those studies using PCR-based assays as their detection method reported a higher prevalence than other less sensitive techniques such as Southern blot or in situ hybridization (21). One of the difficulties with PCR is its extreme sensitivity to contamination that may simply reflect inherent methodological drawbacks. Even careful tissue extraction and handling does not necessarily prevent contamination; it may be virtually impossible to eliminate or account for it using conventional techniques. An overestimation of HPV positivity because of technical reasons may help explain why oral cavity lesions were thought previously to harbor the virus.

Real time quantitative PCR or TaqMan (Perkin-Elmer Corp.) has emerged recently as a relatively easy and reliable method of quantifying the target in question. It has several advantages over conventional PCR: (a) the ability to give a quantity of input template; (b) the absence of post-PCR processing; (c) a minimal chance of contamination because of isolated wells, and (d) allowance for relative quantitation of multiple target templates. We designed primers and probes for the

### Table 1  Prevalence of HPV-16 E6 and E7 DNA in pre-malignant head and neck lesions

<table>
<thead>
<tr>
<th>Histologic Grade</th>
<th>Quantity of HPV-16 DNA (copies/genome)</th>
<th>HPV-16 E6 and E7 prevalence</th>
</tr>
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<tbody>
<tr>
<td>Hyperplasia without atypia</td>
<td>0/34 (0%) n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>0/10 (0%) n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>1/24 (4.2%) E6 = 19.3, E7 = 11.6</td>
<td>n/a</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>0/18 (0%) n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>0/16 (0%) n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>1/34 (2.9%) E6 = 28.4, E7 = 12.4</td>
<td>n/a</td>
</tr>
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</table>

**Note**: E6 and E7 DNA were analyzed using quantitative PCR, and 12 of 14 (85.7%) were positive for the presence of E6 viral DNA (see Fig. 2).
E6 and E7 regions, those coding regions that produce proteins known to be active in inhibiting p53 and Rb, respectively (1, 2).

We were able to identify a population of lesions with significant copy numbers of HPV DNA per genome (>1 copy/10 genome equivalents). Furthermore, we were able to detect and exclude those samples with apparent contamination (HPV DNA present in normal microdissected tissue) and those samples with low viral copy number (<1/10 cells). These cases may also represent patients with low levels of latent HPV in their mucosa.

The question then arises whether those samples with low HPV DNA copy number are significant findings contributing to carcinogenesis; we propose that without sufficient copies of HPV affecting the majority of cells within a lesion, it is unlikely for HPV to play a major role in the earliest stages of tumor initiation and progression. Without at least 1 viral copy per genome, a clonal relationship cannot be established. Even if we had lowered our threshold of detection to 1 HPV viral genome detected per 100 cells, the HPV positivity in premalignant lesions would have increased only to 3 of 102 patients (2.9%), and all of the invasive cancers would still remain negative (data not shown).

In the case when a viral copy number greater than one per genome is detected, it remains unclear whether this is because of one cell with multiple HPV DNA copies (i.e., HPV productive viral infection), or whether it is the result of multiple cells with one viral copy. However, the ability to rule out false-positive and contaminated lesions appears to be the strength of this assay, and it allows us to rule out those lesions that do not meet the minimal requirements for a clonal, HPV-infected cell population.

The assay was validated using 18 invasive tumors found previously to be HPV-positive by other traditional molecular methods. Fourteen of these 18 tumors (77.8%) met our criteria for HPV positivity. Three of the 4 remaining samples contained HPV viral DNA quantities that were below our established threshold for significant infection, and the final sample proved to be negative. The copy numbers of E6 and E7 detected were usually within 1 order of magnitude, and the difference between E6 and E7 values for individual samples were consistent between different replicates. Of note, the E6 copy number tended to be higher, either because of increased efficiency of the E6 amplification primer set or perhaps because of a differential

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Consensus PCR (L1 MY09/MY11)</th>
<th>Type-specific PCR (HPV-16 E7)</th>
<th>Direct sequencing (HPV-16 E6)</th>
<th>Southern blot (HPV-16)</th>
<th>Quantitative PCR (HPV-16 E6)</th>
<th>Quantitative PCR (HPV-16 E7)</th>
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<td>25</td>
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* As detailed in Ref. 3.

** + = specimen positive for HPV-16, − = specimen negative for HPV-16, NA = specimen not available for testing.
stability of this portion of the HPV-16 genome in vivo. Consensus PCR for the L1 MY09/MY11 region detected 1 sample that quantitative PCR determined to be negative, in addition to detecting 3 samples that were below threshold. This evidence demonstrates that quantitative PCR is nearly as sensitive as consensus PCR with the added benefit of being able to exclude samples with insignificant quantities of HPV DNA. The sensitivity of these two techniques is difficult to compare given the unknown biological significance of very low quantities (<1 copy/10 cells) of HPV DNA.

The site of the majority of our lesions were the oral cavity, given that oral premalignant lesions are more common, more likely to be detected on routine clinical exam, and more easily biopsied. Interest in the site of biopsy has come about in light of data demonstrating an increased prevalence of HPV-16 specifically in oropharyngeal carcinomas (3). In our series, we had only 5 premalignant lesions from the oropharynx (1 from the tonsil and 4 from the soft palate) of which none showed the presence of HPV. Similarly, 0 of 12 laryngeal premalignant lesions were positive, although recent reports suggest that HPV may play a more significant role in laryngeal cancer (32). We are unable to make any definitive conclusions regarding the role of HPV in premalignant oropharyngeal or laryngeal lesions given the small samples size tested here. Additional testing of these specific lesions would be beneficial in determining the progression of HPV-16 prevalence in oropharyngeal cancer.

Overall, we found a very low prevalence of HPV-16 in premalignant (1 of 85 patients) and malignant lesions (1 of 34) of the oral cavity. This finding does not necessarily imply that HPV does not play a role in the progression toward cancer at other sites. However, it does show that HPV infection is not prevalent in the development of oral squamous cell carcinoma. Furthermore, this study offers a powerful new technique in the armamentarium of detection assays that allows for sensitive and quantitative detection of HPV, and exclusion of contaminated samples or those samples with low-level presence of HPV with unclear biological significance. This technique may be used to more precisely establish the role of HPV in premalignant lesions, in an effort to guide future therapy toward the prevention and early detection of squamous cell carcinoma of the head and neck.

REFERENCES
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