Human Papillomavirus in Oropharyngeal Squamous Cell Carcinoma: Assessing Virus Presence in Normal Tissue and Activity in Cervical Metastasis

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**Objectives/Hypothesis:** Human papillomavirus (HPV) has been established as an etiologic and prognostic factor in oropharyngeal squamous cell carcinoma (OPSCC). HPV oncogenesis involves expression of E6/E7 oncoproteins, with downstream p53 degradation and pRb inhibition. Although much research has focused on HPV’s oncogenic behavior in primary OPSCC, minimal information exists about HPV in adjacent normal and metastatic tissue.

**Study Design:** Retrospective cohort study

**Methods:** Patient-matched tumor, normal, and metastatic tissue was gathered from 42 OPSCC patients and tested with real-time quantitative polymerase chain reaction (RT-qPCR), in situ hybridization (ISH), and immunohistochemistry (IHC). RT-qPCR was performed using total RNA from fresh-frozen tissues and primers for HPV16 E6, E7, and p16 transcripts. HPV ISH was performed to detect the presence of HPV DNA and IHC to detect p16 protein.

**Results:** Primary tumor, adjacent normal tissue, and tumor metastasis from 17 OPSCC patients were analyzed. When comparing the presence of HPV16 DNA in tumor, metastatic, and normal tissue by ISH, perfect correlation is found at all sub-sites (P < .0001). However, active infections determined by HPV16 E6 and E7 expression using quantitative polymerase chain reaction or p16 detection by IHC, were present only in primary and metastatic tissue (P = .0012, E6; P = .02, E7). No such correlation was found in normal tissue when compared to primary or metastatic tissue.

**Conclusions:** There is a clear pattern of active HPV expression that correlates to disease course. In HPV-positive patients, all sites including primary, metastatic, and normal tissues are DNA positive. Transcriptionally active infections were detected in primary and metastatic tissues, whereas normal tissues appear to have latent infections.

**Key Words:** Oropharyngeal squamous cell carcinoma, human papillomavirus, cervical metastasis, neck metastasis.

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**INTRODUCTION**

Head and neck squamous cell carcinoma (HNSCC) has long been attributed to chronic exposure to environmental carcinogens such as alcohol and tobacco products. These carcinogens have classically been thought to result in the progressive accumulation of adverse genetic modifications leading to loss of cell cycle control and/or derangements in DNA repair. Among the various subsites for HNSCC is the oropharynx (OPSCC), consisting primarily of tonsil and base of tongue tumors. Although the overall incidence of smoking has decreased over the last 2 decades, the incidence of HNSCC has remained stable, correlating with an increased incidence of squamous cell carcinoma in oropharyngeal subsites. This increase has been observed in data extrapolated from the Surveillance, Epidemiology, and End Results program, revealing a higher incidence in young adults (ages 20–44 years). Likewise, the incidence of OPSCC in the United Kingdom has doubled over the 10 years spanning from 1996 to 2006. Again, this population showed prevalence in younger individuals. The increase in incidence, particularly in light of a younger patient age and absence of traditional environmental risk factors, is thought to be due to oncogenic human papillomavirus (HPV). High-risk oncogenic HPV subtypes include HPV 16, 18, 31, 33, and 35. The viral genome codes for early, late, and long control region genes comprising 50%, 40%, and 10% of the genome, respectively. Of oncologic significance are the E6 and E7 oncogenes. E6 oncoprotein is 151 amino acids in length and contains zinc-binding motifs that bind to host cell p53 tumor suppressor proteins.
protein, subsequently inducing degradation via the ubiquitin proteolytic pathway.\textsuperscript{9,10} E7 oncoprotein is 98 amino acids in length and binds with proteins in the retinoblastoma family (Rb, p107, p130). The retinoblastoma protein family is responsible for cell-cycle regulation at various checkpoints, and E7 oncoprotein binding to these genes causes dysfunction of cell-cycle regulation.\textsuperscript{11} E2F, a transcription factor involved in cell-cycle regulation, is regulated by Rb. E2F proteins play a role in cell cycle progression into S phase, as well as differentiation, development, proliferation, and apoptosis of cells. When E7 binds hypophosphorylated Rb, E2F is left unchecked to promote cell-cycle progression.\textsuperscript{3,12} Additionally, Rb acts as a negative regulator of p16, a cyclin-dependent kinase inhibitor, thus when Rb is inactivated by E7, p16 is up-regulated.\textsuperscript{13} p16 has therefore been used as a surrogate marker for active HPV infection within cancer cells, a technique that is currently being investigated.

Additional studies have discovered that HPV infections in HNSCC exist as either DNA positive, latent infections, or transcriptionally active infections, with the latter associated with improved response to treatment. These findings support the practice of further stratifying patient groups into HPV-positive latent and active groups, often using upregulation of p16 (CDKN2A) as a surrogate marker.\textsuperscript{14} In the clinical setting, a combination of tests frequently performed to detect HPV includes in situ hybridization (ISH) to identify HPV DNA sequence and immunohistochemistry (IHC) to detect the expression of HPV oncogenes E6 and E7 and human tissue as previously described.\textsuperscript{15–23} The HPV typing in situ DNA hybridization testing is based on colorimetric in situ hybridization techniques and detects the presence of nucleic acid in cells or tissues. Nucleic acid corresponding to high-risk HPV types (HR-HPV) is visualized by hybridization of labeled DNA probes to target DNA in human tissue as previously described.\textsuperscript{21–23}

**MATERIALS AND METHODS**

**Tumor, Metastatic, and Normal Specimens**

After approval from the institutional review board, primary tumor, metastatic tumor, and normal mucosal samples were prospectively collected during surgery from consecutive consenting patients undergoing surgical treatment of OPSCC with cervical metastasis. The collected normal tissue was harvested during surgery from an immediately adjacent site. This normal tissue was from the same oropharyngeal subsite as the tumor (i.e., tonsil, tongue base), selected on gross examination by the surgeon, and confirmed microscopically by a pathologist. A portion of each tissue was snap-frozen in liquid nitrogen for storage with frozen sectioning and preparation of hematoxylin and eosin slides, and the remainder of the sample was stored using standard FFPE. These slides were then evaluated by pathology to confirm the presence or absence of tumor in each sample. In the former case, the representation of overall tumor composition was also assessed, with >80% of cells being neoplastic considered optimal. A total of 42 patient samples were included in this analysis. Of these, 17 patients yielded metastatic tissue sufficient to provide a research sample in addition to tissue for standard pathology (Supplementary Table I). This results in comparisons of primary and normal (n = 42) containing larger sample numbers than those comparing primary, metastatic, and normal sites (n = 17).

**RNA Extraction**

Total RNA was extracted from portions of the frozen tissue samples using the Purelink RNA Mini Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Extracted RNA was also quantified by NanoDrop ND1000 (Thermo Scientific, Kalamazoo, MI).

**p16 and HPV Oncogene Expression by Real-Time Quantitative Polymerase Chain Reaction**

Expression levels of p16 and HPV oncogenes E6 and E7 were determined using specific primers designed using Primer3.\textsuperscript{20} cDNA was synthesized using the Thermoscript Real-Time Polymerase Chain Reaction (RT-PCR) System (Invitrogen) with 2 μg of total RNA and oligo-dT primers. cDNA quantification was then performed with specific primers using the SYBR green method (Supplementary Table II). The threshold for negative expression of E6 and E7 was determined by comparison to a pediatric tonsil panel collected for medical reasons unrelated to this study and shown to be HPV negative (data not shown).

**HPV Detection by ISH**

The HPV typing in situ DNA hybridization testing is based on colorimetric in situ hybridization techniques and detects the presence of nucleic acid in cells or tissues. Nucleic acid corresponding to high risk HPV types (HR-HPV) is visualized by hybridization of labeled DNA probes to target DNA in human tissue as previously described.\textsuperscript{21–23}

**p16 Detection by IHC**

Detection of the p16 marker p16INK4a by immunohistochemistry was completed using the CINtec cytology kit (mtm laboratories, Heidelberg, Germany). Fresh frozen tumor samples were frozen sectioned and stained according to manufacturer’s protocols with the single modification of post fixation in 4% PFA prior to retrieval.

**RESULTS**

**Detection of HR-HPV by ISH**

Surgically isolated FFPE tissue corresponding to each patient was analyzed using the ISH technique to detect DNA sequence corresponding to high-risk HPV
(HR-HPV) subtypes. Slides were evaluated by pathology to classify the specimen as HR-HPV-positive or negative. This evaluation demonstrated a perfect correlation of HPV status; if the primary tumor was positive, the metastatic tumor and normal mucosal tissue was also positive for HPV DNA (Spearman $r = 1$ in all cases). The same patterns were detected for HPV-negative patients (Supplementary Table III).

**Detection of p16INK4a by IHC**

IHC analysis of fresh-frozen sample was used to detect p16INK4a protein, indicative of active HPV infections. Slides were evaluated by a pathologist to classify the specimen as p16-positive or negative for primary tumor, metastatic tumor, and normal mucosal tissue. Results are summarized for all patients (Supplementary Table III). A significant correlation was found between primary and metastatic tumors by this technique (Spearman $r = 0.54$, $P = .025$). This analysis failed to detect a correlation of p16 expression between these tumor sites and normal mucosal samples, which were negative by this technique. A representative patient sample is described in Figure 1 with p16 detected in primary (Fig. 1A), metastatic tumor (Fig. 1B), and no detection of p16 in normal mucosal tissue (Fig. 1C).

**Gene Expression Measurement Using Real-Time Quantitative Polymer Chain Reaction**

RNA transcript levels were measured using cDNA prepared from fresh frozen tumor tissue and gene specific primers. Following normalization to a constitutively expressed housekeeping gene, expression patterns were analyzed for HPV16 oncogenes E6 and E7 as well as the human gene target p16 (Supplementary Table IV). These continuous data points were used to compare expression patterns of target genes between each of the three subsites, primary tumor, metastatic tumor, and normal mucosa, using Spearman correlation coefficient.

When comparing the gene transcript levels for HPV16 oncogenes, we detected a significant correlation between primary and metastatic tumors for HPV16 E6 (Fig. 2A, $P = .0012$), HPV16 E7 (Fig. 2B, $P = .02$), and human p16 (Fig. 2C, $P = .009$) (Table I).

An additional comparison of HPV16 E7 transcript levels and human p16 was conducted and demonstrated significant correlations for primary tumors (Fig. 2D, $P = .001$) and metastatic tumors (Fig. 2E, $P = .009$) but not in normal mucosal tissue (Fig. 2F, $P = .24$).

No significant correlation was detected when comparing either primary or metastatic tumor to normal mucosal tissues for HPV16 oncogenes E6, E7, or human p16 (Table I).

**DISCUSSION**

The presence of HPV in the oropharynx of the general population is relatively high, with an overall prevalence of 6.9% when analyzing oral rinses. Despite a fairly high number of people carrying the virus, very few actually develop OPSCC, which propounds the idea that virus activity, in concert with underlying genetic and environmental factors, contributes to oncogenicity. Our findings support the idea of latent virus in adjacent normal tissue, as manifested by HPV ISH positivity, without E6, E7, or p16 activity by qPCR. Conversely, we see active virus in tumoral tissue, both primary and metastatic, with positive HPV ISH activity of E6, E7, and p16 by qPCR. We were able to further analyze the role of viral activity in neck metastasis.

The presence of HPV in neck metastasis for patients with HNSCC has been described in the literature. Papillomavirus is also currently being explored as a diagnostic tool for tumor of unknown origin by way of fine-needle aspiration. However, the role of active infection as measured by E6, E7, and p16 levels had not yet been explored when comparing adjacent normal tissue to tissue that is affected by metastatic carcinoma. Our findings represent a clear pattern of active HPV expression that correlates to disease course. In patients with HPV-positive OPSCC, there was perfect correlation between the presence of HPV DNA, as measured by ISH in tumor, normal, and neck metastasis. The converse of this was also true, with all HPV-negative OPSCC patients having no HPV in their tumor, normal, or metastatic tissue. Interestingly, the difference in E6, E7, and p16 expression in normal tissue versus carcinoma, and in normal tissue versus nodal metastasis, has several implications with respect to disease process.

First, the propensity of HPV to be present in the nodal metastasis of an HPV-positive primary tumor may be higher than originally thought. Other studies have shown anywhere from 39% to 100% correlation between HPV-positive primary OPSCC and HPV-positive neck nodes. Perfect correlation was seen in our study.
The increasing incidence of HPV-related OPSCC and the high correlation seen between HPV presence in primary and metastatic tissue creates diagnostic opportunities for clinicians evaluating patients with cervical metastasis from squamous cell carcinoma, particularly carcinoma of unknown primary. This is propounded by the relative lack of HPV in other HNSCC primary sites and their respective cervical metastasis.

Second, in patients with HPV-positive tumors, both the primary and normal tissues are DNA positive, but the normal tissues have low levels of the downstream products of HPV-related oncogenesis: E6, E7, and p16. This signifies the presence of latent virus in adjacent normal tissue. This raises the important question of whether the primary tumor tissue has active HPV because the tumor environment allows or promotes active infection (or perhaps disallows inactivation of infection), or if the presence of the active infection is the trigger that promoted tumor development in that area. Regardless, these data suggest that when the primary tumor metastasizes to a distant location, metastatic tumor cells transport transcriptionally active HPV infection to the metastatic site.

**CONCLUSION**

There is a very clear pattern of active HPV expression that correlates to disease course. In HPV-positive OPSCC patients, all sites including primary, metastatic, and normal tissues are DNA positive. The high correlation of HPV positivity between primary and metastatic disease could be of true benefit when assessing patients with carcinoma of unknown primary. Additionally, transcriptionally active infections were detected in primary and metastatic tissues, whereas normal tissues appear to have latent infections. Demonstration of this could be beneficial when devising vaccination protocols, treatment algorithms, or targeted therapy for patients with HPV-positive OPSCC.

**TABLE I.**

Summary of Spearman Correlation Coefficient Values.

<table>
<thead>
<tr>
<th>Correlation Pairs</th>
<th>No.</th>
<th>HPV16 E6</th>
<th>HPV16 E7</th>
<th>p16 (CDKN2a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor: metastatic tumor</td>
<td>17</td>
<td>Spearman r = 0.7, P = .001*</td>
<td>Spearman r = 0.54, P = .023*</td>
<td>Spearman r = 0.61, P = .009*</td>
</tr>
<tr>
<td>Primary tumor: normal mucosa</td>
<td>41</td>
<td>Spearman r = 0.22, P = .14</td>
<td>Spearman r = 0.25, P = .11</td>
<td>Spearman r = 0.07, P = .61</td>
</tr>
<tr>
<td>Metastatic tumor: normal mucosa</td>
<td>17</td>
<td>Spearman r = 0.35, P = .16</td>
<td>Spearman r = 0.47, P = .06</td>
<td>Spearman r = 0.03, P = .90</td>
</tr>
</tbody>
</table>

*Transcript levels of target genes HPV16 E6, E7, and human p16 were evaluated in primary tumor, metastatic tumor, and normal mucosal tissue by real-time quantitative polymerase chain reaction.

*Transcript levels were found to significantly correlate for all gene targets between primary and metastatic tumor sites. No significant correlations were found between these sites and normal mucosa for any of the gene targets.
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