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Contemporary Review

Human Papillomavirus and Oropharynx Cancer: Biology, Detection and Clinical Implications

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Objectives: To review evidence for the role of human papillomavirus (HPV) in the etiology of oropharyngeal cancers, methods of viral detection, and the resulting clinical implications.

Study Design: Contemporary review.

Methods: Published journal articles identified through PubMed and conference proceedings were reviewed.

Results: HPV-associated squamous cell carcinomas represent a distinct disease entity from carcinogen-associated squamous cell carcinomas. HPV oncoproteins lead to mucosal cell transformation through well-defined mechanisms. Different methods of detecting HPV exist with variable levels of sensitivity and specificity for biologically active virus. Although virus is detected in a number of head and neck subsites, studies demonstrate improved outcomes in HPV-associated carcinoma of the oropharynx only. The cell cycle regulatory protein p16 is upregulated by biologically active HPV and serves as a biomarker of improved response to therapy.

Conclusions: HPV-associated squamous cell carcinoma of the oropharynx is a biologically distinct entity from carcinogen-associated carcinoma. Understanding the molecular mechanisms behind the improved outcomes in patients with HPV-associated oropharyngeal carcinoma may lead to novel therapeutics for patients with carcinogen-associated carcinomas.

Key Words: Human papillomavirus, oropharynx cancer, biology, detection, survival.

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INTRODUCTION

Aside from a few rare causes of genetic predisposition to develop carcinoma, the paradigm of head and neck squamous cell carcinoma (HNSCC) development has centered around long-term exposure to carcinogens that result in multiple insults to one or more cells at the genetic level.[1] These hits induce changes in the expression or function of proteins involved in cell growth, angiogenesis, replication, and cell survival, all changes that contribute to cellular transformation.[1,2] However, over the last 15 years, the incidence of HNSCC has remained stable as the number of people smoking cigarettes has declined.[3,4] This has been attributed to an observed rise in oropharyngeal squamous cell carcinoma (OPSCC) development,[5] linked both epidemiologically and molecularly to human papillomavirus (HPV) infection.[6]

A growing library of evidence details elaborate mechanisms on the molecular level by which HPV may contribute to cellular transformation in squamous mucosal cells. Changes that occur on the molecular level in HPV-infected cells appear to affect the function of several key regulatory proteins commonly disrupted in carcinogen-induced HNSCC.[7,8] That these changes and effects occur by different mechanisms suggest two molecularly distinct forms of upper aerodigestive tract cancer: HPV-associated OPSCC and carcinogen-associated HNSCC. Additionally, of great clinical interest has been the significantly improved survival of patients with HPV-associated OPSCC, suggesting a distinct clinical behavior of these tumors as well.[9,10]

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Given the intensity of research occurring in this field, impressive amounts of clinical and molecular data have been generated in the head and neck cancer literature over the past several years. Here, we provide a basic science review intended for head and neck cancer practitioners. Beginning with basics of HPV infection and lifecycle, we review the current epidemiologic and molecular evidence for the role of HPV in a subset of head and neck cancers, studies indicating an improved response to therapy and survival in these patients, and comment on future directions and implications these data may have on the care of patients with HPV-associated head and neck cancer.

CLINICALLY RELEVANT HPV BIOLOGY

The human papillomavirus is an encapsulated, nonenveloped double-stranded DNA virus of the family Papillomaviridae. This family contains well over 100 genotypes, or subtypes, based on differences in ability to infect mucosal surfaces and DNA sequence.[11] HPV subtypes able to infect mucosal surfaces are further classified into low-risk or high-risk.[11] Low-risk HPV subtypes cause benign neoplasms, such as papillomas. High-risk types have the ability to induce squamous cell immortalization in vitro and can be detected in a subset of malignant neoplasms. Low-risk HPV types 6 and 11 and high-risk types 16, 18, 31, and 33 are the most commonly identified in specimens from the oral cavity or the pharynx.[12] Although a number of HPV subtypes have been detected in HNSCC samples, high-risk types 16, 31, and 33 are linked biologically to the development of oropharyngeal SCC.[7,13,14]

About 8,000 base pairs in length, the circular viral DNA is enclosed in a protein capsule composed of a complex structure of capsid proteins.[15] HPV genomes typically have eighty genes, broadly organized into early (E) or late (L), so named for the temporal relationship of their expression to cell differentiation.[12,16] Expression of early genes E1, E2, E4, E5, E6, and E7 is initiated in the early promoter, named p97 in HPV-16.[12] Initiation of p97 is independent of cellular differentiation; thus, HPV early genes are expressed in undifferentiated, proliferating basal cells.[12] Genes expressed early in the HPV life cycle are generally involved in viral replication and regulation of gene expression.[7,11,12] Initiation of the late promoter, named p670 in HPV-16, results in expression of L1 and L2 capsid proteins.[12] Late gene expression is differentiation dependent and only occurs in differentiating cells in suprabasal epithelial layers.[11] The remainder of the viral genome is composed of the long control region (LCR) that contains elements responsive to cellular and viral transcriptional regulators.[7]

HPV infection begins in and requires basal epithelial cells capable of proliferation.[11] The virus gains access to the basal layer of stratified squamous epithelium through structural breaks in the stratified epithelial superstructure.[13] The mechanism of viral specificity for basal squamous cells may involve viral interactions with heparin sulfate moeties present in the extracellular matrix component of basement membranes.[16] Binding of HPV to the surface of basal cells may be mediated through interaction of capsid proteins with alpha-6 integrin.[17] The virus gains entry into the cell via endocytosis.[12,16,18] Subsequent trafficking to the nucleus and nuclear entry appears to be dependent upon the initiation of mitosis in the infected cells.[16] Once in the nucleus, the virus establishes itself as a persistent infection in the form of a DNA episome. During the maintenance phase of the HPV cell cycle, 20–100 copies of episomal viral DNA per basal cell are sustained via a complex interaction of early proteins expressed from the viral genome, including E1, E2, E6, and E7.[18] As basal cells divide, HPV viral episomes are replicated along with the host DNA. As one daughter cell moves into the suprabasal layers to begin terminal differentiation, the other stays behind in the basal layer, thus establishing a persistent HPV infection.[18] As native, uninfected squamous cells move into the suprabasal layers, mitosis is halted and a program of squamous differentiation begins, ultimately leading to breakdown of the nuclear envelope and cell desquamation. In HPV-infected cells, cell cycle progression continues and cellular differentiation is blocked, in concert with increased expression of E4, E5, E6, and E7.[12,18] Cellular proliferation results, ensuring availability of host replicative machinery for viral DNA replication and virion production. As HPV-infected cells progress from the proliferative phase to the viral amplification and assembly phases, increased expression of E1, E2, E4, and E5 as well as expression of the late genes encoding for capsid proteins occurs.[18] In the middle to upper epithelial layers, viral genomes are amplified and packaged as they await release from desquamated epithelial cells as fully competent virions.[18]

As proteins expressed early in the HPV life cycle, E1 and E2 are involved in viral DNA replication. Together, these proteins form a complex that binds the viral origin of replication in the LCR and recruits cellular polymerases and other host machinery required for viral DNA replication.[7] The E2 protein may also have regulatory effects on the expression of other early genes. E2 has been shown to repress expression of E6 and E7 via inhibition of p97 promoter activation, but recent evidence suggests that this effect only occurs when HPV DNA is integrated into the host genome,[19] as episomal DNA in the region of the p97 promoter in inaccessible due to chromatin structure.[20] Further, activation of promoters other than p97 leading to E7 expression have been identified.[21] The E4 protein, as described above, is expressed later in the HPV cell cycle and is involved in the organization of HPV virions.[7,12,18] The E5 protein is likely involved in DNA synthesis, but unlike E4, is expressed early in the HPV life cycle.[7,12,18]

The E6 and E7 proteins have generated the most interest from investigators given their ability to disrupt the function of known tumor suppressor proteins. The mechanism for this process is shown in Figure 1. E6 binds p53 and, along with the cellular ubiquitin ligase E6-associated protein (E6AP), marks it for degradation via a proteasome dependent pathway.[22] E6 may also activate telomerase in a p53 independent fashion.[23]

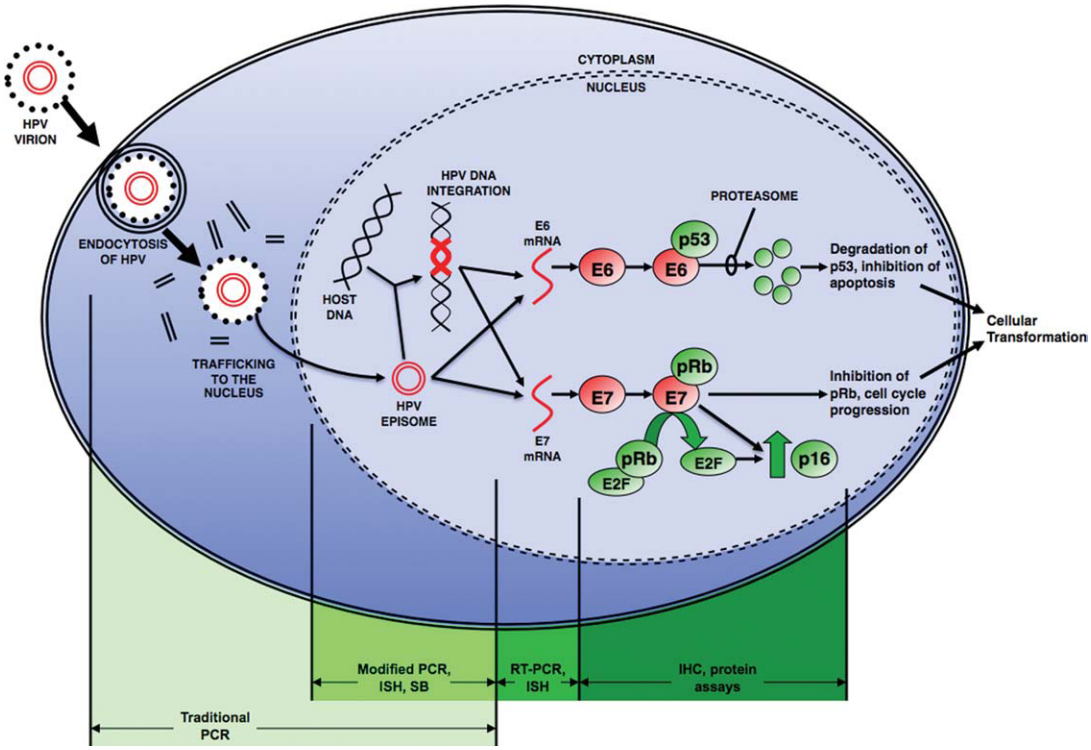

Fig. 1. Schematic of HPV infection of a mucosal cell. After virion entry via endocytosis, the virus establishes a persistent infection as a viral episome or integrates into the host genome. HPV E6 and E7 oncoproteins are expressed from both forms of the viral DNA, which lead to p53 degradation and Rb inhibition, respectively. Methods of HPV, oncogene or p16 detection are depicted with respect to stage of HPV biologic activity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Activating telomerase extends the cell's ability to progress through the cell cycle, and sequestering p53 likely prevents initiation of programmed cell death that would otherwise be activated upon unregulated re-entry into the cell cycle due to the effects of the E7 protein. The E7 protein binds and inactivates proteins in the retinoblastoma gene family of tumor suppressor proteins, including retinoblastoma (Rb), p130, and p107.[24] The hypophosphorylated Rb protein binds and regulates activity of E2F, a transcription factor that induces the expression of factors involved in cell cycle progression. By binding and inactivating hypophosphorylated Rb, E7 releases E2F to promote cell cycle progression.[7,24] Further, E7 directly binds E2F–DNA complexes and stimulates E2F-dependent transcriptional activity independent of the Rb protein.[25] Rb plays a role in the regulation of additional tumor suppressor proteins, including acting as a negative regulator of the expression of p16, a cyclin-dependent kinase inhibitor.[26,27] Interestingly, E7 appears to play a role in HPV immune evasion. Although HPV evades the host immune system largely via residing in the basal epithelium where immune surveillance is difficult for the host, E7 also blocks activity in interferon α and β promoters, decreasing proinflammatory signaling.[28] E7 further plays a role in modulating altered T-cell function in the tumor microenvironment observed in patients with HPV associated carcinomas.[28] These data and others[29] suggest that p53 and Rb-dependent and-independent functions of E6 and E7 likely play a vital role in their oncogenic potential.

MOLECULAR EVIDENCE FOR THE ROLE OF HPV IN HNSCC ONCOGENESIS

It is estimated that 85% of humans will have an HPV infection of any kind during their lifetime.[30] In immunocompetent women, the vast majority of HPV cervical infections are cleared by the immune system and cellular transformation is rare.[30] Despite having a functioning immune system, a subset of patients will develop chronic HPV infections that, in some cases, result in cellular transformation.[28] Similar statistics for presence of HPV and subsequent cellular transformation in the head and neck are unknown. The rationale for the role of HPV in malignant degeneration of epithelial cells originates from the cervical cancer literature, and many of these studies have been repeated in HNSCC models and specimens,[29,31–43] building a case for HPV as an etiologic agent in a subset of HNSCCs.

Viral E6 and E7 oncoproteins are expressed in both high- and low-risk HPV types, but E6 and E7 expressed in high-risk HPV subtypes such as HPV-16 bind their respective tumor suppressor proteins with much higher affinity, affording them increased oncogenic potential.[30,44] Expression of E6 and E7 from a high-risk type DNA is essential for both induction and maintenance of cellular transformation in vitro.[45] In cervical cancer, a crucial step in the progression from dysplasia to carcinoma appears to be integration of viral DNA into the host genome.[46] This occurs randomly over the entire host genome, with a predilection for genomic fragile sites.[46] This integration step has been postulated to

disrupt or affect the expression of critical cellular genes via insertional mutagenesis,[47] but recent literature finds no evidence of this process.[46] Integration of HPV DNA in cervical epithelial cells leads to increased viral oncogene expression in vitro.[48] This increased expression is hypothesized to be due to interruption of the viral E2 gene, releasing p97 promoter activation leading to increased expression of both E6 and E7. However, viral DNA disruption sites have been shown to be inconsistent, occurring anywhere from E1 to the late genes,[46] indicating E2 disruption during integration may be an unreliable event. Further, although integrated viral DNA is detected more frequently in malignant rather than dysplastic cervical specimens, E6 and E7 transcript levels are inconsistently elevated in tumors with integrated viral DNA,[49] suggesting additional levels of transcriptional regulation present in vivo. Independent of relative expression, E6 and E7 proteins expressed from integrated viral DNA transform cells more effectively than E6 and E7 derived from episomal viral DNA, possibly due to a longer half-life of transcripts produced from integrated DNA.[50] The majority of HPV DNA is episomal in premalignant cervical lesions, whereas 90% of cervical carcinomas harbor the integrated form.[30,51,52]

About 25% of all HNSCC specimens contain detectible HPV DNA.[31] Patients with HPV-associated HNSCC tend to be younger and lack the extensive smoking and/or alcohol abuse history common in many patients diagnosed with HNSCC.[53] Evidence suggests that HPV infection and HPV-associated HNSCC are correlated with high-risk sexual behavior.[54,55] Although HPV DNA may be detected at a number of HNSCC sites, it is most commonly found in the oropharynx, and is most commonly high-risk type 16.[31–33] HPV type 16-associated carcinoma is most likely to be located within oropharyngeal lymphoid tissue with an odds ratio of 15 compared to other oropharyngeal subsites, the oral cavity, or larynx.[31,56] Although a variety of HPV types has been detected throughout the head and neck region, high-risk HPVs appear to have tropism for tonsillar epithelium for unclear reasons.[57] Following early reports of an association with HNSCC, numerous in vitro and in vivo studies have revealed viral biological activity in a subset of HNSCC.[7,8]

High-risk HPV can immortalize aerodigestive tract keratinocytes.[34] Inhibition of E6 and E7 expression restores function of p53 and Rb and induces apoptosis in HPV-positive oropharyngeal SCC cell lines.[58] In a mouse model of HNSCC, E7 serves as the major oncogene with a supporting role played by E6.[29] In HNSCC tumor specimens, E6 and E7 transcript expression is present in a subset of HNSCC, indicating biologic activity.[35–43] HPV DNA exists in HNSCC cell nuclei as fully episomal, fully integrated, or mixed.[35,59,60] Viral E6 and E7 mRNAs are transcribed from both episomal and integrated HPV DNA in HNSCC cell lines and tumor specimens.[52,61] As a measure of downstream activity of Rb inactivation by E7, the expression of p16, a gene frequently inactivated in HPV-negative HNSCC,[62] has been thoroughly evaluated. Increased expression of p16 is frequently, but not exclusively, found in HPV-associated HNSCC.[26,38,40,43,63–66] Overexpression of p16 has been attributed mainly to the function of E7 and its interaction with Rb, but recent evidence suggests a supporting role for E6 as keratinocytes that express E6 and E7 induce expression of p16 much more than keratinocytes that express E7 alone.[67]

In support of the role of HPV in malignant transformation in a subset of HNSCC, tumors harboring biologically active HPV DNA have a gene expression profile different from that of tumors lacking HPV DNA.[68] HPV-negative tumors have increased expression of proteins associated with tumor development and progression, such as cyclin D and epidermal growth factor receptor.[28,69] Further, global epigenetic changes and differences in genome stability are observed between HPV-positive and-negative HNSCC.[39,70–72] Within oropharyngeal cancers, increased microsatellite instability and high numbers of chromosomal aberrations can be observed in HPV-negative tumors.[73] Further, recent data suggests that several chromosomal alterations common to both HPV-associated head and neck and cervical carcinomas exist, indicating the presence of genetic derangements necessary for HPV-associated squamous cell carcinogenesis independent of anatomic location.[74] These data suggest that HPV-associated HNSCC differs on the molecular level from HPV-negative HNSCC, suggesting a distinct mechanism of tumor development and further supporting a direct role for HPV.

Development of HPV-negative HNSCC is believed to involve a series of mutations at the genetic level. In mouse models, cellular transformation can be induced after inducing specific genetic aberrations.[2] In HPV-negative HNSCC, these mutations have traditionally been attributed to repeated carcinogen exposure, such as cigarette smoke or betel nut.[75,76] Half or more of all carcinogen-associated HNSCC have an identifiable p53 mutation, whereas p53 mutation in cervical cancer is very rare.[77] This led to investigations regarding the role of p53 mutations in HPV-associated HNSCC development. Indeed, p53 mutation is an uncommon event in HPV-associated HNSCC.[36,66,78–80] Inhibition of p53 by the HPV E6 protein is not equivalent to inactivating p53 gene mutations as some p53-dependent genes are still expressed in the presence of E6.[81] Interestingly, many investigators have observed overexpression of wild-type p53 in HPV-associated HNSCC, a phenomenon usually associated with mutated p53.[43,66,80] Although the exact mechanisms for this finding remains unclear, overexpression of wild-type p53 is of great clinical interest given the protective functions of this key tumor suppressor protein as well as the role it may play in therapeutic responses. The interplay between Rb and p16 is another point on which HPV-associated and carcinogen-associated HNSCC development differs. In carcinogen-associated HNSCC, the p16 protein is commonly inactivated due to a variety of carcinogen induced genetic alterations including deletions, mutations, and promoter methylation.[82] Given that p16 acts to block hyperphosphorylation and inactivation of Rb, loss of p16 leads to loss of functional activity of Rb and cell cycle progression.[57,63] Conversely, as mentioned above, Rb acts as a

TABLE I.
Methods of HPV Detection.

Detection Method	Advantages	Disadvantages
PCR	High sensitivity*	Low specificity†
	Widely available	Cumbersome
DNA ISH	High specificity	Low sensitivity
E6/E7 mRNA	High sensitivity	May require fresh frozen tissue
	High specificity	Cumbersome
E6/E7 protein IHC	High specificity	Questionable sensitivity
		Technically difficult
p16 IHC	Very high sensitivity	Questionable specificity
	Widely available	
Morphology‡	Always available	Imperfect correlation§
	No expense	Questionable reproducibility

†Sensitivity and specificity refer to detection of biologically active HPV.
‡Nonkeratinizing histology.
§Between morphology and biologically active HPV.
PCR = polymerase chain reaction; ISH = in situ hybridization; IHC = immunohistochemistry.

negative regulator of p16 expression. Rb inactivation by E7 in HNSCC with biologically active HPV leads to overexpression of p16.[27,63] Of interest, in a cervical cancer model, p16 transcript expression was enhanced in cells immortalized by HPV much more than expected with Rb inhibition alone, suggesting the presence of Rb-independent effects of HPV on p16 expression as well.[63,83] Further, no point mutations or deletions in the p16 gene were found in these immortalized, HPV-infected cell lines, or in cervical cancer tumor specimens.[83] Supporting the idea of p16 genetic alterations being less prevalent in HPV-associated HNSCC than in carcinogen associated HNSCC, HPV-positive/tobacco-negative HNSCC tumors have much lower rates of p14, p15, and p16 genetic alterations than do HPV-negative/tobacco-positive HNSCC tumors.[84] Despite molecular evidence that overexpression of p16 is due to functional disruption of Rb by E7, there remains a small but clinically important subset of HPV-negative patients that overexpress p16,[9,10] suggesting alternate mechanisms of p16 overexpression.

METHODS USED TO DETECT THE PRESENCE OF HPV BIOLOGIC ACTIVITY IN TUMOR SPECIMENS

Understanding the various methods used to detect HPV in a tumor biopsy or surgical specimen is necessary to appropriately interpret the results of studies evaluating the role of HPV in HNSCC. Available detection methods are based upon the presence of HPV DNA, mRNA transcripts, or translated proteins and are summarized in Table I. Figure 1 shows the methods of HPV detection in relation to HPV infection, physical status, and oncogene expression.

Polymerase chain reaction (PCR) is used to amplify a single sequence of DNA across several orders of magnitude, and can be used to detect as little as one copy of HPV DNA per cell making it extremely sensitive.[7] Specificity for a DNA sequence comes by the use of DNA primers complimentary to the region of interest, and primers can be designed to amplify any region of DNA for which the sequence is known.[85] To detect the presence of HPV in a sample, primers can be used that detect a broad range of HPV subtypes by amplifying a region of DNA common to many HPV subtypes (consensus primers), such as a segment of the highly conserved L1 gene. Conversely, primers designed to amplify a region of DNA unique to one HPV subtype could be used to allow simultaneous identification of the presence and subtype of HPV virus. General use of PCR allows identification of the presence of HPV DNA only, and it gives no information regarding the physical status (episomal vs. integrated) or expression of HPV genes. Further, when used in a heterogeneous tumor sample, it does not allow the investigator to determine if the HPV DNA is present in the population of cancer cells or in the surrounding nonneoplastic/stromal tissue unless laser capture microdissection (LCM) is utilized. Regarding sensitivity, detection with PCR may differ between specimen preparations. PCR may be performed using fresh frozen (FF) tissue or formalin-fixed paraffin-embedded (FFPE) tissue. PCR amplification is more efficient on FF tissue than FFPE tissue.[86] Using fresh-frozen HNSCC tumors that were HPV DNA and E6/E7 oncogene RNA positive as a control, Smeets et al.[38] showed that half of the same tumor population was PCR-positive in FFPE specimens, suggesting decreased sensitivity of the assay in FFPE specimens. However, PCR positivity was detected in a subset of FFPE tumors shown to be HPV DNA and E6/E7 mRNA negative in FF specimens, representing false positives. Given these data, the major advantages of PCR use to detect HPV are very high sensitivity, especially in FF specimens, and widespread availability of the technology. The disadvantages appear to be low specificity for HPV biologic activity within tumor specimens and occurrence of false-positives. PCR is the most commonly method used to detect HPV DNA in a tumor sample.[31,32,87] either alone or in combination with other modalities.

Several authors have utilized PCR technology to characterize the physical status of HPV virus in infected tumor samples.[60,88] The HPV E2 gene is a common break site in the circular viral genome as the virus prepares for integration into the host genome. If the E2 gene is disrupted, it will not amplify via PCR when primers designed to amplify the whole E2 gene are used. When analyzed as a ratio with E6, a gene rarely disrupted by viral integration into the host genome, the E2:E6 ratio can indicate whether the HPV virus present is episomal or integrated.[60,88] Although this technique allows relatively quick assessment of the physical status of the HPV DNA, it is based upon the assumption that the HPV DNA breakpoint is consistently within the E2 gene. Evidence exists in both the HNSCC and cervical cancer literature that HPV DNA breakpoints are

variable and that other genes may be disrupted for integration.[36,46,49,52,89] Evidence of episomal HPV DNA lacking the E2 gene exists as well.[59] An additional use of PCR to determine physical status involves the use of restriction enzymes and inverse PCR.[59] Based on the idea that a circular piece of DNA (episomal HPV DNA) can be amplified in its entirety by using a long-running template PCR with inverse primers, and that linear DNA (integrated HPV DNA) could not be amplified with inverse primers, the physical status of HPV DNA in a sample could be determined by cleaving DNA with restriction enzymes after amplification with inverse primers and comparing the resulting segments based on their expected size. Named rliPCR, this technique allows for determining HPV DNA status as episomal or integrated or both in a single sample. A criticism of using viral integration into the host cell's genome as a surrogate measure of biologic activity is that E6 and E7 mRNA can be transcribed from both episomal and integrated viral DNA,[20,36,49,52,61] and that viral integration into the host genome does not always equate into increased oncogene expression.[49] Examining for only viral integration does not account for the presence of integrated viral DNA that is transcriptionally repressed by episomal derived E2, so called "silent integrants."[90] Viral integration may be a late event in malignant transformation of squamous cells, and may not be necessary for initiation of oncogenesis.[46,59,60]

Further advances in PCR utilization have allowed the development of quantitative real-time PCR (Q-PCR).[91] Via the use of calorimetric markers that accumulate with each cycle of amplification, Q-PCR allows both identification and quantification of the amount of target DNA present in a sample. Thus, this technology allows the determination of both the presence of HPV DNA as well as HPV viral loads. Again, whole-cell HPV DNA is measured, and no determinations about localization, integration, or expression of oncogenes from HPV DNA can be made with Q-PCR. Although assessment of HPV-16 viral load may suggest active replication of HPV in a tumor specimen,[37,38] use of Q-PCR to assess viral load may require LCM to ensure sample purity.[38]

Another method of detecting DNA in a tissue is in situ hybridization (ISH). The use of ISH allows determination of presence as well as localization of HPV DNA in a specimen. Using complimentary nucleic acid probes with either radioactive, dye-, or fluorescent-labeled bases, HPV DNA can be localized via direct microscopic visualization and quantitated colorimetrically.[92] Probes can be designed to detect sequences of DNA common to many HPV subtypes or to sequences of DNA unique to individual subtypes. Under high-power microscopy, the presence of HPV DNA in heterogeneous populations of cells in a sample can be determined. Further, the physical status of HPV DNA can visually be determined with episomal DNA present as diffuse nuclear signal and integrated DNA present as punctate nuclear signal. Although ISH allows localization of HPV DNA, one disadvantage of ISH when compared to PCR may be decreased sensitivity. The limit of ISH sensitivity has traditionally been recognized at 10 viral copies per cell. However, improved reagents and technique have resulted in commercially available ISH kits with the ability to detect as few as one to two copies of HPV DNA per cell.[93] Evaluating the use of ISH in HNSCC specimens, Smeets et al.[38] observed very high specificity for biologically active HPV in FFPE tumor samples, but demonstrated decreased sensitivity of ISH as several tumors shown to be HPV DNA and E6/E7 mRNA positive in FF specimens were ISH negative in FFPE specimens. Similar data was recently published by Shi et al.[43] When compared to E6 mRNA positivity, this study demonstrated the presence of false negatives with HPV ISH in FFPE oropharyngeal cancer tissues. Although ISH may have decreased sensitivity in detecting HPV DNA compared to PCR,[38] it has the added benefit of allowing determination of episomal versus integrated DNA within a tumor cell. However, when using DNA probes, ISH gives no evidence regarding biologic activity, and although viral integration may be a common finding in later stages of cancer progression, episomal viral DNA may be biologically active and crucial to tumor initiation and early development.[39,46,60]

One additional technique used to detect HPV DNA is blot hybridization analysis. The most common technique utilized is the Southern blot (SB).[94] This technique involves the use of restriction enzymes to fragment DNA, electrophoresis to separate DNA fragments by size and transferring the separated DNA to a membrane upon which specific DNA probes can hybridize to DNA of interest. Again, specificity comes from DNA probes that can be designed to detect common or unique HPV DNA sequences. SB analysis has the capability of differentiating between episomal and integrated HPV DNA, and under optimal conditions can be very sensitive detecting one viral copy per cell. However, studies have demonstrated sensitivity of SB far inferior to that of PCR.[79,95]

Although many techniques exist for evaluating the presence and physical status of HPV DNA, none of the above can assess the status of E6 and E7 oncogene expression required to initiate the molecular changes that lead to transformation in squamous cells. The presence of HPV DNA does not necessarily indicate viral gene expression.[36–38,96] E6 and E7 mRNA positivity represents biologically active HPV DNA, regardless of the physical status, and can be directly measured via reverse transcriptase-PCR (RT-PCR). This technique uses reverse transcriptase to create a cDNA sequence from mRNA that is subsequently amplified using traditional or Q-PCR. Although RT-PCR is highly sensitive and specific, performing RT-PCR is time consuming and traditionally requires FF tissue, which is more burdensome to handle and catalog than FFPE tissue. The need for FF tissue is often cited as a reason for an investigator's inability to measure E6 and E7 transcript levels in tumor samples.[38] However, Smeets et al.[38] have recently reported successful identification of HPV-16 E6 mRNA from FFPE tissue samples with perfect sensitivity and specificity when compared to identification from FF tissue. A major limitation of the technique at this point is that it has only been validated in HPV type 16-infected tissues. Similarly, Shi et al.[43] have reported successful extraction and

measurement of E6 mRNA from FFPE tissues. Measurement of E6 and E7 mRNA using RT-PCR is highly sensitive, and offers increased specificity for biologic activity of HPV in a tumor sample. Despite this high sensitivity and specificity for biologic activity, a criticism of RT-PCR to detect HPV oncoprotein mRNA may be that it is cumbersome to perform and would be difficult from a practical standpoint to use in large-scale studies.

Similar to its use for detecting DNA segments of interest, ISH can be used to identify mRNA by using complimentary RNA probes (riboprobes). The technique is largely similar to ISH for identification of DNA, and allows both identification as well as localization of mRNA. RNA ISH has successfully been used to detect transcription of HPV 16 E6/E7 mRNA in tonsillar SCC.[97] Compared to RT-PCR, mRNA ISH would likely have less sensitivity, but would retain its increased specificity for biologically active HPV.

Although identification of HPV oncoprotein transcripts suggests positive viral activity, it does not take into consideration posttranscriptional regulation. Extensive modifications to HPV DNA transcripts in the form of RNA splicing, polyadenylation, and RNA translational control have been described and may impact the assumption that the presence of E6 or E7 mRNA indicates the presence of E6 and E7 oncoprotein. Recent evidence suggests that the HPV E6 and E7 genes are mostly transcribed into bicistronic E6/E7 mRNAs. Splicing of this mRNA leads to mostly E7 oncogene translation, whereas if the mRNA is not spliced, E6 is predominantly translated.[98] This suggests an elaborate posttranscription regulation of oncogene translation. Directly assaying for the presence of HPV E6/E7 oncoprotein establishes that the HPV DNA is being expressed and that the oncoproteins shown to be involved in oncogenesis are present. A number of techniques exist to examine a sample at the protein level, with immunohistochemistry (IHC) the most commonly utilized. IHC can be performed on either FF or FFPE tissues. A primary antibody designed to recognize a protein of interest, followed by an enzyme-linked secondary antibody and substrate, produce a calorimetric reaction that can be visualized with microscopy. Similar to ISH, IHC can both detect and localize proteins. Although not strictly quantitative, semiquantitative analysis can be performed using imaging software or graded scoring systems. Colorimetrically based quantitative protein arrays, such as protein ELISAs and multiplex protein array systems, are based on the same principles of protein:antibody interaction. These newer techniques have not been utilized to date to quantitatively evaluate HPV oncoproteins in tumor samples. Sensitivity of IHC, or how few proteins must be present obtain a positive result, is difficult to assess due to titration of primary antibody as well as variability in specimen fixation methods. However, IHC positivity for E6 and/or E7 in the setting of a negative isotype control would have very high specificity for HPV biologic activity.

The p16 protein, shown to be overexpressed in HPV-positive HNSCC specimens, has been studied principally via immunohistochemistry.[26,40,43,49,63,64,99–102] Many studies show a strong, statistically significant correlation between the presence of p16 and HPV DNA measured by PCR or ISH in HNSCC.[26,43,63,66,80,88,102,103] When examining tumors with biologically active HPV that express E6/E7 transcripts, specificity is reduced, as some tumors do not express p16.[36,43] Further, p16 is overexpressed in a subset of tumors that lack E6/E7 transcript levels or even HPV DNA.[38] As most HPV-positive tumors express p16, sensitivity of p16 for HPV presence is very high. However, given that many studies show elevated expression of p16 in HPV DNA or E6/E7 transcript-negative HNSCC tumors.[36,38,88,102,103] specificity of p16 for HPV biologic activity is in question.

The above techniques evaluate a sample or specimen for the presence or activity of HPV virus on the molecular level utilizing DNA, RNA, or protein detection assays. Changes on a cellular level of cells infected with HPV may produce a distinct and consistent phenotype that can be recognized by histologic examination. El-Mofty et al.[104] examined a large series of oropharyngeal tumors and correlated the presence of HPV DNA with cellular morphology. HPV 16-positive carcinomas are nonkeratinizing and characterized as having well-defined sheets or nests with little stroma and composed of oval to spindle-shaped, basaloid cells with ill-defined borders and hyperchromatic nuclei. Excessive mitoses, cells undergoing apoptosis and comedo-type necrosis are also characteristic. Although these findings support the idea of nonkeratinizing HPV-positive oropharyngeal SCC as a distinct subtype of HNSCC with a characteristic phenotype, classifying tumors based on microscopic appearance is not specific for HPV presence or activity, and has not been validated for interobserver reliability. It has been demonstrated, however, that nonkeratinizing morphology alone predicts strong p16 expression with a positive predictive value of 100%.[14]

Taken together, the above data suggest that there is no perfectly sensitive or specific test to detect biologic activity of HPV in a HNSCC tumor specimen.[43,57,105–107] The ideal test currently described in the literature to determine HPV biologic activity, detection of E6/E7 transcript levels using RT-PCR, is cumbersome, and traditionally has required the availability of fresh tissue. This has led authors to recommend p16 IHC or HPV ISH become standard evaluations for patients with OPSCC.[43] However, protocols have recently been developed that allow reliable detection of E6 mRNA levels in FFPE tumor specimens. Given the ease and widespread use of IHC, staining for the presence of the E6/E7 oncogenes themselves as a marker of HPV biologic activity may be considered. Utilization of these techniques in future studies may assist in revealing the true biologic role of HPV in HNSCC specimens harboring HPV DNA.

CLINICAL AND PROGNOSTIC IMPLICATIONS OF HPV PRESENCE IN HNSCC

A PubMed search was performed to evaluate the role of HPV positivity in survival of HNSCC. Since 1994, 50 studies that investigate the effects of HPV positivity on survival in HNSCC have been published. These studies are listed in Table II. Of these studies, 23 either

TABLE II.
Studies Evaluating Correlation Between HPV and Survival in HNSCC.

Study	Year	N	Tumor Subsite	HPV Positivity (%)	HPV Types Detected	Method Used‡	Tissue Prep§	Treatment‖	Endpoints studied¶	Survival advantage#
All Site Survival Studies*										
Brandwein[117]	1994	55	Oral Cavity	78	—	PCR	FFPE	—	DSS	No
		9	Oropharynx	56						
Clayman[131]	1994	6	Hypopharynx	100	—	PCR	FFPE	S/XRT	DSS	No**
		59	Larynx	41					LRC	No
Chiba[108]	1996	38	Oral Cavity	21	16	PCR	FF	—	DFS	Yes
Haraf[118]	1996	14	Oral Cavity	0	16/33	PCR	FFPE	Mixed	OS	No
		26	Oropharynx	38						
		7	Hypopharynx	14						
		19	Larynx	5						
Snijders[119]	1996	63	All sites	21	16	PCR	FFPE	—	OS	No
Reithdorf[120]	1997	78	Oral Cavity	42	6/11/16/18	PCR	FF	Mixed	OS	No
		9	Oropharynx	44						
		3	Larynx	67						
Paz[121]	1997	15	Tonsil	60	16/6	PCR, SB	FF	—	OS	No
		152	Other site	11					DSS	No
Koch[122]	1999	211	All sites	18	16/33	PCR	FF	—	OS	No
Pintos[123]	1999	29	Oral Cavity	10	—	PCR, SB	FFPE	—	OS	No
		20	Pharynx	30					DFS	No
		52	Larynx	15						
Shima[124]	2000	46	Oral Cavity	74	16/18	PCR, SB	FF	—	OS	No
Schwartz[109]	2001	254	Oral Cavity	16	16+	PCR	FFPE	Mixed	OS	Yes††
									DSS	Yes
Ringstrom[110]	2002	41	Oral cavity	5	16	PCR	FF	—	OS	Yes
		29	Oropharynx	52					DSS	Yes
		4	Hypopharynx	0						
		10	Larynx	10						
		5	Other site	0						
Sisk[111]	2002	32	Mixed sites	47	16/18	PCR	FF	—	OS	Yes
Dahlgren[112]	2003	25	Tonsil	60	16/118/33	PCR	FF	S/XRT	DSS	Yes
Koskinen[60]	2003	5	Tonsil	100	6/16/33/51/52	PCR, ISH	FF	—	OS	No
		15	Tongue	73						
		13	Oral Cavity	54						
		10	Hypopharynx	50						
		18	Larynx	50						
Ritchie[113]	2003	94	Oral Cavity	11	16/18/33	PCR	FFPE	Mixed	OS	Yes
		45	Oropharynx	42						
Azzimonti[125]	2004	25	Larynx	56	16/18	PCR	FFPE	—	OS	No
		9	Tonsil	56						
Baez[126]	2004	36	Oral Cavity	36	16	PCR	FF	—	OS	No
		16	Oropharynx	63					DFS	No
		14	Hypopharynx	36						
		52	Larynx	46						
Dahlgren[114]	2004	85	Oral Cavity	2	16/18/33	PCR	FFPE	S/XRT	DSS	Yes
		25	Base of Tongue	40						
Hoffman[127]	2005	20	Tonsil	55	16/33	PCR, SB	FF	S/XRT	OS	No
		4	Oropharynx	26					DFS	No
		6	Oral Cavity	67						
		24	Hypopharynx	29						
		19	Larynx	26						

(*Continued*)

TABLE II.
(*Continued*).

Study	Year	N	Tumor Subsite	HPV Positivity (%)	HPV Types Detected	Method Used‡	Tissue Prep§	Treatment‖	Endpoints studied¶	Survival advantage#
Kozomara[132]	2005	50	Oral Cavity	64	6/16/18/31	PCR	FF/FFPE	S/XRT	OS	No**
Vlachtsis[128]	2005	90	Larynx	40	16/18	PCR	FF	S/XRT	OS	no
Badaracco[35]	2007	60	Oral Cavity	13	16/33/35/58	PCR	FFPE	—	OS	yes
		2	Nasal Cavity	100					DFS	no
		10	Oropharynx	0						
		8	Tonsil	75						
		5	Hypopharynx	20						
		30	Larynx	13						
Furniss[115]	2007	266	Oral cavity	14	16	PCR	FF/FFPE	—	OS	yes
		78	Oropharynx	19						
		33	Hypopharynx	18						
		90	Larynx	16						
Na[116]	2007	70	Oral Cavity	0	16	PCR	FFPE	Mixed	OS	yes
		38	Tonsil	24						
Sugiyama[129]	2007	66	Oral Cavity	36	16	PCR	FFPE	Mixed	OS	no
Jo[130]	2009	14	Oropharynx	93	16	PCR	FF/FFPE	C, S/XRT	OS	no
		10	Other site	10					PFS	no
Oropharynx Survival Studies†										
Portugal[133]	1997	58	Oral Cavity	7	—	PCR	FFPE	—	OS tonsil	yes
		42	Tonsil	19					OS all sites	no
Gillison[79]	2000	2	Nasopharynx	0	16/18/31/33	PCR/ISH/SB	FF	Mixed	OS all sites	yes
		84	Oral Cavity	12					DSS all sites	yes
		60	Oropharynx	57					OS oropharynx	yes
		21	Hypopharynx	10					DSS oropharynx	yes
		86	Larynx	19						
Friesland[134]	2001	34	Tonsil	41	16	PCR	FFPE	XRT	OS	yes
									DFS	no
Lindel[142]	2001	99	Oropharynx	14	16/33/35/45	PCR	FFPE	XRT/C	OS	no
Mellin[59]	2002	22	Tonsil	55	16/33	PCR	FF	—	OS	no
									DSS	no
Strome[143]	2002	52	Tonsil	46	16/59	PCR	FFPE	—	OS	no
									DFS	no
Klussman[63]	2003	34	Tonsil	53	16/33	PCR	FFPE	S/C/XRT	OS	no
									DFS	no
Li[135]	2003	67	Tonsil	46	16+	PCR	FFPE	Mixed	DSS	yes
Mellin[136]	2003	60	Tonsil	45	16/33	PCR	FFPE	XRT/S	DSS	yes
Wittekindt[100]	2005	34	Tonsil	53	16/18	PCR	FF	—	OS	no
De Petrini[137]	2006	23	Oral Cavity	39	16	PCR	FFPE	—	DSS oropharynx	yes
		21	Oropharynx	52					DSS oral cavity	no
Licitra[88]	2006	90	Oropharynx	19	16	PCR	FFPE	S/XRT	OS	yes
Weinberger[64]	2006	79	Oropharynx	61	16	PCR	FFPE	Mixed	OS	yes
									DFS	yes
Reimers[26]	2007	106	Oropharynx	28	16/33	PCR	FFPE	-	OS	no
									DFS	no
Fakhry[102]	2008	62	Oropharynx	61	16/33/35	ISH	FFPE	IC, CRT/S	OS all sites	yes
		34	Larynx	0					PFS all sites	yes
									OS oropharynx	yes
									PFS oropharynx	yes

(*Continued*)

TABLE II.
(*Continued*).

Study	Year	N	Tumor Subsite	HPV Positivity (%)	HPV Types Detected	Method Used‡	Tissue Prep§	Treatment‖	Endpoints studied¶	Survival advantage#
Hafkamp[103]	2008	81	Tonsil	41	16	PCR/ISH	FFPE	Mixed	OS	yes
									DSS	yes
Worden[138]	2008	26	BOT	62	16	PCR	FFPE	IC, CRT/S	OS	yes
		16	Tonsil	69					DSS	yes
Gillison[9]	2009	323	Oropharynx	64	16+	ISH	-	CRT	OS	yes
									PFS	yes
									LRC	yes
Haughey[10]	2009	174	Oropharynx	72	—	ISH	FFPE	S, C/XRT	OS	yes
									DFS	yes
Ritta[139]	2009	25	Oral Cavity	36	16/6	PCR	FFPE	S+	OS oropharynx	yes
		22	Oropharynx	50					OS other sites	no
		12	Larynx	58						
Sedaghat[140]	2009	49	Oropharynx	53	16	ISH	FFPE	CRT	OS	yes
									DSS	yes
									RFS	yes
Settle[141]	2009	28	Oral cavity	11	16	PCR	FFPE	CRT	OS oropharynx	yes‡‡
		119	Oropharynx	50						
		35	Hypopharynx	6						
		55	Larynx	7						
Shi[43]	2009	111	Oropharynx	66	16	RT-PCR, ISH	FFPE	XRT, CRT	OS	Yes§§
									DFS	Yes

*Studies that evaluate nonoropharynx sites or studies that include oropharynx but do not correlate oropharynx site with survival seperately.
†Studies that correlate oropharynx site with survival.
‡PCR = polymerase chain reaction; ISH = in-situ hybridization; SB = Southern blot.
§FFPE = formallin fixed paraffin embedded; FF = fresh frozen.
‖S = surgery; XRT = external beam radiotherapy; C = chemotherapy; IC = induction chemoptherapy; CRT = concurrent chemoradiotherapy.
¶DSS = disease-specific survival; LRC = local-regional control; DFS = disease-free survival; OS = overall survival; PFS = progression-free survival.
#Statistically significant ($P \leq 0.05$) for endpoint listed.
**Statistically significant for HPV postivity as a negative prognostic variable.
††Statistically significant for HPV type 16 only.
‡‡Includes patients with tissue available for HPV analysis only.
§§Significant improvement in overall survival with RT-PCR only on multivariate analysis.

evaluated only oropharyngeal tumors or evaluated the oropharyngeal subsite separately in survival analysis. Several trends can be observed. PCR is the most common method utilized to detect HPV, but more recent studies have utilized ISH or reverse-transcriptase PCR. HPV type 16 is the most common subtype, detected in all or most of the tumors from each study. Although high-risk HPV can be detected in many head and neck subsites, a survival advantage has been consistently demonstrated with HPV-associated oropharyngeal tumors only.

Of articles that examined sites other than oropharynx or included oropharynx but did not perform a separate survival analysis, 10 demonstrated a survival advantage in patients with HPV-associated tumors,[35,108–116] 15 demonstrated no survival advantage,[60,116–130] and 2 studies indicated a negative correlation between HPV positivity and survival.[131,132] One study revealed a significant increase in overall survival (OS) but not disease-free survival (DFS) in HPV-associated tumors.[35] In studies that evaluated the effect of HPV positivity on survival in OPSCC, 17 studies revealed significantly improved survival[9,10,43,64,79,88,102,103,133–141] and six revealed no improvement in survival.[26,59,63,100,142–143] One study suggested improved OS but not improved DFS.[134] A 2007 meta-analysis by Ragin and Taioli[32] indicated that patients with high-risk HPV-associated HNSCC, independent of site of tumor, have an 18% reduced risk of dying and a 38% reduced risk of disease failure compared to patients with HPV-negative tumors. When broken down by head and neck subsite, this finding appeared to be limited statistically to oropharyngeal tumors.[32] HPV-associated OPSCC had a 28% reduced risk of death and a 49% reduced risk of disease failure compared to HPV-negative OPSCC. No differences in survival were observed between HPV-associated and HPV-negative non-OPSCC. This finding is consistent with the tropism for tonsillar crypt epithelium observed in patients with head and neck HPV infections.[65] Interestingly, the observed survival disparity between Caucasians and African-Americans with similar stage OPSCC appears to be due to higher rates of HPV-associated OPSCC in Caucasians.[141]

Several key articles firmly establish the improved survival of patients with HPV-associated OPSCC treated

with CRT. Fakhry et al.[102] demonstrated an improved response to both induction chemotherapy (IC) and CRT, improved 2-year OS with a 61% lower risk of death, and a 62% decreased risk of progression in 96 patients with advanced (stage III, IV) but surgically resectable HPV-associated OPSCC. Later that year, Worden at al.[138] showed similar findings of improved responses to IC and CRT, improved 4-year survival with an 19% reduced risk of any death, and a 23% reduced risk of disease-specific death in a similar population of 66 patients with advanced stage HPV-associated OPSCC. More recently, Sedaghat et al.[140] demonstrated dramatic improvements in local-regional control, recurrence-free survival, OS, and disease-specific survival in a small (n = 49) population of mixed stage (stages I–IV) HPV-associated OPSCC treated with CRT. At the 2009 ASCO conference, a subset analysis of RTOG 0129 was presented that studied the effects of HPV status on survival outcomes in a large cohort of 323 patients with advanced OPSCC that were treated with CRT.[9] Results demonstrated an improved OS and progression-free survival (PFS) as well as decreased local-regional failure with a 59% reduced risk of death and a 46% reduced risk of disease progression with a median follow-up of 4.4 years. Finally, recent data from a cohort of 111 OPSCC patients treated with either XRT or CRT demonstrated significantly improved OS and DFS in patients with HPV-associated OPSCC.[43]

It has been hypothesized that one of the major factors of improved prognosis in HPV-associated HNSCC is improved sensitivity to radiotherapy. Lindel at al.[142] demonstrated improved OS and DFS by univariate analysis and decreased local-regional failure by multivariate analysis in a cohort of mostly advanced OPSCC treated with radiotherapy alone. Interestingly, p53 immunoreactivity did not correlate to HPV positivity in this study,[142] whereas other studies found p53 to be overexpressed in HPV associated OPSCC.[66,80] Regardless, p53 tends to be wild-type in HPV-associated OPSCC,[36,37] suggesting sensitivity to radiotherapy may be mediated via a functional, nonmutated p53 protein, a protein status not commonly observed in carcinogen-induced HNSCC. This finding may also be true for cellular responses to genotoxic chemotherapeutic agents as well; treatment of cervical cancer cells with cisplatin leads to reduced expression of E6/E7 and induction of apoptosis via p53-dependent and independent mechanisms.[144] In both scenarios, response to therapy is likely linked at least in part to a functional, wild-type p53-inducing programmed cell death in the presence of genotoxic stress.

Improved prognosis with HPV-associated HNSCC is not limited to patients treated with nonsurgical therapy. In 2006, Licitra and colleagues[88] demonstrated improved 5-year OS with a 61% mortality reduction and decreased incidence of tumor recurrence or second primary tumors in 90 patients with mixed stage but mostly advanced HPV-associated OPSCC treated with primary surgical therapy with or without adjuvant radiotherapy. Hafkamp et al.[103] further showed improved 5-year OS, disease-specific survival, and DFS in a cohort of 81 patients with mixed-stage HPV-associated tonsillar carcinomas treated with variable combinations of surgery, radiotherapy, or chemotherapy. In the largest study evaluating survival after surgical therapy, Haughey et al.[10] showed an approximately 60% reduced risk of death in 174 of 204 patients with HPV-associated AJCC stage 3 or 4 OPSCC treated with transoral laser microsurgery for primary tumor resection. These data suggest that the substantially improved prognosis observed with HPV-associated OPSCC may be independent of primary treatment modality.

From a clinical standpoint, patients have a significant improvement in survival when treated with postoperative adjuvant therapy.[10,145] Previously, studies have indicated a survival benefit in patients who receive CRT postoperatively over those that receive radiation alone,[145] at the expense of increased adverse events. Recent evidence from a multicenter surgical study evaluating mostly HPV positive OPSCC suggests that any form of adjuvant therapy positively increases survival, and that in two comparison groups with same risk profiles and no difference in T-stage or p16 status, the addition of chemotherapy to postoperative radiotherapy did not improve the survival benefit seen with radiotherapy alone.[10] Increased radiosensitivity of HPV-associated OPSCC may obviate the needs for postoperative CRT, potentially reducing both acute and long-term adverse events.[146–148] Caution must be used when interpreting the surgical outcomes data due to the relatively small number of cohort sizes and taking into consideration institutional expertise, which may not be generalizable to all surgeons. Future institutional or multicenter trials are needed to help critically evaluate difference in outcomes between surgery with postoperative radiotherapy versus CRT in stage and other risk factor-matched HPV-associated OPSCC.

Consistent with the different molecular profiles associated with the development of carcinogen-associated HNSCC versus HPV-associated OPSCC, smoking and HPV presence appear to have individual effects on the development and prognosis of OPSCC. Hafkamp et al.[103] demonstrated that a nonsmoking status was a much stronger predictor of improved outcome than HPV positivity in tonsillar carcinomas, and that among a cohort of patients with HPV-associated tonsil cancer, nonsmokers do significantly better than smokers. Upon evaluating clinical parameters and molecular biomarkers as predictors on response in OPSCC, Kumar and colleagues[80] found that smoking status did not reach statistical significance for OS or disease-specific survival when accounting for p16 expression, used in this study as a surrogate marker of HPV-16 positivity. The RTOG 0129 study appears to help clarify the effects of smoking and HPV status on survival.[9] Although smoking status alone imparted a worse 2-year OS, smokers with HPV-negative tumors had the lowest survival. Remarkably, patients with HPV-associated OPSCC who smoke had a comparable prognosis to patients with HPV-negative tumors who do not smoke, suggesting that changes on the molecular level as a result of repeated carcinogen exposure may negate the molecular changes associated with HPV-positive HNSCC. Although global genomic instability is present in the tumors of patients

who smoke, an individual candidate protein would include p53, commonly mutated in smokers and rarely mutated in HPV-associated HNSCC.[73,75–77]

Although the molecular basis for the improved prognosis seen in HPV-associated OPSCC is speculated to be due at least in part to an intact p53 gene, the p16 protein has garnered much attention given its known role as a cyclin-dependent kinase inhibitor and its overexpression in HPV-associated OPSCC. Several studies have evaluated the prognostic implications of p16 positivity alone in OPSCC tumors. Reimers et al.[26] demonstrated p16 staining, and not HPV status, to be an independent prognostic factor for improved DFS in 96 patients with OPSCC. Lassen at al.[149] showed improved locoregional control with primary radiotherapy in a large cohort of p16-positive head and neck tumors from various sites. In addition to correlating HPV with survival, the RTOG 0129 study demonstrated that not all p16-positive tumors were also HPV positive, and that patients with tumors staining positive for p16 had a reduced risk of death or tumor progression of higher magnitude than that of HPV positivity.[9] Although some studies have shown no significant correlation between p16 positivity and surgically managed HNSCC,[88,150] Haughey et al.[10] demonstrated, similar to results from the RTOG 0129 study, that not all p16 tumors were also HPV positive, and that patients with tumors staining positive for p16 had a reduced risk of death much greater than that calculated from HPV positivity alone. Further, in a recent study with a cohort of 266 multisite HNSCC patients treated with variety of therapeutic modalities, p16 positivity independently correlated with survival in patients with oropharyngeal, but not nonoropharyngeal, tumors.[99] Whether the relationship between p16 and improved OPSCC survival is due primarily to HPV infection or tissue specific differences between the oropharynx and other subsites remains to be clarified. These data suggest that p16 may be a more predictive of biomarker of improved survival than HPV status.

Although most HPV-associated OPSCC appears to overexpress p16, there is a subset of roughly 10–20% patients with tumors that overexpress p16 that do not harbor detectable HPV. Although this may be an issue of simply not detecting the HPV in a subset of p16-positive tumors, this finding has been repeatedly shown in studies using both ISH[9,10,43,65,102,103] and highly sensitive PCR.[26,38,40,43,88] One key question is whether or not there is a survival difference between patients with HPV-negative and HPV-positive tumors that stain positive for p16. If patients with tumors that overexpress p16 and harbor HPV retain a survival advantage over those that have no detectable HPV, then a rationale for continued HPV specific testing exists. If there is no difference in survival, then for the purposes of determining prognosis, developing risk models, or determining criteria used to deintensify therapy, tumor specimen p16 status in combination with tumor morphology may be all that is necessary for risk stratification. Given the survival advantages observed in both HPV- and p16-positive OPSCC, reassessment of current staging criteria is likely to take place, incorporating one or both of these molecular criteria.[43] Given the significantly improved outcomes for patients with HPV-associated OPSCC with conventional therapies, there is a significant interest in investigating whether treatment can be deintensified to decrease treatment toxicities while still providing equivalent oncologic outcomes. This issue will need to be investigated in carefully designed multicenter clinical trials.

Although there is no doubt that HPV and p16 positivity confers a survival advantage in patients with OPSCC, assumptions about a causal role for HPV in cases of OPSCC have psychosocial implications. In counseling patients with newly diagnosed OPSCC and a negative smoking and alcohol abuse history, discussing the role of HPV can be distressing to both patients and loved ones due to the social stigma surrounding HPV exposure.[151] Persistent oral high-risk HPV infections in one spouse serve as a significant risk factor for oral high-risk HPV infections in the other partner,[151,152] and at least one case of synchronous HPV-associated OPSCC in a husband–wife couple with the same strain of HPV has been documented.[153] Prospective studies evaluating which form of sexual contact leads to high-risk HPV transmission are lacking and may be valuable in counseling patients on which sexual practices pose the greatest risk of transmission. A recent panel discussion held at the National Cancer Institute addressed several key questions regarding patient counseling in the setting of a newly diagnosed HPV-associated OPSCC. Recommendations included no change is sexual behavior for monogamous partners and use of barrier protection to prevent HPV transmission in new partners.[151]

As the head and neck cancer community speculates on the anticipated effects of the HPV vaccine Gardasil (Merck, Whitehouse Station, NJ), a clear understanding of the role of HPV in OPSCC is needed.[6,13] When given prior to exposure to high-risk HPV, Gardasil is a vaccine designed to initiate protective immunity against HPV types 6, 11, 16, and 18 via generation of a humoral immune response. Studies have demonstrated efficacy of this vaccine through 5 years.[154] The need for a booster beyond 5 years is unknown at this time. The vaccine is designed to prevent HPV infection in unexposed patients. The vaccine has no biologic role for treatment of existing HPV-associated OPSCC and is not currently under investigation for this purpose.[151] Conversely, Kenter and colleagues[155] have recently demonstrated clinical efficacy of a synthetic peptide vaccine against HPV E6/E7. Designed to induce $CD4^+$ and $CD8^+$ activity against established high-risk HPV infections, this therapy has been investigated in malignant and premalignant HPV-associated cervical lesions.[155,156] Evaluation of vaccines designed to either prevent HPV infection or induce an immune response against established HPV infections have not yet been evaluated in the head and neck.

As an aside to the biologic role of HPV in OPSCC, a deeper understanding of immune response to HPV will likely provide insight into OPSCC carcinogenesis. As opposed to carcinogen-induced HNSCC, HPV-associated OPSCC cells harbor nonself-viral antigens that have the potential to initiate an innate or adaptive immune

response. Several factors likely play a role in limiting innate responses. HPV infection and replication are integrated into the host squamous cell life cycle and shedding of virions takes place from cells already programmed to differentiate and die, leaving little chance for exposure of HPV to innate defense mechanisms.[28,157] Further, HPV E6 and E7 can inhibit natural killer expression of interferon-γ, further limiting initiation of a cytotoxic immune response.[158] As a consequence, there is little to no evidence of primary infection site inflammation clinically. Interestingly, evidence suggests that interferon treatment of epithelial cells may release E2 inhibition of E6 and E7 expression via clearance of episomal HPV DNA.[19] Likely as a consequence of persistent HPV infection over time, T-cell tolerance to HPV also develops.[157] Reversal of this tolerance to induce clearance of HPV-infected cells via cytotoxic T-cell activity has been the focus of many recent investigations.[159] Additionally, recent work in a murine model of HPV-associated HNSCC revealed loss of tumor response to cisplatin and radiotherapy in immunodeficient mice.[160] These data support the notion of complex interactions between the host tumor microenvironment and the malignant cells themselves, further complicated by the presence of HPV that evades the immune system while expressing oncogenes that may drive carcinogenesis. Despite the immune evasion mechanisms inherent to HPV, the majority of patients with HPV infections do not develop a malignancy, and evidence that elevated levels of tumor associated lymphocytes correlates with decreased nodal metastasis in HPV-associated OPSCC suggests increased immune surveillance in this subset of patients.[161] Although epidemiologic and molecular data suggest a beneficial response to radiotherapy and chemotherapy in patients with HPV-associated OPSCC, controversial issues include the effect of these treatment modalities on immune cell function in the tumor microenvironment given the potential increased immunogenicity of these cancers. Future studies evaluating the role of both the innate and adaptive immune systems in these HPV-associated cancers may help clarify these issues, specifically in the setting of how to best treat distant metastases of HPV-associated OPSCC.

CONCLUSIONS

Epidemiologic and molecular data document the recognition of a unique subset of HPV-associated OPSCC different from traditional carcinogen-induced HNSCC on the levels of risk factors, tumor development, response to therapy, and prognosis. Although the mechanisms of HPV-associated OPSCC tumorigenesis as well as how best to test for the presence and biologic activity of HPV in a standardized fashion is complex and remains a subject of debate, overwhelming evidence demonstrates that the majority of patients with HPV-associated OPSCC have an improved survival. Recognition of this improved response to therapy will likely ultimately lead to deintensifying adjuvant or primary antineoplastic therapy in these patients with the goal of reducing acute and long-term toxicities.

The benefit of further understanding HPV-associated OPSCC oncogenesis lies in how we can translate correlations between alterations on the molecular level and improved survival in HPV-associated OPSCC to other patients. The majority of patients with cancer of the upper aero-digestive tract have HPV negative HNSCC, likely related to carcinogen use. Although molecular differences between HPV-associated OPSCC and carcinogen-associated HNSCC have been revealed, a deeper understanding of which differences lead to improved outcomes in HPV-associated OPSCC may translate into therapeutic approaches aimed at improving the poor prognosis of patients with carcinogen-associated HNSCC.

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