MONOGRAPH

HEAD AND NECK SQUAMOUS CELL CANCER AND THE HUMAN PAPILLOMAVIRUS: SUMMARY OF A NATIONAL CANCER INSTITUTE STATE OF THE SCIENCE MEETING, NOVEMBER 9–10, 2008, WASHINGTON, D.C.

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For the purpose of clinical trials, head and neck cancers have largely been considered as a single disease entity. With the exception of nasopharyngeal cancers, important clinical distinctions between tumor subsites and natural history have often been ignored, given the commonalities of histopathology and responsiveness to treatment. Recent work suggests considerable

differences between some head and neck cancers, beyond that defined by tumor subsite and stage. The common "one size fits all" approach to treatment is neither optimal nor appropriate for all patient subgroups.

One such subgroup can be defined by the identification of human papillomavirus (HPV) DNA in the squamous cell head and neck tumor. HPV-associated cancers tend to occur more frequently in younger, male patients, and most frequently in the oropharynx, a tumor subsite associated with the presence of HPV DNA in up to 60% of cases. Although these tumors are often more poorly differentiated histologically, the patients appear to have a better prognosis after treatment than those whose head and neck cancers are not associated with HPV.

Recognition of this HPV association amounts to the identification of a new disease, a disease with a rapid increase in incidence, and one that poses important challenges for the oncologic community. These challenges are not limited to treatment, but also include early diagnosis, prevention, and public education. Lessons can be learned from our understanding of the importance of HPV in cervical cancer. The potential for sexual transmission of HPV, and therefore of head and neck cancer, and the impact of the recently available HPV vaccine have clear implications. Future study design and data analysis should acknowledge the unique natural history and prognosis of this patient subgroup, and incorporate HPV status into the next generation of clinical trials.

To that end, a National Cancer Institute–sponsored State of the Science meeting on Squamous Cell Head and Neck Cancer and the Human Papillomavirus was convened. On November 9–10, 2008, almost 80 investigators, largely drawn from the NCI Head and Neck Steering Committee and Task Forces, met in Washington, D.C. to focus on the epidemiology, natural history, and diagnosis of HPV-associated squamous cell head and neck cancers. The objectives of this meeting were to review the basic science, epidemiology, and natural history of HPV infection and HPV-associated squamous cell cancers. A review of completed and ongoing head and neck cancer clinical trials that have separately evaluated the HPV-positive subgroup as well as of the currently available diagnostic tools used to define HPV-positivity was planned. The statistical and design issues important in the development of future clinical trials based on HPV-status, and the public health implications of HPV-associated disease were explored.

This monograph summarizes the proceedings of that meeting.

EPIDEMIOLOGY OF HPV-POSITIVE HEAD AND NECK CANCER

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The International Agency for Research on Cancer has concluded that human papillomavirus type 16 (HPV16) is a cause of oropharyngeal cancer based upon a review of the epidemiologic evidence.[1] Critical epidemiologic associations expected for an HPV-caused cancer (eg, with sexual behavior and HPV exposure) have been reported in several case-control studies of oral cancer.[1–11] Moreover, there is increasing evidence that the risk factors and cofactors for HPV-positive and HPV-negative head and neck squamous cell cancer (HNSCC) are sufficiently distinct from one another to conclude, together with clinical and molecular distinctions outlined elsewhere, that HPV-positive head and neck cancer is a distinct disease entity.

Sexual Behavior. Natural history studies and case-control studies of cervical HPV infection and cancer have clearly established HPV as a sexually acquired infection. Lifetime number of sexual partners is the principal risk factor for exposure to HPV, and several case-control studies have reported elevated odds for oral cancer (ie, oral cavity and oropharyngeal cancer) among individuals with a high number of sexual partners.[3,4,8] Strong trends have been observed in particular between number of oral sex partners and odds of oropharyngeal cancer,[3,4] consistent with evidence for oral–genital contact as a principal means of acquiring oral HPV infection. In case–case comparisons, the sexual behaviors of HPV-positive cases were significantly different from those reported by HPV-

negative cases with regard to lifetime number of sexual partners, oral sex partners, and history of oral–anal contact.[3,12] Indeed, when stratified by HPV-tumor status, sexual behavior was associated with the risk of HPV-positive and not HPV-negative cancer.[3] Therefore, sexual behavior has recently been appreciated as a risk factor for head and neck cancer, but is restricted to the HPV-positive form of the disease.

HPV16 Exposure. The sexual behavioral associations noted earlier have been observed to attenuate after adjustment for exposure to HPV as measured by serology, indicating that sexual behavior is a surrogate marker for HPV exposure.[13] Although HPV16 seropositivity is strongly associated with head and neck cancer in numerous case-control studies (and on occasion HPV 35 and 33),[3,4,10] stratified analysis indicates strong associations (from ~4- to 180-fold) with oropharyngeal cancer and weak to null associations with cancers at other anatomic sites (eg, oral cavity and laryngeal cancers).[3,6,10,14] Consistent with these odds ratios is the 14-fold increase in risk for oropharyngeal cancer observed in a nested case control study in Scandinavia, which remains the only study to demonstrate that HPV16 exposure precedes development of disease.[2] By contrast, HPV serology does not appear to be associated with HPV-negative head and neck cancers,[3,14] arguing against a "hit-and-run" mechanism for HPV in these cancers.

Oral HPV Infection. HPV is an epithelium-specific infection that does not disseminate though the bloodstream, so a limitation of HPV serology is that it does not specify the anatomic site of HPV infection. Therefore, the elevated odds of oral cancer observed in association with oral HPV infection are considered more robust evidence for a direct relationship between infection and cancer. Numerous case-control studies have reported elevated odds (from ~2- to 200-fold) of oral and oropharyngeal cancer among individuals with a detectable oral HPV infection.[3,4,7,8,10,11] High-risk, but not low-risk, oral HPV infection is consistently associated with head and neck cancer. Observed associations strengthen considerably when analysis is restricted to high-risk versus low-risk HPV types, HPV16 detection versus all high-risk HPV types, and type-specific HPV16 detection versus multiplex detection. Similarly, associations strengthen when analysis is restricted to certain types of head and neck cancer, such that odds ratios for tonsillar cancer > oropharyngeal cancer > oral cancer > head and neck cancer. Oral HPV infection is not associated with HPV-negative oral cancers.[3,7] Taken together, the data on risk associated with sexual behavior, HPV exposure, and oral HPV detection indicate that sexually acquired oral HPV infection is the principal risk factor for this distinct form of head and neck cancer.

Alcohol and Tobacco Exposure. The majority of head and neck cancers worldwide remain attributable to tobacco and alcohol use, and whether these exposures interact with oral HPV infection to further increase the risk of cancer remains a major topic of debate. Although early research suggested a possible synergy between exposure to tobacco[8] or alcohol[7] and HPV, subsequent work has found no evidence of interaction.[3,6,13] In fact, 2 case-control studies have observed no evidence that tobacco or alcohol use affect risk for cancer among HPV-exposed individuals.[6,13] Complicating factors in these analyses, which may explain these inconsistencies, include the combined analysis of a variable mix of HPV-positive and HPV-negative head and neck cancers as well as imperfect measures of HPV exposure. Seroconversion is not absolute among HPV-infected individuals, and cross-sectional detection of oral HPV infection cannot adequately measure past exposure.

HPV-Positive versus HPV-Negative Tumors. As has been done for clarification of the clinical characteristics and prognostic outcomes for HPV-positive versus HPV-negative cancers, stratification by tumor HPV status will be an important approach in clarifying risk factors, in that associations may be in opposite directions and thus bias findings toward the null. Furthermore, it will be important in these analyses not to use surrogate markers for classification of tumor HPV status (such as HPV serology) because significant misclassification may result. Stratification by tumor HPV status had been used in a single case-control study to compare the risk factors for HPV-positive and HPV-negative head and neck cancer.[3] In this analysis, risk-factor profiles were remarkably distinct. Strong trends were observed between measures of lifetime sexual behavior and marijuana use for HPV-

positive cancers, whereas by contrast strong trends were observed between alcohol, tobacco, poor oral hygiene, and HPV-negative cancers. This study is the first to suggest that cofactors (ie, marijuana for HPV-positive and oral hygiene for HPV-negative), in addition to the principal exposure measures, may differ for HPV-positive and -negative head and neck cancers.

The distinctions in risk-factor profiles for HPV-positive and HPV-negative HNSCC may possibly extend to dietary factors. In a recently reported case-control study, HPV16 serostatus appeared to modify the association between fruit consumption and head and neck cancer.[9] Among HPV16 seronegative individuals, increasing tertiles of fruit intake were associated with decreased odds of HNSCC (as had been previously observed), whereas odds increased with increasing intake among HPV16-seropositive individuals. These trends appeared stronger in an analysis restricted to pharyngeal cancer cases. However, in a case–case comparison, the elevated odds of HPV-DNA positive versus DNA-negative HNSCC one would expect in association with increased fruit consumption were not observed, consistent with an alternate explanation of a differential effect of dietary fruit consumption on seroconversion among HPV exposed individuals.

Oral Cavity and Laryngeal Cancer. Although, in the literature, HPV DNA has been detected in a large proportion of oral cavity and laryngeal cancers, whether HPV is etiologic for these cancers remains unclear. Several studies have reported relatively weak (compared with those for oropharyngeal cancers) but significant associations between HPV seropositivity or oral HPV infection and oral cavity or laryngeal cancers in stratified analysis.[2,6] An international case-control study has estimated that HPV may play a role in approximately 3% of oral cavity cancers.[5] At this time, it is difficult to distinguish between a cause–effect relationship and the possible role of anatomic misclassification of the primary tumor site as the explanation for these findings. Given the apparent proclivity of HPV for transformation of the tonsillar crypt epithelium, ectopic tonsillar tissue in the lateral-posterior tongue or floor of mouth, estimated to occur in 0.4 per 100,000 individuals, may be an additional contributor to these findings.

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HPV AND THE GLOBAL BURDEN OF HEAD AND NECK CANCERS

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Head and neck cancer is the sixth most common cancer worldwide, with an estimated annual burden of 563,826 incident cases (including 274,850 oral cavity cancers, 159,363 laryngeal cancers, and 52,100 oropharyngeal cancers) and 301,408 deaths.[1] The presence of head and neck cancers varies geographically; regions of high incidence include Southeast Asia (particularly the Indian subcontinent where prevalence of tobacco chewing is high), parts of central and western Europe, and Australia.[2] By anatomic site of origin, oral cavity cancers are more common in developing countries, whereas oropharyngeal cancers are more common in developed countries.[2] In the United States, head and neck cancer is the eighth most common among men and 14th most common among women, with 47,560 incident cases (including 7,360 oropharynx cancers) and 11,260 deaths annually.[3]

Human papillomavirus (HPV) infection is an established cause of oropharyngeal cancers, including cancers of the base of tongue, lingual and palatine tonsil, and the pharynx.[4] Several studies have also reported detection of HPV infection in oral cavity and laryngeal cancers.[5] However, because epidemiologic and molecular evidence for an etiologic role in these cancers is weak, quantification of the prevalence of HPV-associated head and neck cancers has been restricted to oropharyngeal tumors. Despite the recognition that HPV is a strong risk factor for oropharyngeal cancers, there is wide variability in the reported proportion of cancers attributable to HPV infection, ranging from 12% to 63%. Based on a multicenter international case-control study, Herrero et al[6] reported that 18.3% of oropharyngeal cancers had evidence of detectable HPV DNA in tumors. Using these estimates, and restricting to subjects who also had evidence of antibodies to HPV early proteins—E6 and E7, Parkin and Bray[2] estimated that approximately 12% of oropharyngeal cancers worldwide are potentially attributable to HPV infection. In a systematic review of PCR-based worldwide published literature, Kreimer et al[5] reported that 35.6% of oropharyngeal cancers were HPV positive. In contrast, recent multicenter studies conducted in the United States indicate that a much higher proportion of oropharyngeal cancer (~63%) is potentially attributable to HPV infection.[7] Geographic differences in HPV exposures may, in part, contribute to this variability. For example, the systematic review by Kreimer et al[5] reported HPV-attributable proportions of 47% in North America, 46% in Asia, 36% in South/Central America, Australia, and Africa, and 26% in Europe. It is likely that the proportion of oropharyngeal cancers that are attributable to HPV infection in a particular region would be altered by the prevalence of smoking and alcohol use and patterns of sexual behaviors. It is widely accepted that an overwhelming majority (90% to 95%) of HPV-associated oropharyngeal cancers is attributable to HPV16,[5] the genotype that is responsible for approximately 50% of all cervical cancers worldwide and targeted in currently available prophylactic HPV vaccines.

Across the range of HPV attributable disease, current data suggest that between 6,000 to 33,000 oropharyngeal cancers worldwide and 800 to 4,600 cancers in the United States are caused by HPV infection (Table 1). The

Table 1. Estimated annual burden of HPV-associated oropharyngeal cancers in the United States and worldwide.

		Number of HPV-attributable cases	
	HPV-attributable proportions	United States: 7360 oropharynx cases annually	Worldwide: 52,100 oropharynx cases annually
International case-control study:			
Herrero et al[6]	18.3%	1325	9378
Parkin and Bray[2]	12.0%	883	6252
Systematic review:			
Kreimer et al[5]	35.6%	2620	18,548
Contemporary U.S. estimates:			
Fakhry et al[7]	63.0%	4637	32,823

Abbreviation: HPV, human papillomavirus.

variability notwithstanding, these estimates indicate a significant role for HPV as a cause of head and neck cancer. In fact, of all HPV-associated cancers—anus, cervix, oropharynx, penis, vagina, and vulva—the burden of HPV-associated oropharyngeal cancers is second only to cervical cancer.[2,8] In the United States, HPV-associated oropharyngeal cancers constitute a significant fraction of all noncervical HPV-associated cancers among women (26%) and a substantial fraction of all HPV-associated cancers among men (76%).[8] HPV-associated oropharyngeal cancers impose a significant economic problem in the United States, with annual estimated costs of approximately $151 million (assuming U.S. $33,000 per case) toward treatment and disease management.[9]

The import of HPV-associated oropharyngeal cancers may have increased substantially during recent calendar periods in the United States.[10,11] According to the U.S. National Cancer Institute's Surveillance, Epidemiology, and End Results data, the incidence of head and neck cancer sites that are potentially related to HPV infection (oropharyngeal cancers; constituting base of tongue, lingual and palatine tonsil, and pharynx) significantly increased between 1973 and 2004, with an annual increase of 0.8%.[10] Across the oropharyngeal cancer subsites, incidence of base of tongue cancers and tonsil cancers (lingual and palatine) increased significantly from 1973 to 2004 (annual increases of 1.27% and 0.60%, respectively), whereas incidence of pharyngeal cancers was stable.[10] In contrast, incidence of head and neck cancer anatomic sites that seem unrelated to HPV infection (oral cavity cancers) declined significantly during 1973–2004 at a rate of 1.85% per year.[10] The increasing incidence for oropharyngeal cancers was observed predominantly among white men (but not among women), at younger ages, and in cohorts born between 1925 and 1940.[10] Notably, there was an equalization of incidence rates for oral cavity and oropharyngeal cancers in the year 2004 (3.2 per 100,000 person-years). Hence, the proportion of all head and neck cancers that are oropharyngeal in origin has increased dramatically, from 18% in 1973 to 31% in 2004. Analogous data are emerging from other regions of the world. For example, in Sweden, incidence of tonsil cancer increased from 1.3 per 100,000 person-years in 1970 to 3.6 per 100,000 person-years in 2002.[12] This increase was evident among both men and women (2.6-fold and 3.5-fold increases, respectively).[12]

The declining incidence of oral cavity cancers may be explained by trends in tobacco and alcohol use during recent calendar periods in the United States.[13] However, the increasing incidence of oropharyngeal cancers among recent birth cohorts suggests that exposure to oral HPV infection and, as a consequence, the proportion of oropharyngeal cancer that is caused by HPV infection have increased significantly over time. Perhaps changes in sexual behaviors during the 1960s, including increasing practice of premarital sex and increasing average number of lifetime sex partners, have led to an increase in oral HPV exposure.[14] Consistent with this hypothesis, Hammarstedt et al[12] reported a 3-fold increase in the proportion of HPV DNA-positive tonsillar cancers from the 1970s to the 2000s in Sweden (23% during the 1970s and 68% during 2000s).

In summary, recent studies show that the worldwide burden of HPV-associated head and neck cancers is considerable, and has been increasing dramatically over the past 2 decades. The recognition of HPV as an etiologic factor for oropharyngeal cancers provides a unique opportunity for prevention, potentially through prophylactic HPV vaccination.[8]

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EPIDEMIOLOGY OF ORAL HPV INFECTION

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Oral human papillomavirus (HPV) infection is newly appreciated as an important cause of oropharyngeal cancer.[1] Despite this risk, the prevalence and risk factors for oral HPV infection in the general population have not been well evaluated.

There is no accepted standard for collection or processing of oral exfoliated cells to detect HPV infections related to head and neck cancer. Variation in DNA purification methods can affect HPV detection, given that polymerase chain reaction (PCR) inhibitors in oral rinse samples are now known to impair HPV detection if DNA is inadequately purified.[2] Suboptimal DNA purification of oral exfoliated cells may cause misclassification of HPV status. In addition, other factors may also affect oral HPV detection because it is unknown whether behaviors such as having just eaten, chewed gum, or brushed teeth might affect detection of HPV DNA in oral exfoliated cells.

The best estimates of oral HPV prevalence come from population-based studies, which report 5% to 10%.[3,4] The majority of oral HPV infections in these population-based studies were of low-risk subtypes. Studies of hospitalized patients without cancer (controls) have comparable or slightly higher estimates of oral HPV prevalence (5% to 18%). In these older hospital-based controls, approximately half of oral HPV infections detected are high risk (3% to 10% prevalence of oncogenic HPV types). Higher oral HPV prevalence has been reported in select populations, including 21% to 37% among women with genital HPV infection, and 20% to 37% among those infected with human immunodeficiency virus (HIV).

The prevalence of oral HPV infection among young adults has not been well evaluated. A recent study of 1235 children and adolescents reported an overall HPV prevalence of 1.9% in youth, with increasing HPV prevalence as children aged from 0.8% in 1- to 4-year-olds to 3.3% in 16- to 20-year-olds.[5] Although genital HPV prevalence usually peaks around age of maximal sexual risk-taking (20s) and then decreases with age, initial studies suggest that oral HPV prevalence may continue to increase with increasing age among adults.[6,7] The pattern of increasing oral HPV prevalence with increasing adult age is unusual for a sexually transmitted infection.

Long-term natural history studies of oral HPV infection have not yet been reported. Although the natural history of cervical HPV infection is well established, it is unclear whether the course of HPV in the oral cavity and oropharynx, which have a distinct immunologic environment, will be similar. One study that collected both oral and cervical samples from the same women reported comparable clearance rates for prevalent oral and cervical HPV infections after 6 months, but this work was limited by short follow-up and small number of HIV-negative participants.[7] The rate of newly detected oral HPV infection was lower than that observed for cervical HPV infection in that report. Another prospective study tested pregnant women and their partners for oral HPV every 6 months for 3 years. In this study, 10% of those with no oral HPV infection at baseline had an incident HPV infection detected within 2 years.[8] Type-specific oral HPV clearance was not reported in this study, but among those who were positive for at least 1 type of oral HPV at baseline, none of the men and only 5% of the women became HPV-negative (ie, cleared all their HPV infections) within 2 years. This suggests the natural history of oral HPV infection might differ from that known in the cervix. Long-term natural history studies of type-specific oral HPV infection are needed to evaluate the incidence and time to clearance of oral HPV infection as well as potential risk factors.

Only a few studies have evaluated factors associated with oral HPV infection. Oral HPV infection is believed to be sexually acquired and oral HPV prevalence is significantly associated with number of recent oral sexual partners in initial studies.[6,9] As sexual behaviors are correlated, prospective studies are needed to ascertain which behaviors are involved in oral HPV transmission and the level of risk associated with these behaviors. Increased risk of persistent high-risk oral HPV infection was associated with persistent oral HPV infection of spouses in another study,[8] supporting sexual transmission of the virus.

Oral HPV prevalence has also been associated with male gender,[6] increasing age,[6,7] and current tobacco use.[5,7,9] Further research is needed to evaluate whether the observed associations may be explained by residual confounding arising from differences in sexual behavior or how these factors may affect oral HPV natural history.

HPV infection at each anatomic site is localized and concordance of oral and genital HPV infection is low.[7] However, women with genital HPV infection do have higher odds of oral HPV infection[10,11] than women without concomitant genital HPV infection. This could be explained by infection at multiple sites from the same infected partner (example: exposure of a penile HPV infection to the oral cavity during oral sex and to the cervix during vaginal sex with the same partner). There is currently no evidence to support autoinoculation of an HPV infection from one site to another site on the body, although this possibility cannot be excluded.

Individuals with human immunodeficiency infection (HIV) are at increased risk for oral HPV infection. Oral HPV prevalence is higher in HIV-positive than that in HIV-negative individuals[6,7] and increases with severity of HIV-related immunosuppression.[6,7] In addition, highly active antiretroviral therapy (HAART) use does not appear to decrease oral HPV persistence.[7] Increased prevalence of oral HPV in HIV-infected individuals could be explained by increased oral HPV infection when exposed, longer time to clearance, and/ or increased reexpression of latent infections in immunosuppressed individuals.

In summary, initial research suggests that oral HPV infection is sexually transmitted and is common among adolescents (~3%) and adults (5% to 10%). Further research on the natural history, risk factors, and vaccine efficacy for oral HPV infection is needed.

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LABORATORY DIAGNOSIS OF HPV-ASSOCIATED HEAD AND NECK SQUAMOUS CELL CARCINOMA. I. OVERVIEW OF METHODS FOR HPV DETECTION

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Methods for the detection of human papillomavirus (HPV) DNA have evolved over time since HPV was first postulated as a cause of cervical cancer in the 1980s. As the sensitivity, specificity, and spectrum of HPV types detected with these methods increased, so too did estimates of the proportion of cervical cancers caused by HPV. These increased from 30% to 60% to >99% with the use of current, broad-spectrum polyermase chain reaction (PCR) methods, thus highlighting the importance of the quality of HPV exposure measurements on ultimate clinical and scientific inference.[1] HPV detection methods changed to accommodate the aims of investigation, from confirming the association with cancer in case-control studies, to screening applications, natural history studies, and studies of HPV prophylactic vaccine efficacy. Currently, a variety of methods are available for the detection of HPV DNA, each with particular strengths and limitations (Table 2). The aims of the investigation should determine the appropriate choice of method used.

Several other factors, in addition to HPV detection methodology, may affect HPV detection results.[2] The type and quality of the sample used for HPV testing is of paramount importance. HPV infects the basal cells of stratified squamous epithelium of the oral cavity and the anogenital tract of both men and women. There is no blood-borne phase of HPV infection, and thus epithelial samples are required for definitive diagnosis. Two types of epithelial samples are generally available: exfoliated epithelial cells or tissue biopsy samples. For oral HPV detection, exfoliated cells are collected by direct swab sampling or by collection of saliva or oral rinse specimens. In general, the most sensitive method for detection of HPV infection in the oral cavity is the oral rinse, likely because of the larger epithelial area sampled.[3] By this method, the HPV detected is present in sloughed epithelial cells. It is possible that infections restricted to the tonsillar crypt epithelium or the basal cell layer may not be detectable.

Table 2. Methods for the detection of HPV in oral specimens.

Method	Assay	Exposure measure	Utility
HPV serology	HPV L1 antibody detection via ELISA or bead array	Cumulative marker of combined exposure (past or present) in the anogenital and/or oral epithelium	Not appropriate for clinical diagnostic or prognostic applications
			Some utility in case-control studies to evaluate etiologic heterogeneity of HPV+ vs. HPV− head and neck cancers
	HPV E6 or E7 antibody via ELISA or bead array	Marker of invasive cancer	Diagnostic and prognostic utility in defining HPV-associated tumor
p16 immunohistochemistry	p16 protein via immunohisto-chemical staining	Marker of high-risk HPV E7 expression	Diagnostic and prognostic utility by defining HPV-associated tumor as clonally HPV-positive
In situ DNA hybridization	Type-specific HPV DNA probe hybridization (usually HPV16 and HPV18)	Cell-localized HPV DNA	Diagnostic and prognostic utility by defining HPV-associated tumor as clonally HPV-positive
HPV quantitation	Type-specific *Taq*Man PCR	HPV viral load	Diagnostic and prognostic utility by defining HPV-associated tumor as clonally HPV-positive
HPV genotyping	Consensus PCR Roche Linear Array INNO-LiPA HPV genotyping test GP5+/6+ with reverse probe hybridization	Multiple HPV-type infections, including high- and low-risk HPV	Primarily for HPV natural history studies

Abbreviation: HPV, human papillomavirus; L1, late protein; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

Oral rinse samples are most useful for studying the natural history of oral HPV infection. Although similar exfoliated samples are preferred for screening applications in the cervix, the utility of oral rinse specimens for early detection of HPV-associated oral cancers has yet to be demonstrated.

Equally important to HPV detection and sampling methodology is the method used for processing and storage of both exfoliated oral epithelial cells and tissue biopsies or surgical tissue samples. For the former, use of SCOPE mouthwash is recommended because it is a validated collection medium that is highly acceptable to the sample donor.[4] A variety of methods are available for the preparation of exfoliated epithelial cells. In general, DNA can be extracted directly from the oral rinse specimen following protein digestion. A protocol based on the PureGene purification kit has been validated.[4] It is important to note that characteristics unique to the oral sample require modifications to the manufacturer's instruction to result in optimal DNA yield and purity. Alternative commercial DNA purification kits are likely to be equally useful, although optimization using appropriate controls and parallel comparison with validated protocols is critical for comparability across studies. If tissue samples are to be collected and stored for HPV testing, the preferred storage condition is snap freezing the specimen in liquid nitrogen with permanent storage at -80°C. If the sample is to be fixed, use of standard 10% buffered formalin will generally preserve the HPV DNA, although degradation is common and highly variable and should influence considerably the choice of HPV detection assays (see Figure 1). Processing of tissue specimens generally requires more rigorous purification, usually involving longer digestion times and organic extraction to maximize both DNA yield and purity.

Serum antibodies are the only systemic marker of HPV infection and may have limited utility. Antibodies to early (E6/E7) viral oncoproteins are often detectable in cancer patients, and may therefore be useful as a specific prognostic marker of HPV-associated tumors.[5] Serum antibodies to late proteins (L1) are insensitive markers of cumulative exposure, and have little diagnostic value. When used in epidemiologic studies of head and neck cancer risk, they can serve as a reasonable marker of HPV exposure; however, extreme caution should be

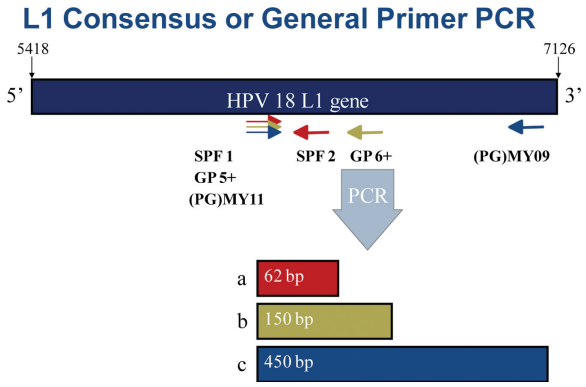

FIGURE 1. Overview of 3 commonly used consensus PCR systems for broad spectrum HPV genotyping: (**A**) SPF10 primers are indicated in red. This system is based on a pool of 10 primers and generates a nearly 62-basepair (bp) fragment and is therefore particularly favored for amplification of highly degraded DNA samples, such as those extracted from formalin-fixed, paraffin-embedded tissue sections. (**B**) GP5+/6+ primers are indicated in gold. This system is based on a single upstream and downstream primer pair and generates a nearly 150-bp product. Type-specific sequence variation in the primer binding region is accommodated by inclusion of degenerate or permissive nucleotides. This system may underestimate type-specific prevalence when multiple genotype infections are common. (**C**) (PG)MY09/11 primers are indicated in blue. The PGMY09/11 system is based on a pool of 18 primers and amplifies an approximately 450-bp product. The MY09/11 version is based on degenerate primers (see GP5+/6+) and has similar limitations in detection of multiple infections. The long product length may preclude amplification from highly degraded DNA samples. A comparison of genotype spectrum detected by these systems is found in Gravitt et al.[2] [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

used when interpreting the results because serologic measures of L1 antibody represent cumulative exposure to all sites (including the common anogenital infections in men and women) and therefore lack specificity to the oral cavity.

As noted previously, the ultimate choice of HPV detection assay among the many available systems is dependent on how the HPV information is to be used. The study of the natural history of HPV infection in the oral cavity requires noninvasive sampling and an agnostic approach to the HPV genotype predicted to be present. At present, target amplification (eg, PCR) methods are most frequently used for this purpose, as reviewed in Gravitt et al.[2] These methods are based on amplification of HPV using type-specific or broad spectrum primer sets. Type-specific PCR methods target a single HPV genotype per test. Thus, if study aims are targeted to specific genotypes only (eg, tracking vaccine-associated HPV infection),

these methods are perfectly suitable. General, or consensus, primer PCR methods rely on pools of primers that are generally targeted to the conserved L1 open reading frame. Three commonly used and validated consensus PCR systems are shown in Figure 1. Using these methods, several HPV types can be amplified in a single test. Therefore, if assessment of the presence of one or more HPV types is the aim of the investigation, use of these primer systems is most efficient.

Although each of the general or consensus primer systems represents a reasonable method for detection of any high-risk HPV, subtle differences may affect results. The efficiency of amplification for any HPV type by consensus primer sets is a function of PCR product size, the homology of the consensus primers to each individual HPV type, and the extent of nonspecific background amplification. All 3 methods target a similar spectrum of HPV genotypes.[2] However, the type-specific sensitivity may differ. In general, degenerate primer systems (eg, GP5+/6+) will have greater heterogeneity in type-specific sensitivity when compared with consensus primer pools (eg, SPF10 and PGMY09/11). This may have a greater impact in the presence of multiple-type HPV infection. HPV types in molar excess with better primer matches in the primer-binding regions may out-compete types with more mismatches, resulting in false-negative results. Such an impact will depend on the prevalence of multiple infections in the population. Initial studies indicate less of a problem in oral than cervical samples in immunocompetent populations. The impact of the different PCR product length is critical when testing DNA extracted from paraffin tissues, in that fixed tissues are highly susceptible to DNA degradation. In this case, the smaller the product sizes, the better. The SPF10 system is preferred for the detection of HPV from fixed tissues.

Many methods are available for the detection of PCR projects, and have been reviewed elsewhere.[6] In general, a second type-specific probe hybridization is required to achieve maximum sensitivity; classification of HPV status based solely on the presence or absence of the expected fragment size on an ethidium bromide gel is not only insensitive, but in the case of consensus primer PCR, relatively nonspecific and should be avoided. The hybridization detection platform for HPV genotyping varies, but includes dot-blot hybridization, reverse line probe hybridization, or higher density, automated platforms such as chip- or bead-based arrays. Because each probe used must be quality controlled and validated with each new lot for optimal performance, use of commercialized assays with standardized protocols is recommended.

If the aim of the testing is for diagnostic or prognostic purposes (eg, defining HPV-associated tumors), the best methods will localize the genome of high-risk HPV to the tumor cell nuclei and/or demonstrate expression of viral oncogenes (eg, E6/E7 mRNA).[7] Methods available for these purposes include in situ hybridization,[8] p16 immunohistochemistry,[8] and PCR quantitation of viral DNA or RNA in microdissected tumor tissue.[9] Although the latter may be the "gold standard" for etiological use, it has limited clinical utility. Studies that can be performed on paraffin-embedded tissues would be preferred. In situ methods are generally type-specific, and therefore require hybridization with multiple probes to get consensus genotyping information. However, since the majority (~90% to 95%) of head and neck cancers are attributed to HPV16, and to a lesser extent to HPV16-related types (eg, types 31, 33, 35), the limitations thus posed are not as severe as in the case of cervical cancer. Strong staining of tumor cells with p16 antibody is a marker of HR-HPV E7 expression (resulting from loss of pRB-mediated negative regulation),[10] and is therefore a good consensus marker of HR-HPV infection. Use of these methods that demonstrate specificity of the signal to tumor cells allows one to distinguish between etiologically relevant HPV detection (clonal presence in all tumor cells) and passenger virus or contamination (low copy detection in only a very few cells).[7]

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LABORATORY DIAGNOSIS OF HPV-ASSOCIATED HEAD AND NECK SQUAMOUS CELL CARCINOMA. II. IN SITU HYBRIDIZATION

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In recent years, the human papillomavirus (HPV) has emerged as an important driving force behind the escalating incidence of oropharyngeal cancer, and its detection in head and neck squamous cell carcinoma (HNSCC) now serves as a highly relevant biomarker.[1] As a biomarker, HPV detection serves a far more comprehensive role than mere prognostication. HPV detection is emerging as a valid method of discerning the presence and progress of disease encompassing all aspects of patient care from early cancer detection, to more accurate tumor staging (eg, localizing site of tumor origin), to the selection of those patients most likely to benefit from specific therapies, to posttreatment tumor surveillance. There has seldom been a greater need for establishing a strategy of biomarker detection that is accurate and faultlessly reproducible from one diagnostic laboratory to the next.

The Diagnostic Language of HPV-Related Head and Neck Cancers. The importance of standardization is not limited to detection methodologies. For HPV-positive head and neck squamous cell cancers, diagnostic language is in great need of a uniform vocabulary that draws attention to its relationship with HPV and yet avoids confusion with more aggressive non-HPV-related cancers.[2] Two microscopic features of HPV-related cancers of the oropharynx have contributed to the difficulty in achieving diagnostic clarity.

First, HPV-related HNSCC is customarily misperceived as a poorly differentiated carcinoma based on the immature appearance of the tumor cells: they are made up of malignant cells that have a high nuclear to cytoplasmic ratio and exhibit little if any keratinization. Although this microscopic appearance departs from that of the nonneoplastic squamous epithelium that lines the oral cavity, it does closely emulate the appearance of the reticulated epithelium—the specialized epithelium lining the tonsillar crypts from which HPV-related cancers probably arise. In other words, HPV-related oropharyngeal cancers are in fact highly differentiated, not poorly differentiated (as so widely assumed).

Second, HPV-related HNSCCs are often aptly described as "basaloid" based on the lobular growth of cells with dense hyperchromatic nuclei and a high nuclear to cytoplasmic ratio. As a diagnostic modifier, however, the term "basaloid" is confusing because it invites an erroneous connection with basaloid squamous cell carcinoma—a subtype of HNSCC notorious for its aggressive clinical behavior. A recent study has shown that the "basaloid" subtype is, in fact, composed of a mixed group of HPV-positive and HPV-negative cancers that widely diverge with respect to clinical behavior.[2] Specifically, the presence of HPV is a favorable prognostic factor that can be used to identify a subtype of basaloid squamous cell carcinomas that does not show the highly aggressive behavior usually associated with this variant. Given this divergence in clinical behavior, pathologists should develop nomenclature that avoids confusing our clinical colleagues. Until consensus panels put forward a classification scheme for oropharyngeal carcinomas that underscores their relationship with HPV, while avoiding confusion with the aggressive basaloid variant, it is our practice to: (1) classify these tumors as nonkeratinizing squamous cell carcinomas, (2) suspend the use of the descriptors such as "poorly differentiated" and

"basaloid," and (3) routinely report on the HPV status of all HNSCC arising in the oropharynx.

Methods of HPV Detection. Although there is a growing consensus for routine HPV testing of all oropharyngeal carcinomas, the optimal method for HPV detection may prove a more contentious matter. A variety of methods are in current use, ranging from consensus and type-specific polymerase chain reaction (PCR) methods, real-time PCR assays to quantify viral load, type-specific DNA in situ hybridization (ISH), detection of serum antibodies directed against HPV epitopes, and immunohistochemical detection of surrogate biomarkers (eg, p16 protein). Standardization of HPV detection in the clinical arena must begin with selection of the best detection platform for universal application. This selection process will be influenced by a variety of concerns relating to sensitivity, specificity, reproducibility, cost, and feasibility.

PCR-Based Detection versus In Situ Hybridization. The preferential use of ISH methods over PCR-based methods is supported both by biological and practical considerations. The reported large variation of HPV prevalence in squamous cell carcinoma of the oral cavity (0% to 100%!) is largely a reflection of the inability of nonquantitative PCR methods to discern virus that is biologically meaningful from virus that is biologically irrelevant. In contrast, punctuate hybridization signals within the nuclei of tumor cells is a pattern of staining seen only following HPV DNA integration into the host genome, and is thus more closely linked with relevant viral infections.[3] The presence of punctuate nuclear hybridization signal abrogates the need for additional sophisticated testing to confirm transcriptionally active HPV (eg, measurement of E6/E7 mRNA expression). Importantly, the improved specificity of HPV detection by ISH does not come at the expense of sensitivity. The introduction of various signal amplification steps has significantly improved the sensitivity of this technique, even to the point of viral detection down to one viral copy per cell.[3]

Compared with PCR-based methods, ISH is a more practical tool for detecting HPV. The development of nonfluorescent chromogens now allows display of DNA hybridization using conventional light microscope. Adaptation of ISH to formalin-fixed and paraffin-embedded tissues has made this technique compatible with standard tissue-processing procedures and amenable to retrospective analysis of archival tissue blocks, whereas most PCR-based methods are optimized in fresh-frozen samples. With these technical adaptations, ISH is a feasible and cost-effective test for most diagnostic laboratories that routinely process formalin-fixed and paraffin-embedded tissue blocks.

P16 Immunohistochemistry versus In Situ Hybridization. In HPV-positive oropharyngeal carcinomas, transcription of the viral oncoprotein E7 is known to functionally inactivate the retinoblastoma (Rb) gene product, causing a perturbation of other key components of the Rb pathway. As one example, functional inactivation of Rb by E7 is known to induce an up-regulation of p16 expression, reaching levels that can be readily detected by routine immunohistochemistry. Accordingly, p16 immunohistochemistry is often advocated as a reliable surrogate marker of HPV-induced neoplasia of oropharynx.[4]

Direct comparison of p16 immunohistochemical staining and HPV-16 ISH for large numbers of HNSCCs reveals a discrepancy rate of about 25%. The discrepancies consistently involve cancers that are negative by HPV16 ISH but p16 positive by immunohistochemistry. In a subset of discrepant cases (about 45%), high p16 expression is attributed to the presence of some other (non-16) HPV type, as confirmed by wide spectrum ISH. The remaining discrepancies likely reflect the imperfection of p16 as a surrogate marker: p16 is often overexpressed in basaloid carcinomas that are not related to HPV infection (eg, breast, lung, and skin). Using E6/E7 mRNA levels as conclusive evidence of HPV involvement, positive p16 immunostaining of HNSCCs is 100% sensitive but only 79% specific.[5] Clearly, an HPV detection strategy is needed that combines the sensitivity of p16 immunohistochemistry with the specificity of HPV-16 ISH.

A Standardized Algorithm for Reliable Detection of HPV in Oropharyngeal Carcinoma. The limitations of any single detection assay may be offset using algorithms that combine the strengths of complementary assays. We use a detection strategy that combines HPV ISH with p16 immunohistochemistry. Use of p16 immunostaining as a surrogate marker is enhanced by its ease of

interpretation. Difficulties in establishing standard thresholds for positive staining that have plagued most other immunohistochemical assays (eg, p53) are minimized by the binomial (rather than graded) distribution of staining: depending on HPV status, p16 staining is either absent or diffusely positive (ie, on or off). Given a sensitivity that approaches 100%, p16 immunostaining is a good first-line assay for eliminating HPV-negative cases from any additional analysis. HPV-16 ISH can be run concurrently with p16 immunostaining or as a second-line assay following a positive p16 result. Given a specificity approaching 100%, a positive HPV-16 ISH reduces the numbers of false-positive cases by p16 staining alone. A p16-positive/HPV-16-negative result singles out a subset of tumors that qualify for rigorous analysis for other (ie, non-16) oncogenic HPV types. For this third-line assay, we use a consensus ISH probe that detects an extended panel of HPV types (ie, 15 different types). Others have advocated PCR-based methods for the detection of transcriptionally active virus. Whatever the method for this third-line assay, the upfront use of p16 immunostaining and HPV-16 ISH accurately establishes the HPV status of the vast majority of oropharyngeal cancers. Although some cases may require a more extended analysis, this algorithm minimizes expenditure of resources by preselecting those cases.

P16 immunohistochemistry and HPV ISH are standardized techniques that are easily applied to formalin-fixed and paraffin-embedded tissues. Test turnaround time is relatively fast—no more than 2 days for diagnostic labs that offer high throughput services. As automated ISH technologies are brought on-line, turnaround time will be further shortened and standardization across various diagnostic laboratories will be enhanced.

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WHAT OTHER BIOMARKERS SHOULD BE CONSIDERED?

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Human papillomavirus (HPV) is nearly ubiquitously present in humans, but only a small fraction of infected individuals develop cancer. Besides cervical cancer, the most widely accepted HPV-associated malignancy, HPV is implicated in the pathogenesis of a subset of oropharyngeal cancers. Molecular markers provide a potential tool to identify the at-risk subpopulation and the presence of early-stage cancers. These molecular markers must therefore be able to distinguish ordinary infections per se from infections that contribute to the development of cancer. In addition, biomarkers that distinguish HPV-positive versus HPV-negative head and neck cancers are needed. The detection of HPV DNA in tumors per se does not prove causal association. This review focuses on these molecular markers and their role in the diagnosis and management of oropharyngeal cancer.

According to the molecular progression model for tobacco-associated head and neck cancers,[1] abrogation of p53 and retinoblastoma (pRb) tumor-suppressor pathways occurs through mutational inactivation of p53 gene and down-regulation of p16 protein, respectively. The molecular events in HPV-induced carcinogenesis lead to functional abrogation of p53 and pRb pathways that is mediated through the expression of the main viral

oncoproteins E6 and E7, respectively. Disruption of the expression of the main viral transcription/replication factor E2, following viral DNA integration, leads to unconstrained expression of the E6 and E7 oncogenes. The E6 and E7 genes of oncogenic HPVs encode oncoproteins that bind and degrade p53 and retinoblastoma (Rb) tumor suppressors, respectively. Most HPV-associated carcinomas harbor wild-type *p53* and *Rb* tumor-suppressor genes. Thus, the tumor-suppressor pathways are intact but dormant in these cells because of the continuous expression of *E6* and *E7* oncogenes. HPV-associated cancers are associated with low pRb protein levels. pRb is a negative regulator of p16 protein at the transcriptional level. Therefore, low pRb levels lead to subsequent p16 up-regulation. Overexpression of p16 protein has been repeatedly found in HPV-associated cancers. The p16 protein functions as a tumor suppressor mainly by binding to the cyclin D1 CDK4/CDK6 complex, preventing phosphorylation of the Rb protein.

We sought to determine the prevalence of biologically relevant human papillomavirus (HPV) in oropharyngeal squamous cell carcinoma (OSCC). We hypothesized that p16 overexpression in OSCC defines HPV-induced tumors with favorable prognosis. We studied a cohort of 107 oropharyngeal squamous cell cancers for HPV-16 DNA viral load by real-time polymerase chain reaction (PCR). In addition, we constructed a tissue microarray composed of these tumors and studied expression of p53, pRb, and p16 proteins using a quantitative in situ method of protein analysis (AQUA). Our results delineated 3 biologically and clinically distinct classes of oropharyngeal squamous cell cancers based on HPV DNA determination and p16 expression status: 1 class of HPV negative/p16 nonexpressing (class I), 1 class of HPV positive/p16 nonexpressing (class II), and 1 class HPV positive/p16 expressing (class III) oropharyngeal tumors. Overall survival in class III was 79% compared with the other 2 classes (20% and 18%, $p = .0095$). Disease-free survival for the class III was 75% versus 15% and 13% ($p = .0025$). The 5-year local recurrence was 14% in class III versus 45% and 74% ($p = .03$). Only patients in class III had significantly lower p53 and pRb expression ($p = .017$ and .001, respectively). Multivariable survival analysis confirmed the prognostic value of the 3-class model. We were able to show that only the HPV positive/p16 expressing tumors fit the cervical carcinogenesis model and they are the ones associated with favorable prognosis.[2]

P16 has also been identified as a useful biomarker for HPV^+ head and neck cancer by other investigators. Strati et al[3] generated a mouse model for HPV-associated head and neck squamous cell carcinoma (HNSCC). HNSCC arising in these HPV-16 transgenic mice shared similar molecular and histopathological characteristics with human HPV-positive HNSCC that distinguish the latter from HPV-negative HNSCC, such as overexpression of p16 and nonkeratinizing histology. The authors also identified minichromosome maintenance protein 7 (MCM7) as a potentially useful biomarker for HPV-positive head and neck cancer.

Smeets et al[4] sought to find a detection algorithm for a biologically and clinically meaningful HPV infection. The authors considered HPV E6 oncogene expression in frozen biopsies as a standard for a meaningful HPV infection and they evaluated the value of the following assays on formalin-fixed paraffin embedded (FFPE) tumor specimens and sera of 48 HNSCC patients: HPV DNA general primer (GP)5+/6+ PCR, viral load analysis, HPV16 DNA fluorescent in situ hybridization (FISH) detection, HPV16 E6 mRNA reverse transcription (RT-PCR), p16 immunostaining, and on corresponding serum samples detection of antibodies against the HPV16 proteins L1, E6, and E7. The most suitable algorithm with 100% sensitivity and specificity appeared to be p16 immunostaining followed by GP5+/6+ PCR on the p16-positive cases. Taken together, p16 protein status can be used as a surrogate marker for a biologically and clinically meaningful HPV infection in FFPE oropharyngeal tumor specimens.

Several investigators used cDNA microarray technology as a tool to identify differences in biomarker expression between HPV^+ and HPV^- cancers. Slebos et al[5] analyzed 36 head and neck squamous cell cancers using Affymetrix Human 133U Plus 2.0 GeneChip and for HPV by PCR and real-time PCR. One of the most significant differentially expressed genes was cyclin-dependent kinase inhibitor 2A (*CDKN2A*), which encodes the p16 tumor-suppressor protein. Genes overexpressed in HPV^+ samples included cell cycle regulators (p16, p18, and CDC7) and transcription factors (TAF7L, RFC4, RPA2, and TFDP2). Pyeon et al[6] performed genome-wide expression profiling of 84 HNSCCs, cervical cancers, and site-matched normal

epithelial samples. HPV status and genotype were determined by hybridization to 70-mer oligonucleotide microarrays containing probes for all 37 known mucosotropic HPV genotypes. HPV+ HNSCCs and cervical cancers were characterized by overexpression of a larger subset of cell cycle regulators than those observed in HPV− HNSCC. The authors noted that the majority of the hallmark differences between HPV+ head and neck cancer and HPV− head and neck cancer, including testis-specific gene expression that are normally expressed only in meiotic cells, were a direct consequence of the viral E6 and E7 oncogene expression.

We sought to identify biomarkers that distinguish our class III oropharyngeal cancers from the other 2 classes.[2] We hypothesized that since HPV16+/p16-expressing tumors represent a distinct molecular and clinical entity, they should harbor distinct protein expression profiles. Our target biomarkers included proteins with well-described roles in cell cycle regulation, angiogenesis, and metastasis. We used AQUA on an oropharyngeal cancer tissue microarray, which allows quantitation and subcellular localization. We found that the expressions of epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and β-catenin were significantly different in HPV16+/p16+ tumors.[7] Using the 3-class model, there was also a significant difference in p16 ($p < .001$), p53 ($p = .026$), and Rb ($p = .001$) expression between groups. For β-catenin there was a significant difference by ANOVA ($p = .009$), with post hoc analysis demonstrating this to be ascribed to class III tumors having elevated expression compared with class II tumors ($p = .001$). For EGFR and VEGF, there were significant differences between groups ($p = .009$ and $p = .028$, respectively). For p14 and Erk2, there was a trend toward difference between groups, although this did not reach statistical significance ($p = .054$ and $p = .074$).

We then sought to determine whether overexpression of EGFR, VEGF, and β-catenin in $HPV16^+$ HNSCC is a direct consequence of viral E6 and E7 protein expression. We used the $HPV16^+$ oropharyngeal cancer cell lines 147T and 090, the HPV-negative cell line 40T, and the cervical cell lines SiHa (bearing integrated HPV16) and HeLa (bearing integrated HPV18) to measure VEGF, EGFR, as well as the cytoplasmic and nuclear β-catenin levels before and after E6/E7 gene silencing. To repress E6 and E7 we used retrovirus-mediated delivery of short hairpin RNAs targeting HPV16 E6 and E7 oncogenes in head and neck cell lines and the E2 repression system in HeLa. Introduction of the bovine papillomavirus (BPV) E2 gene into HeLa cells represses viral oncogene expression, resulting in activation of the p53 and Rb pathways and cellular growth arrest. Infection of 147T and 090 cells with retroviral constructs resulted in severalfold inhibition of viral E6/E7 mRNA, in restoration of p53 and pRB protein expression and substantial apoptosis.[8] Repression of HPV E6 and E7 genes induced a significant reduction in nuclear β-catenin levels. β-Catenin plays a dual role in carcinogenesis. It binds to the cytoplasmic domain of type I cadherins and functions as a component of cadherin–catenin adhesion system. β-Catenin is also the nuclear effector of Wnt signaling pathway. Wnt signaling determines the abundance of nuclear β-catenin. The accumulation of cytoplasmic (signaling) β-catenin leads to its nuclear localization, where it binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and induces expression of target genes. We showed that the principal HPV oncoproteins (E6 and E7) are involved in β-catenin nuclear accumulation and repression of E6/E7 gene expression leads to down-regulation of nuclear β-catenin. Therefore, nuclear accumulation of β-catenin is a direct consequence of E6 and E7 expression. The possibility that nuclear β-catenin is a biomarker of HPV-positive oropharyngeal lesions likely to progress to cancer should be explored.

In summary, the ordered expression of viral gene products can lead to overexpression of multiple molecular proteins or biomarkers. These novel biomarkers may allow the monitoring of essential molecular events in histological or cytological specimens and are likely to improve the detection of lesions that have a high risk of progression in both primary screening and triage settings.

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HPV AS A THERAPEUTIC TARGET

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Human papillomavirus (HPV) infection of cervical epithelium is linked to the generation of cervical cancer. Although most women infected with HPV clear their lesions, the long latency period from infection to resolution indicates that HPV evolved immune escape mechanisms. Dendritic cells (which are targeted by vaccination procedures) incubated with HPV virus-like particles induce an HPV-specific immune response. Langerhans cells (LCs), which are located at the sites of primary infection, do not induce a response, implicating the targeting of LC as an immune escape mechanism used by HPV. LC incubated with HPV virus-like particles up-regulate the phosphoinositide 3-kinase (PI3-K) pathway and down-regulate mitogen-activated protein kinase (MAPK) pathways. With the inhibition of PI3-K and incubation with HPV virus-like particles, LC initiate a potent HPV-specific response. PI3-K activation in LC defines a novel escape mechanism used by HPV, and PI3-K inhibition may serve as an effective clinical target to enhance HPV immunity.[1]

As an alternative to targeting the PI3-K targeting, we also explored other potential LC activating pathways. Demonstrating that Toll-like receptor 7 (TLR7) and TLR8 are expressed on human LC, we hypothesized that imidazoquinolines would activate LC infected with HPV16, leading to the induction of a HPV16-specific cell-mediated immune response. Surprisingly, both phenotypic and functional hallmarks of activation are not observed when LCs are infected with HPV16 virus-like particles (VLPs) and treated with imiquimod (TLR7 agonist). However, we found that LCs are activated by 3M-002 (TLR8 agonist) and resiquimod (TLR8/7 agonist). LCs infected with HPV16 VLP and subsequently treated with 3M-002 or resiquimod highly up-regulate surface activation markers, secrete pro-inflammatory cytokines and chemokines, induce CCL21-directed migration, and initiate an HPV16-specific $CD8^+$ T-cell response. These data strongly indicate that 3M-002 and resiquimod are promising therapeutics for treatment of HPV infections and HPV-induced cervical lesions.[2]

To induce HPV-specific T-cell responses through the use of therapeutic vaccines, we explored the option that heterologous prime-boost regimens offer potential advantages over homologous vaccine administration, including enhanced immune responses and lack of vector neutralization by host antibodies. Therefore, HPV vaccines based on Venezuelan equine encephalitis virus replicon particles (VRPs) and recombinant vesicular stomatitis virus (VSV) vectors, both expressing mutated E7/E6 fusion proteins from HPV16 or HPV18, were tested in homologous or heterologous prime-boost regimens in mice to assess antitumor immunity and in Rhesus macaques to assess levels of immunogenicity in primates. Both VRP/VRP and VSV/VSV immunization elicited strong antigen-specific T-cell responses in mice as assessed by interferon (IFN)-γ ELISPOT, although responses after VRP/VSV heterologous vaccination were significantly higher. Antitumor immunity was assessed by prophylactic and therapeutic vaccination in mice against HPV16-transformed tumors. Full protection from tumor challenge was observed after immunization with VRP/

VRP, VSV/VSV, and VRP/VSV regimens. Therapeutic immunization of tumor-bearing mice showed 75% to 90% rejection after treatment with VRP/VRP or VSV/VSV and 80% to 100% rejection with VRP/VSV regimens. Macaques were primed with 3 inoculations of VRP at 0, 4, and 16 weeks, or with 2 inoculations of VSV at 0 and 8 weeks. Following each priming series, T-cell responses measured by IFN-γ ELISPOT to E6 and E7 peptides were low, but detectable. At 21 weeks following the priming series, macaques were immunized with the heterologous vaccine. VRP boosting in VSV-primed macaques returned T-cell responses to postprime levels. However, VSV boosting in VRP-primed macaques dramatically induced IFN-γ responses that were at least 10-fold greater than postpriming responses. In conclusion, the strong in vivo antitumor responses in mice and the robust T-cell responses in nonhuman primates after heterologous VRP prime/VSV boost immunization provide strong justification for further development of these vectors as therapeutic vaccines for HPV-associated disease.

Despite the fact that HPV specific T-cells can potentially be induced by the above-mentioned strategies of PI3-K inhibition, TLR 8 activation or heterologous prime-boost vaccination strategies, induced HPV specific T-cells might still have difficulty entering the HPV induced lesions/tumors. It is known that lymphotoxin-beta receptor (LTBR) signaling plays an important role in the formation of lymphoid structures, where T-cells are more effectively primed. LIGHT, a ligand for LTBR and herpes virus entry mediator (HVEM), restores lymphoid structures in $LTB^{-/-}$ mice, establishes lymphoid-like tissues inside tumor sites via its interactions with LTBR on stromal cells and recruits naïve T-cells into the tumor. LIGHT coordinately induces activation and expansion of incoming T-cells through HVEM, thereby generating stronger anti-tumor immunity. We hypothesized that intratumoral therapy with recombinant adenovirus carrying LIGHT (Ad-LIGHT) induces tumor-specific T-cell responses in vivo, which can eradicate well-established HPV-induced tumors in mice. To test therapeutic efficacy of Ad-LIGHT in an HPV16-induced mouse tumor model, B6 mice were challenged with C3 tumors and tumor growth was monitored. On day 14 and day 17 after tumor challenge, Ad-LIGHT or Ad-Control particles were injected intratumorally at 10^{10} virus particles per mouse. Tumors, lymph nodes, and spleens were harvested on day 27 to measure antigen-specific T-cell responses. Additional mice were maintained to monitor tumor growth and survival. Analysis of the spleens and lymph nodes with HPV16 E7(49–57) tetramers revealed that although none of the Ad-Control treated mice had detectable E7(49–57)-recognizing T-cells, approximately 1% of the $CD8^{+}$ cells from lymph nodes and spleens of Ad-LIGHT–treated mice were directed against E7(49–57) and secreted IFN-γ. Consistently, tumor growth correlated inversely with the number of functional tumor-specific T-cells. Our data show that Ad-LIGHT therapy can induce functional tumor-specific T-cells within the tumor tissue and this correlates inversely with tumor growth.

In conclusion, our combined data set shows that HPV may serve as a therapeutic target because there are opportunities to reverse a major T-cell immune escape mechanism of HPV that involves the targeting by the virus of human LC. This reversal involves blocking the PI3-K pathway or the activation of the TLR8 pathway in LC. In addition HPV specific T-cells can be very significantly induced by heterologous prime/boost strategies involving 2 different vector systems and HPV specific T-cells can be lead efficiently into HPV induced lesions by the forced expression of LIGHT.

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PROPHYLACTIC VACCINES FOR CERVICAL CANCER

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Two prophylactic human papillomavirus (HPV) vaccines have received regulatory approval and are commercially available in many countries. These vaccines have the potential to substantially reduce HPV-related morbidity and mortality. HPV2 is the bivalent HPV16/18 vaccine (Cervarix; GlaxoSmithKline Biologicals, Rixensart, Belgium), and HPV4 is the quadrivalent HPV6/11/16/18 vaccine (Gardasil; Merck, Whitehouse Station, NJ). The major components of both vaccines are noninfectious virus-like particles (VLPs), which are synthesized to resemble the outer capsid (shell) of the virus. Each VLP is composed of multiple copies of a single viral protein (L1). Eukaryotic cells are used to transcribe and translate the HPV L1 gene into L1 proteins that upon contact with each other self-assemble into VLPs. Whether L1 VLPs are manufactured in eukaryotic cells or are from live virus, they are highly immunogenic and capable of stimulating production of type-specific neutralizing antibodies that protect the host from reinfection with the same HPV type.[1] In addition to VLPs, the vaccines also contain an adjuvant, which is a compound that enhances the immune response to specific antigens. The adjuvant in the HPV4 vaccine is aluminum hydroxyphosphate sulfate (alum), and in the HPV2 vaccine it is aluminum hydroxide, 50 g 3-O-deacylated-4-monophosphoryl lipid A (AS04). Both vaccines require 3 intramuscular injections, with recommended schedules of day 1, month 1 (HPV2) or month 2 (HPV4), and month 6. Neither of the vaccines is therapeutic[2,3] and thus neither is expected to alter the course of established vaccine-type HPV infections, lesions, or cancers.

Randomized controlled trials[2,4–7] have demonstrated (with remarkable consistency) that these HPV VLP vaccines achieve high levels of prophylactic efficacy across geographically diverse female populations. In phase III trials of young women who were negative for vaccine-type HPV antibodies and DNA at baseline, the HPV4 vaccine prevented 95% of HPV16/18-related precancerous lesions of the uterine cervix,[2], 96% of HPV6/11/16/18-related genital warts, and 91% of HPV6/11/16/18-related precancerous lesions of the vagina or the vulva,[6], and the HPV2 vaccine prevented 90% of HPV16/18-related precancerous lesions of the uterine cervix.[7] These findings are clinically important because HPV-16 and HPV-18 cause about 70% of cervical cancers worldwide[8] and HPV-6 and HPV-11 cause at least 70% of genital warts.[9] Immunogenicity analyses show that over 99% of vaccinees seroconvert and that 3 doses of vaccine stimulate levels of antibodies to vaccine-type HPVs that are higher than that reported for natural infections.[4,5] An antigen challenge study showed that young women who received a booster dose of HPV4 vaccine 4.5 years after completing the 3-dose regimen, developed antibody titers for all 4 vaccine-type HPVs that were higher than or equivalent to titers observed 1 month after completing the initial series.[10] These data are significant because they indicate that the vaccine induces B-cell memory, a hallmark of vaccines that provide long-lasting immunity. A vaccine that provides durable protection is much more beneficial than one that requires additional boosting years after the initial series is completed.

In the efficacy trials, both vaccines were found to be generally well-tolerated, with the most common vaccine-related side effects being pain and erythema at the site of injection. Notably, the range and intensity of side effects were not more common among those with prior exposure to vaccine-type HPVs, and did not increase with each subsequent dose.[2,6,7] In addition to safety data collected in the clinical trials, public health agencies throughout the world are monitoring population-level safety as HPV immunization programs become established in their countries. For example, the Centers for Disease Control (CDC; http://www.cdc.gov/vaccines/recs/acip/slides-oct08.htm#hpv) recently analyzed safety data based on the administration of millions of HPV4 vaccine doses in the U.S. general population. These analyses also showed a high level of vaccine safety and moreover, did not support anecdotal concerns that HPV vaccination was associated with an increased risk of Guillain-Barré Syndrome, transverse myelitis, or death. Although results from clinical trials and population-level safety analyses are very reassuring, the CDC and other vaccine surveillance groups will continue to monitor general population data to determine whether HPV vaccination is associated with extremely rare or delayed adverse outcomes. Although the HPV2 vaccine has not yet been approved for use in the United States, data from a population-level

vaccine program in the United Kingdom indicate that the safety profiles of HPV2 and HPV4 are similar. Both vaccines are contraindicated for individuals with hypersensitivity, including those with severe allergic reactions to active and inactive ingredients included in the vaccine. Neither vaccine is currently recommended for pregnant women because there are insufficient safety data for this population.

Although the vaccine efficacy trials did not enroll girls <15 years of age, immunogenicity and safety studies that included girls and boys, 9 to 15 years of age, showed that over 99% seroconverted for all vaccine-types of HPV. The antibody titers for these children were as high as or higher than observed in the immunogenicity studies of older adolescent females and women.[11,12] Safety profiles were also similar for children and young women. Thus, recommendations for targeted vaccination of approximately 9- to 13-year-old girls were based on data from these child-to-young adult bridging studies, as well as the knowledge that HPVs are acquired primarily through sexual contact (which on average begins around age 16), and the observation that HPV vaccination is most beneficial when administered to those without vaccine-type HPV infections. The vaccine is approved for females 9 to 26 years of age in many countries, including the United States. Based on limited safety and immunogenicity data, some countries approved vaccine for females through 45 years of age and for boys (9 to 15 years of age).

In summary, the HPV2 and HPV4 vaccines are generally safe and extremely effective in preventing HPV-16 and HPV-18 infections and HPV16/18-related precancerous cervical lesions. In clinical trials, HPV4 vaccine was also shown to prevent HPV6/11/16/18-related precancerous vulvar or vaginal lesions, and genital warts. Administration of vaccine before sexual debut most likely provides maximal benefit and currently, 10- to 13-year-old females are the focus of HPV immunizations programs in many countries. Although there are limited follow-up data on vaccinated cohorts, demonstration of durable protection throughout the course of the trials, and of immune memory with antigen challenge 4.5 years after the completion of the initial vaccine series, supports longer-term protection. Only time will tell if the protection is sufficiently durable to eliminate the need for routine boosting years after the initial vaccine series is completed. Several additional questions concerning the use of these vaccines in boys, older adults, and populations with a high burden of HIV are being addressed in ongoing trials. Other issues related to implementation of immunization programs, feasibility of 2 versus 3 doses of vaccine, herd immunity, and cost are being evaluated in demonstration projects and mathematical models. Whether prophylactic administration of HPV vaccine will prevent oropharyngeal HPV-16 and HPV-18 infections and the associated cancers remains to be determined but there is no reason to suspect that it will not.

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BURDEN OF HPV INFECTION AND DISEASE IN MALES: OPPORTUNITY FOR PREVENTION

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There is increasing interest in understanding the burden of human papillomavirus (HPV) infection and disease among men. In earlier work, much of this interest focused on the role of men in the transmission of HPV to women, and its contribution to the propagation of cervical cancer. Over the past several years there has been rapid development and now recognition that HPV causes disease in men.[1,2] In addition, there is now evidence that the currently licensed HPV vaccine for females confers protection against HPV 6, 11, 16, 18 infection and external genital lesions (EGLs) in males ages 16 to 26 years.[3] With vaccination, we have a strong prevention candidate for men, (pending regulatory approval). However, we know little of the underlying natural history of infection and progression to disease in men: such as the diversity of HPV types that cause EGLs, proportion of infection at a variety of anatomic sites that progress to cancer, the rate of progression, and differences in the epidemiology across the lifespan. In addition, we know little of the biology of HPV infections related to EGLs in men, such as viral load and integration status of different HPV types and types and grades of EGLs. This natural history information is necessary to estimate the population effectiveness of deploying the quadrivalent vaccine to men, to inform future generation vaccine development (expansion of HPV types included in vaccine), and to inform development of improved, and novel strategies of infection and disease prevention in men, interventions that may ultimately impact disease burden in men as well as women.

HPV Infection–Related Disease in Men. HPV infection causes cancerous and noncancerous lesions in men. Several cancers of the anogenital tract and upper aerodigestive tract, and their precursor lesions in men are now known to be caused by infection with the sexually transmitted HPV.[1] Worldwide approximately 85% of anal cancer cases are attributable to this infection. Approximately 50% of cancers of the penis, 33% to 72% of oropharyngeal cancers, and 10% of cancers of the larynx are attributable to HPV infection.[1] There is a growing body of literature focused on anal precancerous lesions in high risk male populations. In contrast, relatively little information has been published focused on HPV-related pre-neoplastic lesions of male external genitalia, oropharynx, and other anatomic sites among predominantly heterosexual men. No studies to date have been conducted assessing the natural history of HPV related precancerous genital lesion development in men. In addition, although HPV-related cancers in men occur at a median age of 60 years and older, few male HPV natural history studies have included older men for HPV evaluation, limiting our understanding of HPV over the life span and in the years preceding onset of cancer.

At genital sites, HPV causes 2 categories of external genital lesions (EGL): condyloma (genital warts) and penile intraepithelial neoplasia (PIN). Genital warts are a common condition reaching peak incidence in men ages 20 to 29 years. HPV types 6/11 are the most commonly detected in genital warts, although 20% to 50% appear to have coinfection with oncogenic HPV types.[4] Unlike the United Kingdom, where data are available to estimate genital wart incidence, there are no comparable data systems in most other countries, including the United States. Therefore, outside of the United Kingdom, the incidence of genital warts is unknown. Although in most cases genital warts are a benign condition, the diagnosis and treatment of these lesions is associated with psychosocial distress and anxiety for the male as well as high medical expenditures for the health provider system. This is attributed in large part to the inadequate response and genital wart recurrence after apparent clearance. In the United States, a common treatment is to prescribe self-treatment with Imiquimod, which has a treatment efficacy of 40% in circumcised men and 62% in uncircumcised men.

PIN lesions are thought to be precancerous lesions from which penile cancer arises. Although rare, penile cancer is associated with a high morbidity and mortality. There is large variation in the incidence of penile cancer, with low rates observed in the United States (~1/100,000) compared with Brazil, which has among the highest rates worldwide (~5/100,000). Using 2 different cancer registry data sets from the United States (SEER and NAACCR), we have shown that Hispanics have a significantly higher incidence of penile cancer compared with other racial/ethnic groups.[5] No

studies have estimated the prevalence of PIN, or examined progression of HPV infection to PIN, PIN incidence, or the factors associated with PIN development. Few studies have examined PIN HPV type distribution,[6–8] with most studies only testing for HPV 16 and 18. One study found a high proportion of HPV 16 and 18 coinfection with HPV types 5 and 8, cutaneous carcinogenic viruses, suggesting multiple viral pathways to disease. This finding was corroborated by others who examined HPV type distribution in penile carcinoma tumor specimens. Data from Stoler and colleagues suggest that PIN may be underidentified, or misidentified as genital warts on visual inspection in clinical practice,[6] demonstrating the need in research studies to obtain biopsy specimens so that histological criteria are utilized in defining lesions and lesion material is available to identify HPV type(s) present (causal infections). This misclassification of PIN as genital warts upon visual inspection may underlie the relatively poor genital wart treatment efficacy observed in the clinic. No study to date has defined the rate at which HPV infection progresses to EGLs, the proportion of infections that progress, nor the specific type, and combination of types within the lesion responsible for EGLs. Because PIN and genital warts are reservoirs of infection for transmission, these lesions have significance for the disease status of the male as well as his female sexual partner.

HPV Infections in External Male Genitalia. Until recently, in the literature there has been tremendous variability in the methods used to assess male HPV status, resulting in poor comparability across studies and populations. Over the past several years, our group developed, and validated, methodologies to establish the reproducibility of genital HPV status assessment in men.[9] In general, genital HPV infection prevalence among healthy men appears to be as high as or higher than what has been observed among women. Based on our systematic review of the literature, genital HPV infection has been detected in up to 73% of healthy men.[10] In recent reports from our cross-sectional study of U.S. men, 51.2% were positive for at least one known oncogenic or nononcogenic HPV type and an additional 14.3% were positive for an unclassified HPV infection.[11] Among asymptomatic heterosexual men, the penile shaft, coronal sulcus/glans penis (including prepuce in uncircumcised men), and scrotum are the sites that contribute to >95% of genital HPV infection detected.[9] Study results from our ongoing Human Papillomavirus Infection in Men (HIM) study found HPV DNA in 50.5% in men. The proportion of low-risk types was 38.5%, and unclassified infections were found in an additional 14.7%,[12] similar to our previous results among U.S. men.[11] In subsequent analyses in which we sequenced amplicons that did not hybridize with any of the 37 HPV types on the linear array system, we observed presence of cutaneous HPV sequences. In specimens obtained from the surface of genital warts and lesions, we observe nearly 7% "unclassified" infections, suggesting that cutaneous HPV types are present on the penile skin, but may have a lower rate of progression to disease compared with mucosal HPV types.

Few studies have determined the correlates of genital HPV infections in men.[10] In these studies, consistent positive associations between measures of sexual history, including lifetime and recent number of sexual partners and sexual frequency, and HPV detection have been observed in the literature. Conversely, circumcision is consistently (4 of 6 studies) associated with reduced detection of HPV infection in men.[10,13] In the HIM study, HPV prevalence was significantly lower among circumcised men.[12] Less consistently (2 of 6 studies), condom use has been associated with reduced risk of HPV detection in men.[10] Most studies find no association between age and HPV prevalence in men.[10] In the HIM study, we also found no relation between age and HPV prevalence.[13]

Prospective Natural History Studies of Genital HPV Infection. Small prospective studies report rates of HPV acquisition and clearance in sexually active men. However, the small sample sizes and short and inconsistent follow-up periods have resulted in imprecise estimates of HPV incidence, duration, and antibody response in men.[10] In our recent study of U.S. men,[14] the probability of acquiring an HPV infection among heterosexual men ages 18 to 44 years was 29% per year, an estimate similar to that reported for young males attending university,[15] and females of a similar age range.[16] Unlike what has been observed among women, we did not detect a clear age pattern in rates of HPV acquisition in men.[14] In the HIM study, the

probability of acquiring an HPV infection among heterosexual men ages 18 to 70 years was 39% per year. The incidence of a new HPV infection was 5.2, 1.2, 6.0, and 2.4 per 1000-person months for HPV types 6, 11, 16, and 18, respectively, whereas the 12-month cumulative risk of acquiring any of these 4 vaccine types was 13%. As observed in our smaller U.S. study, the overall rate of acquiring any, oncogenic, and nononcogenic new HPV infections did not vary by age group (18–30, 31–44, and 45–70 years). The lack of an association with age suggests that the relatively constant HPV prevalence observed in cross-sectional studies may be attributable to acquisition of new infections.

HPV Antibody Response in Men. Most information regarding antibody development in response to HPV infection is derived from studies of women. Collectively, results of these studies indicate that antibody responses to HPV capsids are of low titer, slow to develop, and are only detectable in roughly 50% of women with HPV DNA detected in the genital tract by polymerase chain reaction (PCR). In women there is delayed seroconversion (12–18 months) and lower seroconversion among those with transient compared with persistent infection. Few studies have evaluated HPV antibody status in men, with most focusing on a single evaluation of HPV 16 sero-status. In our recent review of the literature,[10] 14 studies of HPV seroprevalence met our inclusion criteria, 11 of which evaluated HPV 16 seroprevalence. HPV 16 seropositivity was higher in STI clinic populations (19% to 48%) compared with 7.9% in males participating in the National Health and Nutrition Examination Surveys (NHANES) from 1991 to 1994.[17] Eight of 9 studies that compared seroprevalence in men and women reported a higher seroprevalence in women than in men. For example, among women participating in NHANES, 17.9% were HPV 16 seropositive.[17] Very few studies evaluated HPV 6 and 11 seroprevalence, and only one study evaluated HPV 18 seroprevalence. HPV-6 or 11 seroprevalence ranged from 26% to 41%. The single estimate of HPV 18 seroprevalence was 19%. A recent population-based study of HPV 6, 11, 16, and 18 seroprevalence reported peak seropositivity among men 40 to 49 years of age for types 6 and 11 of 15.4% and 9.1%, respectively, and among men 50 to 59 years of age for types 16 and 18 of 14.3% and 8.2%, respectively.[18] In general, HPV seroprevalence in males was associated with increasing age and sexual behavior. In our recently completed cross-sectional study of men in 2 U.S. cities, the combined seroprevalence to HPV 6/11, 16, or 18 was 21% among men ages 18 to 40 years.

Host immunity is believed to play a central role in the control of HPV infections, as evidenced by the high rates of infection and HPV-associated disease in immunosuppressed populations, such as HIV infected individuals. The precise role of humoral immunity in immune protection is unclear. Studies in women failed to show that preexisting type specific capsid antibody protects against subsequent infection with the same type. However, the design of those studies makes it difficult to distinguish reinfection from reactivation, and the studies were not powered to address the relationship between antibody titer and protection. Animal studies and vaccine trials in humans clearly show that capsid antibody can confer protection against HPV infection. In addition, the duration of vaccine mediated immunity appears to last >5 years, but it is unknown whether immunity will be lifelong.

CONCLUSIONS

HPV infection is common in men and is readily transmitted, influencing disease rates in both males and females. Now that HPV vaccine efficacy has been demonstrated in males, the cost-effectiveness of vaccinating males under different scenarios needs to be evaluated. The efficacy of the quadrivalent HPV vaccine to reduce infection and lesions caused by HPV at a several different anatomic sites continues to be tested among men internationally. Vaccination of males may become inevitable if and when vaccination of females fails to control disease because of suboptimal adherence to vaccine recommendations. From a disease transmission perspective, female-only vaccination may work well for controlling cervical cancer should we achieve broad vaccine dissemination. However, both the realities of not achieving broad vaccine dissemination among females and the interest in preventing male disease caused by HPV may force us to consider other strategies such as vaccinating males.

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TUMOR HPV STATUS AND SURVIVAL

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The current literature supports the conclusion that tumor human papillomavirus (HPV) status is an important and independent predictor of overall and disease-specific survival for head and neck squamous cell carcinomas.

The potential prognostic significance of HPV in head and neck cancer was originally suggested in single-institutional case series, the majority of which reported tumor HPV status to be a favorable biomarker for survival outcomes, particularly for oropharyngeal cancers. In these original reports, patients with HPV-positive tumors were estimated to have a 50% to 80% reduction in risk of disease-failure when compared with the HPV-negative patient.[1–4] It may be appropriate to be skeptical of such retrospective analyses because of the significant heterogeneity in patient populations with regard to sample size, methods for tumor HPV classification, tumor stage, tumor treatment, and variable inclusion of other prognostic factors in the analysis. However, the consistent findings reported from study to study despite this heterogeneity testify to the strength of the effect.

In a recent meta-analysis of these case series,[5] patients with HPV-positive head and neck squamous cell carcinomas had an 18% (hazard ratio [HR], 0.85; 95% confidence interval [CI], 0.7–1.0) reduction in risk of death and a 38% (HR, 0.62; 95% CI, 0.5–0.8) reduction in risk of disease failure when compared with the HPV-negative patient. When stratified by anatomic site of the primary, the survival benefit was restricted to HPV-positive oropharyngeal cancers. Patients with HPV-positive oropharyngeal cancers were estimated to have a 28% (HR, 0.72; 95% CI, 0.5–1.0) reduced risk of death and a 49% (HR, 0.51; 95% CI, 0.4–0.7) reduced risk of disease failure when compared with patients with HPV-negative oropharyngeal cancers. This meta-analysis had some limitations, as the estimates were derived from unadjusted hazards

and were not based on individual patient data. Additionally, no attempt was made to classify studies based upon method used for tumor HPV classification, categorization likely resulting in false-positive classification that would bias results toward the null. However, the inclusion of patients with recurrent or metastatic disease in the survival analysis may have biased results away from the null, by worsening survival outcomes preferentially in the HPV-negative group. Nevertheless, the meta-analysis is a useful summary of the existing literature from retrospective, case series.

Because of the initial reports noted earlier, the Eastern Cooperative Oncology Group (ECOG) incorporated an analysis of the effect of tumor HPV status on survival outcomes in a phase II trial of investigational therapy in patients with oropharyngeal and laryngeal cancers, ECOG 2399.[6] In this trial, patients were treated with paclitaxel and carboplatin induction followed by radiation concurrently administered with weekly radiosensitizing paclitaxel. Tumor HPV status was determined via a combination of HPV in situ hybridization and PCR, and 40% of all cancers and 63% of oropharyngeal cancers were found to be positive. After a median survival of 39 months, a patient with HPV-positive tumors had an improved overall survival and after adjustment for age, tumor stage and ECOG performance status had a 73% (HR, 0.27; 95% CI, 0.10–0.75) reduction in risk of progression and 64% (HR, 0.36; 95% CI, 0.15–0.85) reduction in risk of death when compared with the HPV-negative patient.[6] This was the first study to demonstrate tumor HPV status to be a strong, independent and favorable prognostic biomarker in the context of a prospective analysis in a uniformly staged and treated patient population. Retrospective analyses of prospectively acquired data are ongoing to determine whether similar survival differences can be observed in the context of large, randomized controlled clinical trials.

The survival benefit for the HPV-positive patient reported in ECOG 2399 was observed in the context of aggressive, multimodality therapy. It is important to note, however, that at this time it is unclear to what extent the survival benefit for the HPV-positive patient depends on therapeutic choices. The magnitude of the survival difference observed in the ECOG trial has similarly been observed in studies in which oropharyngeal cancer patients were treated with surgery with or without adjuvant radiation[7] or radiation with or without surgery.[8] In most of these studies, the 5-year overall survival for the HPV-positive patient is approximately 80% to 85% and for the HPV-negative patient between 30% and 35%. In fact, the data, in total, suggest that the survival benefit may be observed independent of the specific therapy administered, as long as it is within the current standard of care. Therefore, some patients with HPV-positive tumors may be unnecessarily exposed to treatments (induction chemotherapy followed by concurrent chemoradiation), which significantly increase morbidity compared with radiation therapy alone.

In addition to observational studies and clinical trials, the survival benefit observed for the HPV-positive patient may also be apparent at the population level, according to a recent analysis of over 47,000 incident cases of oral cancer reported to the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute.[9] In the United States during the period from 1973 through 2003, the incidence rate for cancers at sites etiologically related to HPV infection significantly increased, whereas significant declines in incidence were observed for oral cancers not etiologically related to HPV. In concert with this increase, from 1973 to 2003, significant improvements in absolute, 2-year overall survival were observed for patients treated with radiation therapy with local or regionally-advanced HPV-related cancers, but not for patients with HPV-unrelated cancers, respectively. This finding is consistent with the interpretation that recent improvements in the survival for patients with head and neck squamous cell carcinoma may in part be attributable to a shift in the underlying etiology of the disease and its inherent responsiveness to therapy.

The underlying biological reasons for the improved survival for the HPV-positive patient are not entirely clear, but appear to be multifactorial: (1) two prospective clinical trials have observed HPV-positive tumors to have a significantly improved response to chemotherapy when compared with HPV-negative tumors[6,10]; (2) patients with HPV-positive tumors also appear to have a lower risk of second primary cancers[7,11,12]; (3) tumor HPV status inversely correlates with several poor prognostic biomarkers, such as a history of smoking, high EGFR expression[10,13] inactivating p53 mutations,[14,15] and nuclear survivin expression.[15] It is important to

note, however, that some of these factors are so tightly correlated (eg, p53 mutation and HPV-negative tumors) that it is difficult to discern whether or not each remains independently associated with survival after accounting for the other. For instance, one study has suggested that smoking was not a significant prognostic factor after accounting for HPV status,[10] whereas another found HPV status had a minimal effect on survival outcomes among smokers, but smoking status had important impact among HPV-positives. HPV-positive nonsmokers have best outcome.[11] Similarly, tumor HPV status was suggested to account for the survival benefit observed among patients without p53-mutant tumors.[7] Correlative studies within large clinical trials conducted by the cooperative groups will be required to gain further insights into the molecular underpinnings of the survival difference, and will be important to the future potential of molecularly targeted therapies specific to the HPV-positive and HPV-negative patient.

In summary, at this time data support the conclusion that tumor HPV status is an important prognostic biomarker for head and neck cancers, particularly oropharyngeal cancer. Whether HPV status is associated with survival among the small proportion of nonoropharyngeal cancers (<5% or oral cavity or larynx cancers) that may be etiologically associated with HPV is unclear, and may be difficult to evaluate, given the sample size that would be required. Nevertheless, it is clear that tumor HPV status should be incorporated, as a minimum, as a stratification factor in clinical trials including patients with oropharyngeal cancers. The Radiation Therapy Oncology Group has embraced this recommendation. In the ECOG, disease-specific trials for the HPV-positive versus HPV-negative patient are in development.

Hence, the method for classification of tumors as HPV-positive or negative is of clinical importance. Based on the ECOG experience, tumor HPV status as determined by in situ hybridization is currently the standard of care.[6] This assay correlates strongly with the analysis of viral oncogene expression in fresh-frozen tumors,[16] and has been predictive of survival outcomes, whereas HPV presence by PCR was not.[17] Quantification of viral load or p16 immunohistochemistry appear to be useful in discriminating the false-positive from true-positive case when PCR is used.[17] P16 immunohistochemistry has been suggested as a potential alternative surrogate marker for HPV status, and this is likely sufficient in the context of retrospective clinical trial analysis. However, the ramifications of clinical decision made from HPV typing of an individual patient must be considered. In the setting in which clinical trials will probably move toward deintensification of therapy for the HPV-positive patient, one must avoid under treatment of the HPV-negative patient as a result of misclassification. High specificity and a high-positive predictive value are required. Assays for p16 are highly variable with regard to antibody used and the definition of a positive test. Additionally, unacceptable misclassification rates from 10% to 20% would be expected.

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PANEL DISCUSSION: COUNSELING THE PATIENT WITH HPV-RELATED ORAL CANCER

Moderator: Andy Trotti, MD
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Panelists: Maura Gillison, MD, PhD, Ohio State University Comprehensive Cancer Center, Columbus, OH; Patti E. Gravitt, PhD, Johns Hopkins School of Public Health, Baltimore, MD; Erich M. Sturgis, MD, MPH, The University of Texas M. D. Anderson Cancer Center, Houston, TX; Marshall Posner, MD, Dana-Farber Cancer Institute, Boston, MA; Aimee Kreimer, PhD, National Cancer Institute, Bethesda, MD; Susan Rosenthal, PhD, Columbia University Medical Center, New York, NY.

Recognition that a sexually transmitted viral infection, human papillomavirus (HPV), is causing oropharyngeal cancers has only recently been disseminated. Epidemiologic and medical information on this topic is rapidly evolving. The relationship between HPV and oropharyngeal cancer has provoked a broad range of medical questions as well as sensitive psychological, sexual, and social issues. The incidence rates for these cancers are clearly on the rise in the United States; and they affect a younger population largely without traditional risk factors for head and neck cancer. Therefore, community-based providers of cancer care will have an increasing likelihood of encountering patients with a diagnosis of HPV-positive head and neck cancer each year. Understanding and communicating the complexities of the infection, the development of cancer, the prognostic implications, and treatment options is a challenge for both clinicians and their patients.

The purpose of this panel was to discuss the best currently available medical advice for practitioners to offer to patients in response to common but difficult-to-answer questions. Since clinicians have limited experience with fielding these questions, it was suggested that a panel of experts actively involved in HPV research may be useful in articulating their opinions or the best available answers at this time.

The panel was composed of expert clinicians, clinician-scientists, epidemiologists, and laboratory scientists. The format was a case-based discussion of questions a clinician may commonly encounter in counseling patients. The resulting discussion is therefore designed to serve both the medical and lay communities.

A Summary of the Issues and Discussion. Based on the case presentation, a range of issues were discussed, beginning with the methods of spread of the virus. Dr. Kreimer reviewed the HPV types that are associated with benign and malignant disease, as well as what is known about the latency period. The HPV type of principal concern for oral cancers is HPV16, and it is currently estimated that infection precedes development of cancer by at least 10 years. She also covered the prevalence of HPV in the general population and compared oral (<10%) and genital (~25% in women and 60% in men) rates, noting there are far fewer data on the oral rates.

Dr. Sturgis discussed the general lack of clinical features of the initial infection and showed examples of benign papillomas. He noted that the initial infection in most cases is completely asymptomatic. Dr. Gravitt discussed the need for a standard clinical test (eg, oral swab) to test for presence of the virus. Currently, there is no accurate test to assess oral infection, nor are the cancer implications of an oral infection sufficiently understood.

Dr. Gillison discussed sexual behaviors capable of viral transmission, including oral sex and oral to oral (kissing) contact. She described how she responds to sensitive questions from

patients and partners, for example, whether the newly diagnosed patient should refrain from sex or certain types of sex. For committed and monogamous partners, she recommends no change in behavior. For new partners, she recommends consistent use of barrier methods during oral and vaginal sex because this is known to decrease rates of transmission of a number of sexually transmitted infections including HPV. She noted how sexual issues can add stress to an already stressful situation related to a cancer diagnosis and treatment. It is important to address these issues openly. She recommends patients and partners seek professional relationship counseling in some cases.

The topic of cancer screening for current or past partners was addressed by several of the panelists. Current female partners should undergo routine gynecologic screening as practiced in the general population per American Cancer Society guidelines. There is no evidence to support routine oral screening exams of partners, unless they are unduly concerned. In such cases, an examination and discussion of oral cancer risk factors by an otolaryngologist will help inform and reassure.

Screening policies and technology for detecting HPV-related oral cancer were also covered. Dr. Rosenthal noted there is currently no oral equivalent of a pap smear for HPV-related oral cancer at this time. A number of studies are under way, including a "brush" test of the oral cavity and tonsil region and oral rinses studies. Dr. Sturgis noted that it is much harder to thoroughly examine the crypts of the tonsils and base of tongue than the cervix. More studies are needed on the efficiency and sensitivity of sampling techniques.

The role of the Gardasil (Merck) vaccine was discussed. The Gardasil vaccine is not a treatment for an established cancer. It is for the prevention of viral infection and must be administered before exposure to the virus, which means before one becomes sexually active. It has a tremendous potential to reduce the rates of oral cancer in future generations, but it has no benefit and is not under study as a cancer treatment.

The observation that HPV-related oral cancers are much more common in males than females was discussed. A number of hypotheses to explain this are currently under consideration including gender differences in immune surveillance, local tissue factors, the generally higher number of sex partners among men, and changes in sexual practices since the 1960s. There are little to no data to support any of these hypotheses currently.

Dr. Gillison briefly reviewed the growing body of data showing that HPV-related cancers are associated with a very good prognosis. However, most of the panel felt strongly that this does not mean that these patients can be treated with less aggressive treatments as part of the standard of care at this time. Reducing treatment intensity may carry an increased risk of not curing the cancer. For patients seeking less aggressive treatments, it is strongly recommend that patients enroll in clinical trials testing these questions.

Dr. Posner noted the NCCN Guidelines describe a number of options based on stage and other factors. Although there are no data yet to support a change in therapy based on HPV status, he does consider HPV status in relation to the volume of cancer on an individual basis to select less aggressive treatments among the currently available options.

The role that smoking may play in development or treatment outcome of HPV-related cancer was reviewed. Although tobacco does not seem to be a strong cofactor for the development of HPV-related cancer, preliminary data suggest a history of tobacco use may negatively affect what is an otherwise excellent prognosis for HPV-related cancers.

Dr. Sturgis discussed standard follow-up recommendations after cancer treatment. There was consensus that patients with both HPV-related and unrelated cancers currently follow the same program, as outlined in the NCCN Guidelines. These involve head and neck exams every 3 to 4 months and an annual chest X-ray.

Dr. Posner addressed the risk of second cancers in HPV-positive versus -negative patients. Although only a few long-term studies are available, the data suggest there is a somewhat higher rate of second cancer development in an HPV-positive patient population compared with the general population, but this is not as high as cancer rates in HPV-negative patients, which may be up to 30% to 40% risk at 10 years. More data are needed.

The panel concluded that the recent recognition of HPV-related cancers as a distinct entity raises a number of important social, scientific, and therapeutic issues that have only begun to be addressed in a systematic manner. This meeting and others will help define the most important studies to be conducted in the near future, and help establish the standards of care for this unique population.

PANEL DISCUSSION: CLINICAL CONSIDERATIONS IN THERAPEUTIC TRIAL DESIGN BASED ON HPV STATUS

Moderator: David J. Adelstein, MD
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Panelists: John A. Ridge, MD, PhD, Fox Chase Cancer Center, Philadelphia, PA; Kian Ang, MD, PhD, The University of Texas M. D. Anderson Cancer Center, Houston, TX; Gregory Wolf, MD, University of Michigan, Ann Arbor, MI; Arlene Forastiere, MD, Johns Hopkins University School of Medicine, Baltimore, MD; David Brizel, MD, Duke University Medical Center, Durham, NC

Based on the recognized favorable prognosis after treatment for patients with human papillomavirus (HPV)-positive oropharyngeal cancer, the question of how to proceed in clinical trial design was posed to the panel. It was acknowledged that current information does not support therapeutic recommendations based on HPV status. It was also agreed that future clinical trials for patients with oropharyngeal cancer should at least be stratified by HPV status, with most attendees favoring separate clinical trial initiatives for the HPV-positive and HPV-negative groups.

The debate focused on the appropriate treatment approach for these good-prognosis HPV-positive patients, in whom prospective and retrospective studies suggest a 2-year survival in excess of 80%. To convincingly demonstrate any statistically significant improvement in this outcome, a very large randomized phase III trial would be required. Given current rates of clinical trial accrual in the United States, such a study might require 8 to 10 years to complete, and the questions asked by such a trial would likely be irrelevant by the time they were answered. It was repeatedly noted that the excellent results achieved in this patient population come only at the cost of the significant short- and long-term morbidity associated with current multimodality treatments. Considerable sentiment was expressed favoring a change in the focus of future clinical trials for these patients. Rather than attempting to improve survival endpoints, the question might become one of reducing treatment-related morbidity in this patient population. Treatment deintensification was suggested as an investigational strategy, with the hope that toxicity could be reduced without compromising outcomes. A phase III noninferiority trial was deemed impractical given the large number of patients required. Instead, there was near consensus that a large randomized phase II study design, with a standard control arm would be a reasonable way to proceed.

An alternative approach was also strongly voiced. Although patients with HPV-positive oropharyngeal cancer have an excellent prognosis, not all are cured. Future clinical investigation should focus on those patients not cured with current treatments and should attempt to improve their outcomes. Although deintensification to modify toxicity may be a valid philosophy for patient populations, any individual patient seeks the best chance for cure. Ideally we should try to identify the poor prognosis HPV-positive patients for whom current therapies fail, so that alternative and more successful treatment approaches can be chosen. The importance of smoking in the HPV-positive patient population was noted as a potential prognosticator. It was also acknowledged that as we further subdivide these patients population, the timely completion of clinical trials becomes more difficult.

There was considerable discussion focused on possible ways to deintensify treatment, and to improve results in those poor prognosis HPV-positive patients. It was recognized that there is, at present, insufficient information to make a fully informed decision. In particular, it is important to understand the patterns of failure in the HPV-positive patients after treatment. Distant disease failure might demand more focus on systemic treatment, perhaps with a reduction in the intensity of locoregional management. A preponderance of locoregional failure, however, would preclude that strategy.

Several suggestions for future study were made. A standard control arm such as radiation and high-dose single-agent cisplatin was felt to be important in any study design. Other phase II arms suggested included exploring a lower dose of radiation therapy with cisplatin, a standard dose of radiation therapy with reduced, modified, or no chemotherapy, induction chemotherapy followed by radiation, or radiation with a biologic agent. The inherent difficulty of assessing toxicity endpoints was acknowledged.

The panel discussion was followed by a workshop focused on the specifics of clinical trial design for previously untreated patients based on HPV status. A proposal was introduced by the Radiation Therapy Oncology Group for

a multiarm randomized phase II study in HPV-positive patients. Potential treatment arms were discussed and the importance of limiting this study to the good prognosis, nonsmoking HPV-positive patient population was stressed.

Given the infrequency of HPV positivity at other primary tumor sites in the head and neck, it was felt reasonable to combine the patients with hypopharyngeal and laryngeal cancer and the HPV-negative oropharyngeal cancer patients for clinical trial design. Several potential phase II approaches were discussed and were felt to be reasonable, in an effort to improve outcomes in this patient population. Reintroduction of surgery at an earlier stage in patient management was also suggested.

Further development and refinement of these potential HPV-based treatment designs will be undertaken by the cooperative groups. Formal proposals are anticipated in the near future.

MEETING SUMMARY

The meeting closed with a summary of current accomplishments and goals for the future. HPV-positive squamous cell cancer of the oropharynx represents a new and emerging disease, with a natural history and prognosis, which differs from that of HPV-negative oropharyngeal cancer, and from squamous cell cancers originating in other head and neck sites. It was recognized that this disease must be studied separately from the HPV-negative cancers and that further attempts should be made to identify those clinical and molecular features that might predict treatment success. Additional short-term goals include the standardization and dissemination of laboratory methodology for HPV testing, and the further development of HPV-based clinical trials. Intermediate and long-term goals include a better characterization of the epidemiology of oral HPV infection and its implications for the development of malignancy, as well as the implementation of screening and prevention strategies. The implications of the potential for HPV vaccination and its impact on the incidence of this disease were noted.

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