CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

The Epidemiology and Risk Factors of Head and Neck Cancer: a Focus on Human Papillomavirus

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ABSTRACT

Head and neck cancer was the eighth leading cause of cancer death worldwide in 2000. Although the incidence of head and neck squamous cell carcinoma (HNSCC) in the United States is relatively low, survival is poor and has not improved for several decades. While tobacco and alcohol are the primary risk factors for HNSCC development, epidemiological studies report a strong association with human papillomavirus (HPV) in a subset of HNSCC. More than 95% of cervical squamous cell carcinomas are linked to persistent HPV infection; evidence demonstrates that HPV is a necessary carcinogen. Not all HPV-positive HNSCC express the viral oncogenes (*E6* and *E7*), which suggests that HPV may function as a carcinogen in a smaller proportion of HNSCC. This review presents our current understanding of the relationship between HPV and HNSCC, and describes future research directions that may lead to a better understanding of the involvement of HPV in head and neck cancer.

KEY WORDS: human papillomavirus, oral cancer, risk factors.

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(1) INTRODUCTION

Head and neck cancer includes malignant tumors arising from a variety of sites in the upper aero-digestive tract. Analysis of these tumors reveals a heterogeneous neoplastic process involving numerous sites with unique sets of epidemiologic, pathologic, and treatment considerations. The most common histologic type is squamous cell carcinoma, which occurs in the oral cavity, oropharynx, hypopharynx, and larynx. Therefore, the term 'head and neck squamous cell carcinoma' (HNSCC) is frequently used to imply squamous cell carcinomas involving these anatomical sites.

(1.1) Epidemiology of HNSCC

Incidence

In 2000, head and neck cancer was ranked as the eighth leading cause of cancer death worldwide. Approximately 481,100 new cases developed, and 320,000 persons died of this disease, resulting in an average mortality rate of 7.3 and 3.2 *per* 100,000 males and females, respectively, and an average incidence rate of 8.8 and 5.1 *per* 100,000 males and females, respectively (Shibuya *et al.*, 2002). Between the years 1993 and 1997, there was extensive variation in HNSCC incidence by both sex and geographic region. Although the highest incidence worldwide was reported in Somme, France, for males, with an average rate of 43.1 new cases *per* 100,000 (95% confidence interval [CI] = 39.9-46.3), females in this region had a significantly lower incidence rate of 4.7 cases *per* 100,000 (95%CI = 3.7-5.7) (Parkin *et al.,* 2002). The highest worldwide incidence of this disease for females was reported in Bangalore, India, with an average rate of 11.2 cases *per* 100,000 (95%CI = 10.4-12.0) (Parkin *et al.,* 2002). The lowest worldwide incidence for males was reported in Quito, Ecuador, with an average of 2.4 new cases *per* 100,000 (95%CI = 1.7-3.2) (Parkin *et al.,* 2002). Although females in Ecuador had similar rates during this time frame, with 1.8 new cases *per* 100,000 (95%CI = 1.2-2.3), the lowest worldwide incidence for females was reported in Kangwha County, Korea, with an average rate of 0.5 *per* 100,000 (95%CI = -0.2-1.2) (Parkin *et al.,* 2002). In comparison, the incidence of head and neck cancers in the United States, reported between 1993 and 1997, was more than three times lower for males (12.2 *per* 100,000; 95%CI = 12.0-12.5), and more than two times lower for females (4.8 *per* 100,000; 95%CI = 4.7-5.0), than the highest reported worldwide rates for both genders (Parkin *et al.*, 2002).

Although the incidence and mortality rates for oral and oropharyngeal cancers are lower in the United States than the average worldwide rates ([incidence] males, 5.0 *vs.* 8.8 *per* 100,000, and females, 2.6 *vs.* 5.1 *per* 100,000; [mortality] males, 2.8 *vs.* 7.3 *per* 100,000, and females, 1.3 *vs.* 3.2 *per* 100,000) (Parkin *et al.*, 2002; Shibuya *et al.*, 2002), approximately 30,990 new cases and 7430 new deaths were expected in 2006. This constitutes the eighth most common cancer among US men (3% of all sites) (Jemal *et al.*, 2006). The National Cancer Institute's Surveillance, Epidemiology and End Results Program (SEER) reported that between 1997 and 2002, the median age of oral and oropharyngeal cancer diagnosis was 63 years, and the median age at death was 68 years (Ries *et al.*,

2005). As is seen worldwide, males in the US tend to have a higher incidence than females; however, due to a more recent downward trend of incident cases in males, the disparity between the sexes has decreased progressively. The age-adjusted incidence for males declined from 21.2 to 15.9 *per* 100,000 (mean, 18.8 *per* 100,000) between 1975 and 2002, while for females, the age-adjusted incidence decreased less, from 7.1 to 6.5 *per* 100,000 (mean, 7.2 *per* 100,000) (Ries *et al.*, 2005). There continue to be marked differences in head and neck cancer incidence by race. African-American males have a higher incidence of oral and oropharyngeal cancers than do Caucasian males (age-adjusted rates [1992-2002]: 20.7 *vs.* 16.2 *per* 100,000, respectively), and mortality rates are approximately twice as high (age-adjusted rates [1992-2002]: 8.2 *vs.* 4.2 *per* 100,000, respectively) (Murdock and Gluckman, 2001; Ries *et al.*, 2005).

Relative Survival Rates

The worldwide five-year relative survival rate from oral cancer is generally less than 50%, although females tend to have a higher relative survival rate than males. In the US, approximately 84% of all patients survive more than one year, but the five-year relative survival rate for all races between 1995 and 2001 was approximately 59% (58% for males and 62% for females). In contrast, African-Americans have a five-year survival rate of 39.5% (34% for males and 52% for females), which is significantly lower than that of 61.8% in Caucasians (61% for males and 63% for females) (Ries *et al.*, 2005). This poor five-year survival rate has remained unchanged for more than three decades. According to the SEER data, between 1995 and 2001, the five-year relative survival rate for patients with localized disease (with no evidence of spread) was 82%; patients diagnosed with regional disease (with spread to nearby lymph nodes and other organs) had a lower five-year relative survival rate (51%) than did those with distant disease (with spread to distant organs and lymph nodes; 27.6%).

A review of the stage distribution among cases of oral and oropharyngeal cancer from 1995 to 2001 revealed that, for all races, the majority of patients (61%) were diagnosed with either regional or distant disease (males = 68%, females = 60%). A greater difference in stage distribution can be observed between races (regional and distant disease: Caucasians, 58% [60% males; 52% females]; African-Americans, 75% [79% males; 65% females]). We and others have observed that patients who survive their initial primary tumor quite often develop multiple recurrences as well as additional primary tumors for which the five-year survival rate appears to be significantly lower than in patients diagnosed with a single primary tumor (Cooper *et al.*, 1989; Panosetti *et al.*, 1989; Leon *et al.*, 1999; Haughey *et al.*, 1992; Rafferty and O'Dwyer, 2001).

(1.2) Tobacco and Alcohol Use

In 1957, cigarette smoking was first identified as an independent risk factor for oral and oropharyngeal cancer (Wynder and Bross, 1957). Later, the use of tobacco products (*i.e.*, smoking cigarettes, pipes, and/or cigars, dipping snuff, or chewing tobacco) was confirmed, along with the use of alcohol, to be the two major risk factors for the development of these cancers (IARC, 1986; Choi and Kahyo, 1991; Brennan *et al.*, 1995; Macfarlane *et al.*, 1995; Franceschi *et al.*, 1999). Numerous studies to date have shown that tobacco and alcohol use increases the risk of HNSCC in a dose-response fashion (Lewin *et al.*, 1998; Talamini *et al.*, 2002), and the joint effects have been shown to be synergistic rather than additive (Olsen *et al.*, 1985a,b; Blot *et al.*, 1988; Lewin *et al.*, 1998; Talamini *et al.*, 2002; Castellsague *et al.*, 2004). A recent matched case-control study of 375 participants (Castellsague *et al.*, 2004) reported a higher prevalence of current and former smokers among the cases (85.4%) than among the controls (69.9%). After adjustment for alcohol consumption, all measures of tobacco smoking, amount, duration, cessation, and type of tobacco were shown to be strongly associated with oral and oropharyngeal cancer. Additionally, measures of alcohol drinking status, duration, amount, and cessation were also associated with oral and oropharyngeal cancer development. The authors reported a significant supra-additive combined effect between smoking and alcohol consumption (never-smokers who never drank, OR = 1.0 [reference]; never-smokers who ever drank, OR = 1.7 (95%CI = 0.8-3.4); ever-smokers who never drank, OR = 1.6 (95%CI = 0.5-4.7); ever-smokers who ever drank, OR = 12.7 [95%CI = 5.5-29.1; p-value = 0.008]).

It is important to note that, although the majority of cases of oral and oropharyngeal cancer are attributed to tobacco and alcohol use, one study of oral cancer cases, ages 45 years and younger, reported that 25% did not have a history of tobacco or alcohol use (Llewellyn *et al.*, 2003). Other studies have suggested that there may be a distinction between the tumors that develop in smoker/drinkers and those that develop in non-smokers/non-drinkers (Koch *et al.*, 1999; Wiseman *et al.*, 2003). The study of 1648 cases of head and neck cancer (Wiseman *et al.*, 2003), which included 40 cases with no history of tobacco or alcohol use, reported these non-smoker/non-drinkers to be primarily female (78%), with a mean age of 60 years (range, 27-90 yrs). The average ages of the non-smoker/non-drinker cases were comparable with those of the general study population (range, 18-97 yrs), but the general study population was primarily male (68%). The authors also reported that patients who were non-smokers/non-drinkers tended to have tumors of the oral cavity (primarily tongue). Similar findings were reported previously in the smaller study (Koch *et al.*, 1999), in which the population consisted of 46 non-smokers, 233 smokers, and 29 former smokers diagnosed with head and neck tumors. In contrast to the latter study, the non-smokers were defined as never having used tobacco on a regular basis, whereas in the other study, non-smokers were defined as never having used tobacco in their lifetime. Females constituted a disproportionately larger percentage of non-smokers than of smokers (p-value = 0.17) or former smokers (p-value = 0.09). Although the mean age at diagnosis was similar for all three groups (non-smokers = 58.6 yrs, smokers = 60.6 yrs, and former smokers = 69.5 yrs), non-smokers consisted of a wide range of ages (17-93), while former smokers and smokers tended to consist of older individuals, 33-84 yrs and 46-85 yrs, respectively. The tumors in non-smokers also tended to occur in the oral cavity (primarily the tongue) ($p < 0.001$), while former smokers and current smokers appeared to have more tumors of the larynx, hypopharynx, and floor of the mouth. Despite the similar findings in both studies, careful consideration should be made in comparisons of these and other studies, because of variation between studies in the classification of non-smokers/non-drinkers. It was also interesting to note that, in the Koch study, although the oral cavity tumors identified in non-

smokers were primarily of the tongue, the oral cavity tumors in smokers involved the floor of the mouth ($p < 0.001$). This evidence of variation between anatomical sites of tumors occurring in smokers and non-smokers suggests that there may be different underlying mechanisms contributing to HNSCC development in these individuals.

(2) HUMAN PAPILLOMAVIRUS AND HNSCC

(2.1) Background

Human papillomaviruses are 8-kb, circular DNA viruses that specifically target the basal cells of the epithelial mucosa (zur Hausen and de Villiers, 1994). The HPV family is comprised of more than 100 genotypes, classified in accordance with the type of epithelial cells infected and the ability to effect cellular transformation. Viral types such as HPV1 infect cutaneous epithelial cells, whereas HPV6, 11, 16, and 18 infect mucosal epithelial cells of the oral cavity, oro-pharynx, ano-genital tract, and uterine cervix. The ability of HPV to transform epithelial cells is divided into high-risk and low-risk types. Low-risk types are associated with benign lesions such as warts, while infections with high-risk types progress to malignant lesions.

The HPV genome is comprised of several early and late genes, as well as a non-coding region, all of which play roles in viral replication, transcription, and carcinogenesis. The late (L) open reading frames encode the L1 and L2 capsid proteins and are transcribed only in productively infected cells. The early (E) open reading frames encode the E1, E2, E5, E6, and E7 proteins. The E1 and E2 proteins regulate viral replication as well as the expression of the other early viral genes. At least 3 proteins (E5, E6, and E7) coded by the high-risk HPVs are considered oncogenic, due to their transforming and growth-stimulating properties. These proteins have the ability to de-regulate tumor suppressor function by binding to and abrogating the functions of the p21, p53, and pRb proteins, resulting in defects in apoptosis, DNA repair, cell cycle control, and eventually leading to cellular immortalization (Munger *et al.*, 1989; Hubbert *et al.*, 1992; Boyer *et al.*, 1996). The non-coding long control region (LCR) contains binding sites for the *E2* and *E1* gene products, located just upstream of the P_{97} promoter sequence, which controls the transcription of the *E6* and *E7* oncogenes. There is a dose-dependent regulation of *E6* and *E7* expression by *E2*. High levels of E2 protein result in the repression of *E6* and *E7* expression (Steger and Corbach, 1997).

From our knowledge of cervical cancer, persistent infection with high-risk HPVs is required for cancer development (zur Hausen, 2000; Bosch *et al.*, 2002; Bosch and de Sanjose, 2003; Schiffman *et al.*, 2005). Linearization and integration of the circular HPV genome into the host chromosome usually occur as a late event (*i.e.*, in advanced pre-cancers and the majority of invasive carcinomas) (Cullen *et al.*, 1991; Hudelist *et al.*, 2004; Pett *et al.*, 2004). HPV integration is thought to be random throughout the host genome, with a predilection for chromosomal fragile sites (Wentzensen *et al.*, 2004); however, with respect to the viral genome, integration occurs with a break in the *E1/E2* gene sequence. The disruption of the viral *E2* sequence releases the HPV oncogenes from repression, resulting in overexpression of *E6* and *E7* and leading to the alteration of key tumor suppressor pathways. This overexpression is a consistent finding in cervical cancers, and is necessary for the maintenance of the malignant phenotype (Jeon *et al.*, 1995).

The *E5* open reading frame is transcribed from the episomal form of the viral DNA, and the gene sequence is usually deleted when HPV integrates (zur Hausen and de Villiers, 1994; DiMaio and Mattoon, 2001). Therefore, the E5 protein is thought to exert its carcinogenic effects during the early stages of the viral infection, and this viral oncoprotein may not be required for the maintenance of the malignant phenotype. Nonetheless, the E5 protein has been shown to stimulate cell growth through the activation and up-regulation of the epidermal growth factor receptor (EGFR), initiating signaling cascades leading to the overexpression of proto-oncogenes, as well as the repression of cyclin-dependent kinase inhibitor 1A (*CDKN1A*/p21) expression (Tsai and Chen, 2003).

HPV E6 protein has demonstrated growth-stimulatory abilities, acting through a different mechanism. In conjunction with another cellular protein, E6-associated protein (E6-AP), the E6/E6-AP complex binds p53 and targets the molecule for proteosome degradation (Werness *et al.*, 1990; Scheffner *et al.*, 1993). In doing so, the effects of p53 loss are observed in these cells, such as the inhibition of p53-mediated apoptosis, and an inefficient G_1/S checkpoint in cells with DNA damage, all of which contribute to defects in cell cycle regulation and, eventually, to chromosomal instability in the infected cells (Kessis *et al.*, 1993; White *et al.*, 1994). The E6/E6-AP complex also prevents ubiquitination and degradation of *src* family tyrosine kinase, *BLK*, which results in stabilization of the activated form of this kinase, thereby stimulating mitosis (Oda *et al.*, 1999). The HPV type 16 E6 protein has been reported to activate or inhibit many additional cellular targets (Mantovani and Banks, 2001; Munger and Howley, 2002), a few of which include a signal transduction protein, paxillin, the telomerase reverse-transcriptase gene *hTERT*, the MYC oncoprotein, the interferon regulatory factor 3 (IRF3), a transactivator of interferons, and the single-strand DNA repair protein XRCC1 (Klingelhutz *et al.*, 1996; Reznikoff *et al.*, 1996; Tong and Howley, 1997; Ronco *et al.*, 1998; Iftner *et al.*, 2002).

The HPV E7 protein binds and degrades the tumor suppressor protein, pRB, by ubiquitin-mediated degradation (Boyer *et al.*, 1996). Destabilization of pRB causes release of E2F from pRb/E2F complexes. This permits E2F, a transcriptional regulator of cell proliferation genes, to transactivate S-phase-related genes. The functional inactivation of pRB by E7 leads to overexpression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ (Khleif *et al.*, 1996). The detection of $p16^{INK4a}$ expression is considered to be a surrogate marker for HPV infection. *E7* also exhibits kinase activity by forming indirect complexes with cyclins A and E (Arroyo *et al.*, 1993; McIntyre *et al.*, 1996), which are thought to play a role in driving cellular hyperproliferation. Similar to *E6*, there are several additional cellular targets of *E7* (Munger and Howley, 2002), some of which include the transcription factor JUN (Nead *et al.*, 1998), TATA box-binding proteins (Massimi *et al.*, 1997), and the cyclin-dependent kinase inhibitors p21 and p27, which induce cell proliferation (Zerfass-Thome *et al.*, 1996; Funk *et al.*, 1997; Jones *et al.*, 1997).

Due to the ability of high-risk HPV *E6* and *E7* to de-regulate the cell cycle and stimulate growth, these oncoproteins have also been shown to predispose a cell to genomic instability (White *et al.*, 1994). More recent studies have demonstrated that HPV *E6*- and *E7*-expressing cells develop chromosome segregation

defects, due to numerical centrosome abnormalities (Duensing *et al.*, 2001; Duensing and Munger, 2002). The presence of multipolar spindles has been recognized as a hallmark feature of high-risk HPV-associated lesions of the cervix, and may result from the development of abnormal centrosome numbers. Centrosomes duplicate once *per* cell cycle. However, in cells expressing the HPV E7 protein, this viral oncoprotein has been shown to uncouple centrosome duplication from the cell cycle, leading to abnormal centrosome and centriole numbers (Duensing *et al.*, 2000, 2001). An additive effect has also been seen when both high-risk HPV *E6* and *E7* are expressed in cells *in vitro.* Relaxation of the G_2/M checkpoint is observed, which leads to an increase in the frequency of cells entering mitosis with multipolar spindles. Additional evidence suggests that not only may this G_2/M checkpoint failure be due to the abrogation of p53 function, but also that alternative mechanisms cannot be excluded, since *E6* and *E7* have also been reported to de-regulate G_2/M-phase proteins, such as Plk1, Aurora A kinase, Cdk1, and Nek2 (Patel *et al.*, 2004). These combined effects are thought to promote mitotic defects, aneuploidy, and, eventually, chromosomal instability (Duensing *et al.*, 2000; Duensing and Munger, 2002, 2003; Plug-Demaggio and McDougall, 2002).

(2.2) Evidence for the Role of HPV in HNSCC Carcinogenesis

Almost all cases of invasive cancers of the cervix, most other ano-genital tract cancers, and approximately 20-25% of head and neck cancers contain oncogenic HPV viruses (predominantly types 16, 18, 31, and 45 for cervical and other ano-genital tract cancers, and type 16 for oropharyngeal cancers) (zur Hausen, 1996; Muñoz *et al.*, 2003). Several high-risk HPV types have been detected in head and neck cancers, even though there is a predominance of HPV type 16 (Hodge *et al.*, 1985; Hoshikawa *et al.*, 1990; Niedobitek *et al.*, 1990; Blot *et al.*, 1994; Ostwald *et al.*, 1994; Shindoh *et al.*, 1995; Haraf *et al.*, 1996; Paz *et al.*, 1997; Mineta *et al.*, 1998; Adams *et al.*, 1999; Nishioka *et al.*, 1999; Badaracco *et al.*, 2000; Gillison *et al.*, 2000; Mellin *et al.*, 2000; Sisk *et al.*, 2000; Venuti *et al.*, 2000; Klussmann *et al.*, 2001; Lindel *et al.*, 2001; Mork *et al.*, 2001).

HPV DNA Prevalence

The involvement of HPV in oral and oropharyngeal carcinogenesis was first proposed by Syrjanen *et al.* (1983). Although numerous studies have reported HPV DNA in normal and pre-neoplastic oral mucosa, as well as oral and oropharyngeal carcinomas, many of these studies were small hospital-based cross-sectional studies. Often, the collection methods for tumor and normal control specimens were different, impeding the investigators' ability to draw firm conclusions as to the prevalence of HPV DNA in oral lesions. More recently, larger studies of HPV DNA prevalence in the head and neck mucosa have showed that HPV may be an additional independent risk factor for a subset of HNSCC (Schwartz *et al.*, 1998; Smith *et al.*, 1998, 2004; Mork *et al.*, 2001; Herrero *et al.*, 2003; Hansson *et al.*, 2005).

A meta-analysis of reports from 1982-1997, examining the risk of HPV detection in normal oral mucosa, pre-cancerous oral tissue, and oral carcinoma, showed that the probability of HPV being detected in the oral mucosa increased with increasing degree of dysplasia (Miller and Johnstone, 2001). In a total of 4680 samples from 94 studies, these investigators reported that the pooled probability of detecting HPV in normal mucosa was 10% (95%CI = 6.1-14.6). The likelihood of detecting HPV in benign leukoplakia was 22% (95%CI = 15.7-29.9), in intra-epithelial neoplasia 26.2% (95%CI = 19.6-33.6), in verrucous carcinoma 29.5% (95%CI = 23.0-36.8), and in oral squamous cell carcinoma 46.5% (95%CI = 37.6-55.5). The pooled probability of detection of any high-risk HPV was 2.8 times more likely than for a low-risk subtype (0.24 [95%CI = 0.16-0.33] and 0.09 [95%CI = 0.06-0.13], respectively). HPV16 and 18 were detected in 30% of oral squamous cell carcinomas (OSCC), while other high-risk types were detected in less than 1% of these tumors. Although the likelihood of high-risk HPV being detected may be higher in samples of squamous cell carcinoma, there was substantial heterogeneity in detection rates between studies. This may be attributed to several factors, including: variations in prevalence between geographic locations of the performed studies, and between head and neck subsites (Kreimer *et al.*, 2005a); multiple HPV detection methods (polymerase chain-reaction [PCR], *in situ* hybridization [ISH]. and Southern hybridization); the use of type-specific *vs.* universal primers (primarily HPV type 16 and/or 18 *vs.* L1 consensus primers); the use of different consensus primer sets (*e.g.*, MY09/11 *vs.* GP5+/6+); and variable sample sources and collection methods (swabs, brushings, mouthwash/gargle, and biopsies). A review of the literature (Miller and White, 1996) revealed that HPV DNA was detected at a higher frequency by more sensitive assays, such as PCR (37.1%), than by moderately sensitive assays, such as Southern blot hybridization (27.2%), or by low-sensitivity assays, such as *in situ* hybridization or immunohistochemistry (25.2%) ($p < 0.005$). Although the wide variation in HPV prevalence may be narrowed with the increasing use of PCR as a more sensitive HPV detection method, variability in HPV prevalence still exists among these studies, and may be due to differences in the sensitivity of the various PCR primer sets used. The MY09/11 and GP5+/6+ primers are the most frequently used to amplify HPV DNA in cervical samples. A recent study compared the sensitivity of detecting HPV in oral *vs.* cervical samples with MY09/11 or GP5+/6+ primers (Remmerbach *et al.*, 2004). The authors reported that, although both primer sets were in agreement when used in cervical DNA samples, the GP5+/6+ primers were more sensitive than MY09/11 for HPV detection in oral DNA samples.

To reduce the variation in the literature of HPV DNA prevalence in the oral and oropharyngeal mucosa, one recommendation may be to design more sensitive PCR primers. There is increasing evidence that HPV infection may occur frequently in the normal oral mucosa (Lambropoulos *et al.*, 1997; Terai *et al.*, 1999; Kansky *et al.*, 2003), but this does not mean that the presence of the virus predicts progression to malignancy, since the majority of HPV infections may be transient rather than persistent.

Genital HPV Infection and Risk of HNSCC

Early studies examined the incidence of second cancers after an initial diagnosis of cervical carcinoma *in situ* (Bjorge *et al.*, 1995) and invasive cervical and anal cancers (Rabkin *et al.*, 1992), and have showed that there is an increased risk of head and neck cancer as well as other HPV-associated ano-genital cancers in these patients. This correlation between HPV-associated ano-genital cancers and HNSCC has more recently been strengthened by two larger studies (Frisch and Biggar, 1999; Hemminki *et al.*, 2000). Using data from the SEER registry between 1973 and 1994, these investigators identified 72,066 patients with HPV-

associated ano-genital cancers or cervical carcinoma *in situ*, and 422,023 patients with first cancers of the colon, stomach, or breast (Frisch and Biggar, 1999). The study was designed to determine whether there was a risk of tonsillar or other HNSCC among patients with HPV-associated ano-genital cancers. The risk of another ano-genital cancer among patients with an HPV-associated ano-genital cancer was high (relative risk [RR] = 3.6, 95%CI = 3.1-4.1), and the risk of tonsillar cancer (RR = 4.3, 95%CI = 2.7-6.7) or other HNSCCs (RR = 2.3, 95%CI = 1.7-3.0) was also increased. Patients with cancers unrelated to HPV had a relative risk close to 1.0. Similarly, in a subsequent study with data from the Swedish Family Cancer Database between 1958 and 1996 (Hemminki *et al.*, 2000), the occurrence of second primary cancers in the upper aero-digestive tract among 135,386 women who were initially diagnosed with cervical or *in situ* cervical carcinoma, as well as the occurrence of first primary cancers among their husbands, was assessed. This study revealed that female cervical cancer patients had elevated standard incidence ratios (SIR) for second cancers at upper aero-digestive sites. The overall SIR for females with carcinoma *in situ* was 1.68 (range between sites: 1.10-2.43, with the highest SIR attributed to the larynx), and for females with invasive cervical cancer, the overall SIR was 2.45 (range between sites: 1.05-4.98, with the highest SIR attributed to the hypopharynx). Husbands of cervical cancer patients also had elevated SIRs of cancers in the upper aero-digestive tract. Husbands of women with carcinoma *in situ* had an overall SIR of 1.43 (range between sites: 0.93-2.39, with the highest SIR attributed to the tonsils). Husbands of women with invasive cervical cancer had an overall SIR of 1.37 (range between sites: 0.93-2.72, with the highest SIR attributed to the tonsils), and the higher incidence of tonsillar cancer among the husbands of the latter group was higher among men whose wives were younger than 50 years at diagnosis (Hemminki *et al.*, 2000). Although neither of these studies investigated the prevalence of HPV in upper aero-digestive tumors, the findings provide some additional support for the role of HPV in tonsillar carcinogenesis.

HPV *E6/E7* mRNA Expression

In cervical carcinomas, it is believed that all HPV DNA-positive tumors express *E6* and *E7* mRNA (von Knebel Doeberitz *et al.*, 1988; Nakagawa *et al.*, 2000). In contrast, in HNSCC, HPV DNA positivity may not always mean that the virus is transcriptionally active, since studies have showed that only about 11-50% of HPV-positive HNSCC express *E6* and *E7* mRNA transcripts (van Houten *et al.*, 2001; Wiest *et al.*, 2002; Braakhuis *et al.*, 2004). Since the pathogenicity of HPV relies on the expression of the viral E6 and E7 oncoproteins, the lack of viral oncoprotein expression in some HPV DNA-positive head and neck tumors suggests that HPV infection may not be etiologically linked to tumor development in these cases. In support of this notion, one study of HPV *E6/E7* mRNA expression in 28 HNSCC cases showed that, of the subset of HPV-positive tumors that express *E6* and *E7* transcripts, 12 (48%) lacked *TP53* mutations in 11 of 12 (92%) of the cases, while 12 of 16 (75%) of the HPV-positive tumors that did not express viral transcripts had mutations in *TP53* (Wiest *et al.*, 2002). Similar findings have been reported by other investigators (van Houten *et al.*, 2001; Braakhuis *et al.*, 2004).

HPV Serology

The immune response to HPV infection involves both the cell-mediated (cytotoxic T-cell: CTL) and humoral (serum antibody) responses. The cell-mediated immune response to HPV infection may be important for infection control. This is evidenced by the high prevalence of HPV-associated malignancies in individuals with impaired cellular immunity, such as those with HIV infection and transplant recipients (Brown *et al.*, 2000; Frisch *et al.*, 2000). Serum antibodies to HPV capsid proteins (VLP: virus-like particles) are thought to be a marker of lifetime HPV infection (Kirnbauer *et al.*, 1994; Carter *et al.*, 1996), and also provide an HPV type-specific protection from re-infection, since passive transfer of sera from VLP-vaccinated mice to naïve mice is enough to generate a protective immune response (Marais *et al.*, 1999). Antibodies against HPV E6 and E7 proteins are markers for an invasive HPV-associated cancer (Meschede *et al.*, 1998; Stanley, 2003).

Not all women with persistent cervico-vaginal HPV infection seroconvert (*i.e.*, test positive for HPV-specific VLP antibodies) (Carter *et al.*, 2000; Ho *et al.*, 2004), and not all patients with HPV-associated cancers have detectable HPV antibodies (only ~ 50-60% of cervical cancer patients). Therefore, serum antibody production may be a limited biomarker for HPV infection and carcinogenesis (Mann *et al.*, 1990; Gaarenstroom *et al.*, 1994; Silins *et al.*, 2002). Nonetheless, it has been shown to be significantly associated with head and neck cancer development. In a nested case-control study, which examined the relationship between HNSCC and HPV infection, 292 patients with HNSCC and 1568 matched controls were tested for HPV antibodies to the *L1* and *L2* sequences of HPV16, 18, 33, and 73 (HPV DNA detection by PCR was performed on only 160 HNSCC cases) (Mork *et al.*, 2001). These authors reported a higher prevalence of HPV16 seropositivity in cases than in controls (12% *vs.* 7%). After adjustment for smoking, positive HPV16 serology was found to be significantly associated with HNSCC (OR 2.2, 95%CI = 1.4-3.4). Another large multicenter case-control study of oral and oropharyngeal cancer also reported that HPV16 VLP (L1) antibodies were associated with oral cavity and oropharyngeal cancers (oral cavity, OR = 3.5, 95%CI = 1.1-4.8; oro-pharynx, OR = 3.5, 95%CI = 2.1-5.9) (Herrero *et al.*, 2003). In contrast, although also associated with risk of cancer in the oral cavity and oro-pharynx, antibodies to HPV E6 and E7 proteins were more strongly associated with tumors from the oro-pharynx than from the oral cavity (oral cavity, OR = 2.9, 95%CI 1.7-4.8; oro-pharynx, OR = 9.2, 95%CI = 4.8-17.7). HPV serology to VLP or E6 and E7 proteins may not be site-specific, and may not allow for inferences on the causality of head and neck tumors. However, a case-control study by Schwartz *et al.* (1998) reported that HNSCC cases were more likely to be seropositive for HPV16 VLP (OR = 2.3, 95%CI = 1.6-3.3). In addition, this association was strengthened if HPV16 DNA, rather than HPV6 or 11, was detected in the tumor (HPV16 DNA, OR = 6.8, 95%CI = 3.0-15.2; HPV6 or 11, OR = 1.2, 95%CI = 0.2-3.8) (Schwartz *et al.*, 1998). A comparison of HPV serological markers with viral load in oral and oropharyngeal biopsies revealed that the prevalence of HPV VLP antibodies among HPV DNA-positive cases was higher among those with a high viral load in biopsy specimens (adjusted OR = 14.6, 95%CI = 6.0-35.6), than in those cases with a low viral load (adjusted OR = 2.7, 95%CI = 1.1-6.9) (Kreimer *et al.*, 2005b). Similarly, serum antibodies to HPV E6 and E7 proteins were strongly associated with biopsies with a high viral load (adjusted OR = 36.0, 95%CI = 14.1-92.0), but

not with biopsies with a low viral load (adjusted OR = 2.8, 95%CI = 0.8-10.0). This suggests that the use of HPV viral load in conjunction with serological markers (particularly for HPV E6 and E7 protein expression) may serve to identify a subset of HPV-associated head and neck tumors in which HPV is biologically active.

HPV and Smoking

Although HPV DNA in head and neck tumors is not found exclusively in non-smokers, studies have showed that HPV-positive tumors tend to be located in the oro-pharynx in non-smokers, but not all the reported associations were statistically significant (Snijders *et al.*, 1996; Fouret *et al.*, 1997; Koch *et al.*, 1999; Badaracco *et al.*, 2000; Bouda *et al.*, 2000; Gillison *et al.*, 2000; Klussmann *et al.*, 2001; Lindel *et al.*, 2001; Miller and Johnstone, 2001; Mork *et al.*, 2001; Herrero *et al.*, 2003; Smith *et al.*, 2004). For example, one study of the relationship between smoking status and HPV DNA was reported for 187 HNSCC cases, of which 10 (5.4%) patients were non-smokers (Fouret *et al.*, 1997). Tumors from 50% (5/10) of non-smokers (95%CI = 1.9-8.1) and 8.5% (15/177) of smokers (95%CI = 0.5-1.4, p = 0.003) were HPV DNA-positive. All cases in non-smokers were HPV16-positive, while, in smokers, 80% (12/15) were positive for HPV16, 13% (2/15) for HPV31, and one case was positive for an uncharacterized HPV type. Although the finding in this study was statistically significant, this result should be interpreted with caution, due to the wide confidence interval for non-smokers.

The combined effect of smoking and HPV serum antibody status in patients with HNSCC has been investigated. A recent multicenter study reported that, for tumors arising in the oral cavity, when compared with non-smokers who were seronegative for HPV16 E6/E7 antibodies, non-smokers seropositive for HPV16 E6/E7 had an increased (OR 6.7, 95%CI = 2.6-17.3) but similar risk in comparison with smokers who were HPV16 E6/E7-seronegative (OR = 6.7, 95%CI = 5.4-8.4) (Herrero *et al.*, 2003). Smokers who were seropositive for HPV16 E6/E7 had an even higher risk for oral cavity tumors (OR = 13.0, 95%CI = 7.2-23.5). Similarly, for the tumors arising in the oro-pharynx, HPV16 E6/E7-seropositive non-smokers had a higher risk of developing these tumors (OR = 64.5 95%CI = 18.3-226.7) than did HPV16 E6/E7-seronegative smokers (OR = 11.2 95%CI = 5.9-21.4), but a risk similar to that in HPV16 E6/E7-seropositive smokers (OR = 56.2 95%CI = 22.5-140.4) when compared with HPV16 E6/E7-seronegative non-smokers. The findings from this study suggested an additive effect of smoking and HPV infection in both the oral cavity and oro-pharynx, indicating an absence of synergism between HPV and smoking. This study also demonstrated that tumors originating in the oro-pharynx may be more likely to arise in HPV-infected non-smokers than in HPV-infected smokers. Another study examined the joint effect of the HPV VLP serology and smoking (Schwartz *et al.*, 1998), and also reported an additive effect of smoking. When compared with HPV VLP-seronegative non-smokers, HPV VLP-seropositive smokers had a higher risk for HNSCC development (OR = 8.5, 95%CI = 5.1-14.4) than did HPV VLP-seronegative smokers (OR = 3.2, 95%CI = 2.0-5.2) and HPV VLP-seropositive non-smokers (OR = 1.7, 95%CI = 1.1-2.6). In addition, this study did report a multiplicative (synergistic) effect of smoking and alcohol with HPV VLP serology. One other study evaluated the joint effects of tobacco and alcohol with the presence of high-risk HPV DNA in oral exfoliated cells from patients with head and neck tumors and tumor-free controls (Smith *et al.*, 2004). This study also reported a synergistic effect of heavy tobacco and alcohol use with a positive high-risk HPV DNA status (synergy index = 6.0, 95%CI = 1.1-32.1). When the interaction effects between the presence of high-risk HPV DNA and heavy tobacco or alcohol use alone were compared, there was no statistically significant effect modification from the joint exposures with tobacco (synergy index = 4.5, 95%CI = 0.7-27.4). This suggested that the combined risk of head and neck cancer might be limited to an additive effect of tobacco and high-risk HPV types. For the interaction effect with alcohol and high-risk HPV, the risk of head and neck cancer was statistically significantly higher in heavy alcohol users with high-risk HPV, than in never-drinkers who were negative for high-risk HPV (synergy index = 7.4, 95%CI = 1.7-33.4). Since the synergistic effect observed for heavy tobacco and alcohol exposures with high-risk HPV was similar to that of heavy alcohol exposure alone, and there was an additive effect observed only with heavy tobacco exposure, the authors commented that the combined synergistic effect of tobacco and alcohol use with high-risk HPV may be contributed only by alcohol exposure. The variation in results reported by this and the previously discussed study by Schwartz might be explained by the following differences. Further studies are needed to define the specific molecular mechanisms underlying this interaction.

HPV Integration

The ideal molecular model for HPV pathogenesis in a squamous cell carcinoma has been developed from studies of cervical cancer. Since the prevalence of integrated viral DNA increases with each progressive stage of abnormal HPV-infected cervical epithelia, the majority of cervical tumors exclusively harbor integrated viral sequences. This suggests that the tumorigenic potential of the virus increases when HPV integrates, that viral integration occurs as a late event, and that the resulting overexpression of the HPV oncogenes is crucial for the carcinogenic process. Some have suggested that HPV integration may disrupt tumor suppressor genes or activate proto-oncogenes *via* insertional mutagenesis. This has been observed in a few studies of cervical carcinomas, in which HPV integration sites have been mapped to tumor suppressor genes, such as *FHIT* at 3p14, the *MYC* locus at 8q24, and several loci within the *hTERT* promoter region at 5p15 (Durst *et al.*, 1987; Wilke *et al.*, 1996; Ferber *et al.*, 2003a,b). It is not clear whether viral integration targets these specific cancer-related genes, but the HPV integration sites seem to correspond to common fragile sites (*e.g.*, FRA3B and FRA8C). Other studies of HPV integration in cervical carcinomas have also reported a high frequency of integration into common fragile site regions (Cannizzaro *et al.*, 1988; Popescu and DiPaolo, 1989; Smith *et al.*, 1992; Wilke *et al.*, 1996; Thorland *et al.*, 2003; Wentzensen *et al.*, 2004). These are regions of DNA that are late-replicating, with loose chromatin structure. Fragile sites are 'hot spots' for DNA breakage, and their expression has been proposed to be linked to the development of cancer (Popescu, 2003). A few studies have mapped specific viral integration sites in HNSCC (Kahn *et al.*, 1994; Steenbergen *et al.*, 1995; Wiest *et al.*, 2002; Ragin *et al.*, 2004). For example, we mapped HPV16 integration sites in a cell line derived from a HNSCC of the base of tongue to chromosomal bands 9q31 and 6p21. Common fragile site induction in normal peripheral blood cells revealed that the sequences involved in these HPV integration sites are prone to breakage (Ragin *et al.*, 2004). There is evidence demonstrating that smoking induces DNA breaks in human cells (Nakayama *et al.*, 1985; Luo *et al.*, 2004). Therefore,

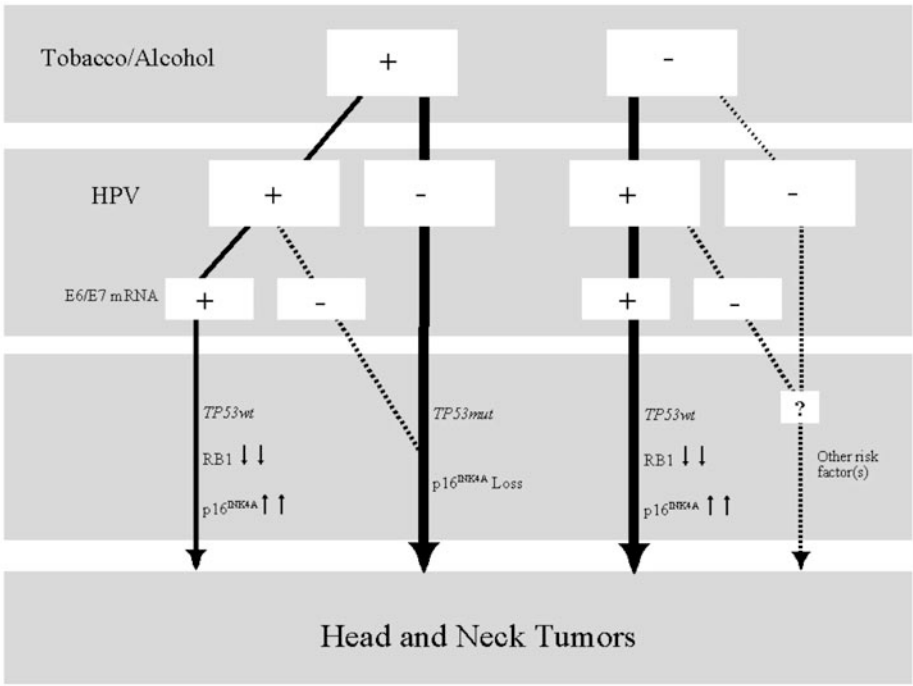

Figure. The multifactorial model of HNSCC carcinogenesis. Tobacco- and alcohol-related head and neck tumors are predominantly negative for HPV. However, there may be a subset of HPV-positive tumors in which the virus may (in *E6/E7* expressors) or may not (in *E6/E7* non-expressors) play a role in carcinogenesis. In the absence of tobacco and alcohol use, head and neck tumors may or may not be positive for HPV. The virus may function as a carcinogen in those tumors that express *E6/E7*. Risk factors other than HPV may account for the development of HPV-negative as well as HPV-positive, E6/E7 non-expressing tumors.

there is a strong likelihood that HPV-infected head and neck tumors from smokers may have a higher frequency of HPV integration. To our knowledge, no studies have tested this hypothesis, but some studies have reported a strong association of smoking with cervical cancer development (International Collaboration of Epidemiological Studies of Cervical Cancer, 2005). Others who have compared HPV-positive women with CIN1 to HPV-positive women with CIN3 have showed that cigarette smoking was associated with CIN3, suggesting that HPV infection may interact with smoking and lead to neoplastic progression (Ho *et al.*, 1998). Although reports have showed that HPV-positive head and neck tumors are more likely to occur in non-smokers, HPV has been detected in tumors from smokers (Gillison *et al.*, 2000). Therefore, defining a relationship between the integration of the virus and the effects of environmental influences, such as smoking, may be essential for gaining insight into the biology of HPV-associated HNSCC development.

From our knowledge of cervical cancers, it would be easy to assume that viral integration correlates with the natural progression of HPV carcinogenesis in HNSCC. That is, a persistent infection in the oral and oro-pharyngeal mucosa results in a pre-neoplastic change that may lead to integration of the virus and subsequent oncogene overexpression. The physical state of HPV in pre-cancerous oral and oro-pharyngeal mucosa has not yet been clearly delineated. Although integrated HPV DNA has been detected in carcinomas from the floor of the mouth, tonsils, and larynx (Kahn *et al.*, 1994; Steenbergen *et al.*, 1995), and the prevalence of HPV integration in tumors from these sites varies from 21 to 43% (Venuti *et al.*, 2000; Rodrigo *et al.*, 2002; Hafkamp *et al.*, 2003). There are also variations in the reported physical state of the virus among tumors from different head and neck sites (Venuti *et al.*, 2000; Mellin *et al.*, 2002; Koskinen *et al.*, 2003). One study reported that at least 43% of laryngeal cancers positive for HPV16 carry integrated forms of the virus (Venuti *et al.*, 2000), while others have showed evidence of integrated or episomal forms only, or a combination of both, in tumors from various sites within the oral cavity and pharynx (Watts *et al.*, 1991; Koskinen *et al.*, 2003). Studies of HPV-positive tonsillar carcinomas have reported that these tumors carry the viral genomes primarily in the episomal form (Mellin *et al.*, 2002), and although some of these tumors do not contain integrated HPV DNA, expression of the viral oncogenes can still be detected. This would suggest that, at least for HPV-positive tonsillar carcinomas, viral integration may not be a necessary step for carcinogenesis. Nonetheless, further studies are needed for a clear definition of the relationship among HPV infection, persistence, and integration in oral and oropharyngeal cancers.

(3) CONCLUSIONS

To demonstrate causality, we again refer to cervical cancer as the model of HPV carcinogenesis. Studies that support a causal relationship between HPV and cervical cancer include: a consistent detection of HPV DNA in tumor specimens; *E6/E7* viral oncogene expression in cervical lesions; the requirement of cervical carcinoma cell lines to express *E6/E7*, to maintain a malignant phenotype; interaction of viral oncoproteins with growth-regulating proteins (p53, pRb, etc.) of the host cell; and epidemiological data highlighting HPV infection as a risk factor for the development of cervical cancer. A comprehensive review of important studies demonstrating the causal relationship between HPV and cervical cancer development has been published (Bosch *et al.*, 2002). For the over 50 case-control studies that have been conducted worldwide between 1985 and 1990, with both PCR- and non-PCR-based methods for HPV detection, there is striking consistency in elevated odds ratios for the association of HPV with invasive cervical cancer, ranging from > 1.0 to infinity. More recently, case-control studies conducted after 2000 have reported the odds ratios for HPV associations with high-grade cervical lesions, carcinoma *in situ*, and invasive cervical cancers to be greater than 50 (Herrero *et al.*, 2000; Thomas *et al.*, 2001).

The tumorigenic potential of HPV in HNSCC is evident. Although a viral association within a subset of HNSCC has been shown, the molecular and histopathological characteristics of these tumors have yet to be clearly defined. The current evidence suggests that tumors caused by HPV express *E6/E7*

mRNA and have a wild-type *TP53* gene sequence, and that immunostaining is reduced for pRB and strongly positive for $p16^{INK4a}$ (Wiest *et al.*, 2002; Braakhuis *et al.*, 2004; Ragin *et al.,* 2006). Evidence supports the idea that HNSCC is a multifactorial disease with at least two, possibly distinct, pathways, one driven by smoking and alcohol consumption, and another driven by HPV (Fig.). There have been several studies in which HPV infection and smoking are not mutually exclusive. We and others have observed that HNSCC from smokers may contain transcriptionally active HPV (Braakhuis *et al.*, 2004; Ragin *et al.*, 2004; Ferris *et al.*, 2005). Further studies are warranted for a clearer definition of whether these tumors may have developed due to the interactions between these two risk factors. If HPV16 is involved in the development of some HNSCC, the implementation of a vaccine program for HPV 16 and 18 may prove to be beneficial in preventing not only cervical cancer, but possibly HPV16-positive head and neck tumors as well.

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