Daniel L. Miller · M. Sharon Stack
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Human Papillomavirus (HPV)-Associated Oropharyngeal Cancer

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Chapter 1
Epidemiology of HPV-Associated Oropharyngeal Squamous Cell Carcinoma

Susanne M. Gollin

1.1 Introduction

Head and neck squamous cell carcinoma (HNSCC) arises from the squamous epithelial cells that line the moist, mucosal surfaces within the head and neck region (reviewed by Gollin 2014). This region includes the oral cavity (gums, lining of the lips and cheeks, the floor of the mouth, front two-thirds of the tongue, the hard palate, and the retromolar trigone); the salivary glands; the nasal cavity and nasal sinuses; the pharynx, which is comprised of three sections: the nasopharynx (the upper section of the pharynx behind the nose), the oropharynx (the middle pharynx including the base of the tongue (the back one-third of the tongue), the soft palate (the back part of the roof of the mouth), the tonsils, and the side and back walls of the throat), and the hypopharynx (the lower part of the pharynx that connects to the esophagus); and the larynx (the voicebox and the epiglottis, which covers the larynx to prevent food from entering the airways leading to the lungs). This chapter focuses primarily on squamous cell carcinoma of the oropharynx or OPSCC, the majority of cases of which are caused by human papillomavirus (HPV). This chapter reviews the biology, prevalence, and transmission of HPV infection, the incidence of OPSCC, risk factors for HPV-associated OPSCC, and strategies for preventing HPV infection and HPV-driven cancers.

This chapter is dedicated with gratitude to Prof Dr. Harald zur Hausen and Dr. Ethel-Michele de Villiers for hosting the author for 3 months to examine HPV in oropharyngeal cancer in their laboratory at the German Cancer Research Centre (DKFZ) in Heidelberg, Germany.

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1.2 The Biology of HPV Infection

Human papillomavirus (HPV) is listed among the most powerful human carcinogens (IARC 2011). Further, HPV is the most common sexually transmitted infection in the United States (U.S.) (CDC 2014) and worldwide, and is therefore, a major public health concern. HPV is a large group of viruses in the family *Papillomaviridae* that infect the basal layer of either the cutaneous or mucosal epithelia of vertebrates and cause neoplasia, benign papillomas (condylomas or warts), or persist asymptomatically (zur Hausen and de Villiers 1994; Bernard et al. 2010). More than 150 human HPV types have been identified and subdivided based on their L1 nucleotide sequence being at least 10 % dissimilar from another HPV type. HPVs are subdivided into high-risk and low-risk types, depending on whether they can transform the cells they infect. The following discussion will be limited to human mucosal HPVs. The high-risk types include the most common high-risk types, HPV16 and HPV18, as well as HPV types 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70 (Rautava and Syrjänen 2012). Persistent high-risk HPV infection is a necessary, but not sufficient cause of approximately 5 % of human cancers (Wang et al. 2015). The low-risk types include the more common HPV6 and 11 types, which cause genital condylomas, but are also seen in benign oral lesions. The less common low-risk HPV types include types 42, 43, and 44 (Rautava and Syrjänen 2012).

HPVs are comprised of a double-stranded circular DNA genome ~8 kb in length that codes for eight genes and a noncoding region that regulates viral replication and controls cellular and viral transcription. All protein-coding genes are located on the same DNA strand (Rautava and Syrjänen 2012; reviewed in Blitzer et al. 2014). The genes are divided into early (E) and late (L) genes, E1, E2, E4, E5, E6, E7, L1, and L2, with the late genes encoding the major and minor capsid proteins, respectively. The capsid is the protein shell that surrounds the viral DNA. HPV can integrate into the host cell chromosomes and/or persist in episomal form. The circular genome is thought to linearize and integrate as a late event during infection, breaking in the E1/E2 gene region, with disruption of the E2 gene, releasing repression of the viral genome, leading to overexpression of the viral E6 and E7 genes necessary for maintenance of the malignant phenotype (reviewed in Ragin et al. 2007). The E5, E6, and E7 proteins are most important for oncogenic transformation. The E5 protein plays a role during the early stages of carcinogenesis, and appears to increase cellular EGFR signaling, resulting in upregulation of viral gene expression and cellular proliferation. In general, the E6 protein from high-risk HPVs activates a number of cellular proteins, including the cellular ubiquitin ligase E6AP, which targets the TP53 protein for degradation, resulting in loss of TP53-mediated processes, including TP53-mediated apoptosis, cell cycle checkpoints, the DNA damage response, and chromosomal stability. Low-risk E6 does not degrade TP53. The E7 protein promotes proliferation of HPV-infected cells by degrading the RB1 protein, releasing the E2F transcription factor and driving expression of S-phase cell cycle genes and their proteins,

including *CDKN2A* and its protein, $p16^{INK4a}$, which serves a surrogate marker for HPV expression. E6 and E7 gene expression not only knocks out the two most important cellular tumor suppressor pathways, RB1 and TP53, but also affects expression of multiple tumor suppressor genes, DNA damage response genes, and oncogenes, leading to oncogenic transformation.

One of the earliest next generation sequencing studies of HNSCC demonstrated that a core set of biochemical pathways is altered in both HPV-positive and HPV-negative tumors, including the TP53, RB1/cell cycle regulatory, and PI3K/AKT/mTOR pathways (Lechner et al. 2013). Rampias et al. (2009) inhibited HPV16 E6 and E7 expression by specific transfection of two human OPSCC cell lines harboring integrated HPV16 (147T and UPCI:SCC090) with specific shRNA and demonstrated restoration of the TP53 and RB1 pathways and significant cellular death via apoptosis (Rampias et al. 2009). These results further substantiated the causal relationship between HPV and OPSCC and further confirmed the significance of cell lines as important preclinical models for investigation of molecular characteristics and mechanisms of disease in HPV-positive OPSCC. The controversies associated with another HPV-infected cell line, HPV18-positive HeLa cervical cancer cells should not derail utilization of cell cultures as critically important preclinical models for cancer research investigations, including those noted above as well as examination of the effects of new therapies on HPV-positive OPSCC cells.

As described above, in some cases, HPV linearizes and integrates into the genome. Several questions arise related to HPV integration. (1) Does the virus integrate randomly throughout the human genome or at specific sites? (2) Does integration play a role in carcinogenesis as it does for cervical cancer? (3) Is viral integration influenced by cigarette smoking? (4) Do viral load, gene expression, and integration site(s) play a role in OPSCC aggressiveness, response to therapy, and prognosis? In an effort to answer these questions, we and others have carried out or participated in studies of HPV-driven OPSCC. Most of these questions remain unanswered, but the cell culture of multiple OPSCC naturally infected in vivo with HPV has enabled us to develop cell lines and investigate the answers to these questions.

The OPSCC cell line, UPCI:SCC090 has been investigated for more than 10 years, and has been distributed by our group and the German Cell Repository (DSMZ) to scores of laboratories around the world. The UPCI:SCC090 cell line was developed by culturing a recurrent tumor (T2N0) from the base of tongue of a 46-year-old-Caucasian man with a history of smoking, drinking, a positive family history of cancer, and previous treatment for OPSCC (White et al. 2007). A second cell line, UPCI:SCC152 was developed 1 year later from a recurrent tumor in the hypopharynx of the same patient as UPCI:SCC090. He subsequently died as a result of OPSCC, 4 years and 3 months after initial diagnosis. A third HPV16-positive OPSCC cell line, UPCI:SCC154 was developed by our group from a T4N2 base of tongue/soft palate tumor removed from a 52-year-old-Caucasian man who smoked and drank (White et al. 2007). He was alive at most recent followup, 10 years and 2 months after surgery. The HPV16-positive

UPCI:SCC070 cell line was derived from a T4NB SCC of the retromolar trigone from a 34-year-old-Caucasian woman who had a prior HNSCC surgically removed 7 years earlier. She died of other causes 15 years and 2 months after the surgery that led to the cell line.

Mapping of the HPV16 integration sites in the human genome in the UPCI:SCC090 cell line was first reported by Ragin et al. (2004) who found consistent integration sites among the UPCI:SCC090 cell line, the tumor from which the cell line was developed, and the tumor from which the UPCI:SCC152 was later developed, suggesting that the tumors were clonal in origin (Ragin et al. 2004). The integration site mapping was carried out using fluorescence in situ hybridization (FISH), spectral karyotyping (SKY), and restriction fragment oligonucleotide PCR followed by sequencing and BLAST searching for the map location of the sequence. Ragin et al. (2004) also confirmed by FISH analysis that the map locations coincided with common chromosomal fragile sites. We observed multiple tandemly arranged viral copies within the genome in five chromosomal locations, (3q, 6p21, 9q31, 13q, and in a der(?)t(1;8)). Further, Ragin et al. (2004) found that the E6 and E7 genes were not only expressed from the chromosomally integrated HPV16, but also from episomal HPV genomes. In a complementary study, Ferris et al. (2005) detected that the HPV16 DNA (European variant E-G131G) in UPCI:SCC090 is integrated in a head to tail tandem repeat, contains a 163 bp deletion of the LCR (nucleotides 7658–7818), that the HPV16 E6 and E7 genes are highly expressed, that about 100–150 copies of viral DNA are present as estimated by qPCR, and that integration does not disrupt the HPV16 E2 gene. Olthof et al. (2015) investigated viral load, gene expression and mapped the HPV16 integration sites in a series of seven OPSCC cell lines, including UPCI:SCC090 and UPCI:SCC152 (Olthof et al. 2015). The two cell lines from the same patient revealed identical numbers of six HPV16 FISH signals/nucleus and two different PCR methods both identified HPV16 integration at 9q22 as well as on chromosomes 3 and 6 as shown previously by Ragin et al. (2004) and Akagi et al. (2014). Viral load in UPCI:SCC090 was determined to be 739 HPV16 copies/β-globin copy and UPCI:SCC152 was found to have 210 HPV16 copies/β-globin copy (Olthof et al. 2015). Akagi et al. (2014) determined by qPCR that UPCI:SCC090 harbors 182 viral copies, an estimated 483 copies by whole genome sequencing (WGS), and detected 33 viral-cellular breakpoints by WGS, with viral integrations at 3p12, 6p21 and 9q22, usually adjacent to regions of copy number alterations, including >90-fold amplification of host DNA (Akagi et al. 2014). Gene disruption by viral integration was seen in multiple loci in UPCI:SCC090, including amplification of two oncogenes, *FOXE1* and *PIM1*, and *C9orf156* (Ragin et al. 2004; Akagi et al. 2014). Most importantly, the Akagi et al. (2014) study showed loop structures containing integrated HPV (including the HPV origin of replication) and the adjacent chromosomal sequences, which they call viral-host DNA concatamers, that are proposed to facilitate replication and reintegration; structural and/or copy number variations adjacent to and usually including the HPV integrants; viral breakpoints distributed across the viral

genome; and E6 and E7 viral oncogene retention and expression as viral-host fusion transcripts in the HPV-positive tumors and cell lines they examined.

Independent examination of OPSCC showed that both HPV-positive and negative tumors have decreased expression of six large genes located at common chromosomal fragile sites, although the HPV-positive tumors showed greatly decreased expression of these genes (Gao et al. 2014a). Dividing the HPV-positive OPSCC into those with greatly decreased expression of these genes compared to those without revealed a higher incidence of local recurrence and distant metastasis and a statistically shorter time to recurrence in the tumors with decreased expression of the six genes (Gao et al. 2014b). Several of these genes have been shown to be tumor suppressor genes, including *FHIT* and *PARK2,* and others are suspected to be tumor suppressor genes as a result of their functions and reduced expression in tumors (*DLG2, NBEA, CTNNA3, DMD*) (Gao et al. 2014b), although this remains controversial. Whether this indicates that HPV integration disrupts these large genes mapped to common chromosomal fragile sites is under further investigation in both OPSCC and other HPV-driven cancers. The results of these studies suggest that HPV integration may occur at chromosomal fragile sites in the human genome followed by evolutionary selection of tumor cells in favor of cell proliferation over cell death and therapeutic resistance, as occurs in most cancers. Comparable to deletion of tumor suppressor genes in cancer, insertional disruption of tumor suppressor genes by HPV may serve to tip the balance, leading to favorable selection of cells expressing not only multiple copies of the HPV-encoded E6 and E7 oncogenes, but physically disrupted tumor suppressor genes.

A recent study of 3,667 HPV integration sites in 26 cervical intraepithelial neoplasias, 104 cervical carcinomas (CC), and five cell lines using whole genome sequencing (WGS) with >30× coverage and high throughput viral integration detection (HIVID), which is even more sensitive than WGS in identifying viral integration sites, supports and extends the list of HPV integration sites previously identified in both OPSCC (described above) and CC (Hu et al. 2015). Further, they showed breakpoints and/or altered expression of a number of large genes, including *FHIT, DLG2,* and *DMD*. These investigators also observed that viral breakpoints could occur anywhere in the viral genome, but were more likely to occur in E1 instead of E2. Most importantly, they showed a significant enrichment of microhomologies (MH) between the human and HPV genomes at or near integration sites, suggesting that the MH-mediated DNA repair pathways, fork stalling and template switching (FoSTeS) and MH-mediated break-induced replication (MMBIR) may mediate the viral integration process. Elements in the human genome that are often seen at sites of genomic instability, including satellite and SINE-Alu repeats were highly enriched near integration sites, suggesting that chromosomal instability may lead to stalled replication forks and breaks, resulting in activation of these MH-based DNA repair pathways, with HPV integration facilitated by the MH between the viral and human genomes (Hu et al. 2015).

Whether smoking plays a role in viral integration is not entirely clear, but plausible. We have reported direct evidence that low levels of cigarette smoke condensate (CSC) in amounts substantially less than the quantity extracted from

one cigarette, cause DNA double strand breaks (DSBs), resulting in irreversible changes in the chromosomal constitution of cultured human cells (Luo et al. 2004). We also showed that the reactive oxygen species (ROS) scavenger, 2′ deoxyguanosine 5′-monophosphate (dGMP) prevents CSC-induced DSBs, anaphase bridge formation and genomic imbalances. Further, exposure to tobacco carcinogens increases the potential for chromosome breakage at fragile sites (Stein et al. 2002). These investigators showed that active smokers exhibit a significantly higher frequency of fragile site expression, including FRA3B, which maps to the *FHIT* gene, compared to nonsmokers and lung cancer patients who have stopped smoking. Thus, active tobacco exposure increases chromosomal fragile site expression (Stein et al. 2002). It is plausible that viral integration may be facilitated by the presence of tobacco-induced DSBs. Cigarette smoking was shown to be an independent risk factor for oral HPV incidence/acquisition (Kreimer et al. 2013; Fakhry et al. 2014), as discussed further in the section, The Prevalence of HPV Infection, of this chapter. Tobacco use has been associated with local and systemic immunosuppression (Sopori 2002; Lee et al. 2012) and appears to alter the biologic features of HPV infection, including persistence and reactivation of infection (Kero et al. 2014a). Further studies are warranted to examine the role of tobacco in the natural history of oral HPV infection and progression to OPSCC.

Although we have made tremendous progress in our understanding of HPV integration and gene expression, additional studies of the mechanisms involved in chromosomal breakage, viral acquisition, and integration and its effects on the host genome are indicated in both cell lines and tumors. The roles that viral load, gene expression, and integration site(s) play in OPSCC aggressiveness, response to therapy, and prognosis remain under active investigation and discussion. In spite of a large body of solid research in the literature, the relationship between viral integration, viral gene expression, and chromosomal instability was questioned by Mooren et al. (2013). The issue is whether chromosomal instability precedes and facilitates viral integration or whether viral gene expression results in chromosomal instability, as it does in cellular model systems transfected with HPV E6 and/or E7 genes. Mooren et al. (2013) concluded that HPV-positive tonsillar SCC is more often genetically stable than HPV-negative lesions, and that these tumors are associated with a favorable prognosis. They found that chromosome instability, which may precede viral infection and/or facilitate viral integration, is associated with an unfavorable prognosis, particularly in the HPV-positive patient group (Mooren et al. 2013). Duensing and colleagues created cellular model systems comprised of normal neonatal human foreskin keratinocytes transfected with either HPV E6 or E7 and found that HPV16 E7 induces centrosome abnormalities including multipolar spindles, which result in aberrant chromosome segregation and aneuploidy. These viral proteins also stimulate DNA replication stress, which can result in DNA damage and structural chromosomal instability, TERT upregulation as well as anaphase bridge formation (Duensing and Munger 2002; reviewed in Korzeniewski et al. 2011). Mooren et al. (2013) questioned whether aberrantly high levels of viral gene expression in the model systems are representative of the possibly lower expression levels of the E6 and E7 genes in tumors,

which may not necessarily result in the chromosomal instability seen in the model systems. They observed that the majority of HPV-positive carcinomas with chromosomal instability and a worse prognosis arise in smokers. These tumors may initially be smoking-induced, resulting in chromosomal instability, which facilitates viral integration into the genome.

Spardy et al. showed that human keratinocytes expressing high-risk HPV16 E7, but not E6, have an activated Fanconi Anemia (FA) DNA repair pathway (Spardy et al. 2007). FA is a heterogeneous DNA damage response disorder (Kee and D'Andrea 2012). FA patients have biallelic germline mutations in one of the 16 known FA genes, are hypersensitive to DNA interstrand crosslinking agents (including the chemotherapeutic agents, mitomycin C and cisplatin), express chromosomal instability, developmental anomalies in multiple organ systems, early-onset bone marrow failure, and a significant predisposition to cancer (Mouw and D'Andrea 2014). Many of the SCC in FA patients have been considered to be HPV-driven, but the contribution of HPV to SCC in FA patients is not entirely clear at this time, since conflicting results have been published in the literature (van Zeeburg et al. 2008; Alter et al. 2013; Mouw and D'Andrea 2014). The cellular hallmark of FA is a high frequency of chromosomal aberrations, pointing to a defect in the DNA damage response (Mouw and D'Andrea 2014). Spardy and colleagues reported that cells deficient for the FA pathway are prone to HPV-induced chromosomal breakage (Spardy et al. 2007). These results suggested to the research team led by Dr. Susanne Wells that HPV infection in the context of FA pathway deficiency results in increased epithelial hyperplasia, genomic instability, and malignant progression. They then confirmed this hypothesis and showed that these findings could be attenuated by complementation of *FANCA*-deficient cells by reintroduction of *FANCA* expression (Hoskins et al. 2009). Park et al. (2013) showed that FA protein deficiency in mice predisposes HPV16 E7 transgenic mice to OPSCC by promoting DNA damage by inactivation of Rb1 and other pocket proteins, which clarifies the mechanism by which DNA repair deficiency would increase susceptibility to high-risk HPV E7-driven cancer (Park et al. 2013). They also showed that FA deficiency does not predispose E6 transgenic mice to OPSCCs, suggesting specificity in the interaction between FA deficiency and the HPV E7 oncogene (rather than the E6 oncogene) in causing OPSCC. Further studies are warranted to examine the relationship between DNA damage response defects, chromosomal instability, HPV infection, integration, viral load, and viral gene expression in OPSCC.

1.3 Transmission of HPV Infection

Exposure to HPV is quite common, and high-risk HPV infections are often asymptomatic. Most HPV infections are cleared by the immune system; the individual is not aware he or she had the infection and does not develop visible lesions or cancer. Although the lifetime oral exposure rate is not known, an estimated 65–100 % of sexually active adults are thought to have been exposed to HPV (Pytynia et al. 2014).