High-Risk Human Papillomavirus in Head and Neck Squamous Cell Carcinoma

by

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PREFACE

A version of CHAPTER II (High-risk Human Papillomavirus Detection in Oropharyngeal, Nasopharyngeal, and Oral Cavity Cancers: Comparison of Multiple Methods) is accepted for publication, JAMA Otolaryngology-Head & Neck Surgery, Published online October 31, 2013. The authors are: Heather M Walline, Christine M Komarck, Jonathan B McHugh, Serena A Byrd, Matthew E Spector, Samantha J Hauff, Martin P Graham, Emily Bellile, Jeffrey S Moyer, Mark E Prince, Gregory T Wolf, Douglas B Chepeha, Francis P Worden, Matthew H Stenmark, Avraham Eisbruch, Carol R Bradford, and Thomas E Carey.

CHAPTER III (Integration of High-Risk Human Papillomavirus into Cellular Cancer-Related Genes in Head and Neck Cancer Cell Lines) has been submitted for publication. The authors are: Heather M Walline, Christine M Komarck, Jonathan B McHugh, Alice L Tang, John H Owen, Bin T Teh, Erin McKean, Thomas Glover, Martin P Graham, Mark E Prince, Douglas B Chepeha, Steven B Chinn, Robert R. Ferris, Suzanne M Gollin, Thomas K. Hoffmann, Henning Bier, Rudd Brakenhoff, Carol R Bradford, and Thomas E Carey.

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LIST OF ABBREVIATIONS

APOT ..... Amplification of Papillomavirus Oncogene Transcripts

BPV ..... Bovine Papillomavirus

CDK ..... Cyclin-Dependent Kinase

ChemoRT ..... Chemoradiation

CIN ..... Cervical Intraepithelial Neoplasia

CRPV ..... Cottontail Rabbit Papillomavirus

DIPS-PCR ..... Detection of Integrated Papillomavirus Sequences-PCR

DNA ..... Deoxyribonucleic Acid

dsDNA ..... Double-Stranded Deoxyribonucleic Acid

E6AP ..... E6-Associated Protein

EBV ..... Epstein-Barr Virus

EGBD ..... Extra Genital Bowen's Disease

EGFR ..... Epidermal Growth Factor Receptor

EV ..... Epidermodysplasia Verruciformis

FFPE ..... Formalin-Fixed, Paraffin-Embedded

FISH ..... Fluorescence In Situ Hybridization

GAPDH ..... Glyceraldehyde 3-Phosphate Dehydrogenase

HNSCC ..... Head and Neck Squamous Cell Carcinoma

HPV ..... Human Papillomavirus

hrHPV..... High-Risk Human Papillomavirus

HSIL .....High-Grade Squamous Intraepithelial Lesion

IHC ..... Immunohistochemistry

INDEL..... Insertion or Deletion

ISH..... In Situ Hybridization

IVC..... Invasive Vulvar Cancer

lrHPV.....Low-Risk Human Papillomavirus

LSIL..... Low-Grade Squamous Intraepithelial Lesion

MALDI-TOF..... Matrix-Assisted, Laser Desorption/Ionization-Time of Flight

MHC ..... Major Histocompatibility Complex

NASBA..... Nucleic Acid Sequence Based Amplification

NPC .....Nasopharyngeal Carcinoma

nt.....Nucleotide

OCSCC .....Oral Cavity Squamous Cell Carcinoma

OPSCC .....Oropharyngeal Squamous Cell Carcinoma

ORF ..... Open Reading Frame

PCR..... Polymerase Chain Reaction

PCR-MA .....Polymerase Chain Reaction-MassArray

qPCR-MA.....Quantitative PCR-MassArray

qRT-PCR..... Quantitative Reverse Transcription-Polymerase Chain Reaction

Rb .....Retinoblastoma

RNA.....Ribonucleic acid

RT-PCR ..... Reverse Transcription-Polymerase Chain Reaction

SCC ..... Squamous Cell Carcinoma

SKY ..... Spectral Karyotyping

SNP ..... Single-Nucleotide Polymorphism

TCGA ..... The Cancer Genome Atlas

URR ..... Upstream Regulatory Region

VIN3 ..... Vulvar Intraepithelial Neoplasia 3

ABSTRACT

High-risk human papillomavirus (hrHPV)-driven carcinogenesis is the predominant etiologic factor in oropharyngeal squamous cell carcinoma (OPSCC). Most HPV-positive OPSCCs respond well to therapy, prompting interest in reducing treatment intensities, but approximately 20% fail to respond to therapy and recur for unknown reasons. The prognostic value of HPV in OPSCC warrants a universal standard for hrHPV assessment, and evaluation of factors that may differentiate responsive from non-responsive tumors is needed to determine the optimal treatment for patients.

We compared hrHPV detection by PCR-MassArray, $p16^{INK4a}$ immunohistochemistry, and HPV in situ hybridization in oropharynx, nasopharynx, and oral cavity tumors to determine the optimal assessment of hrHPV. HPV copy number, viral oncogene expression, integration sites, and integration transcripts were examined in seven HPV-positive HNSCC cell lines from patients who progressed, plus five responsive and five recurrent OPSCC tumors.

Using combined PCR-MA with L1 consensus PCR and sequencing for resolving discordant results, we found PCR-MA to have the greatest sensitivity and specificity of the methods evaluated, making it optimal for HPV detection in combination with p16 for correlative viral activity. Of 338 tumors, 183/212 (86%) of oropharynx, 9/18 (50%) of nasopharynx and 28/108 (26%) of oral cavity tumors were positive for hrHPV.

All of the HPV-positive cell lines and tumors evaluated expressed HPV E6 and E7 oncogenes and exhibited alternate splicing, indicating active viral oncogenesis. Each of the

HPV-positive cell lines, which came from non-responsive outlier tumors, and the five recurrent tumors exhibited HPV integration into cancer–associated cellular genes. Each of the responsive tumors demonstrated viral integration into non-genic chromosome regions, with only one integration into a cancer-related gene. Integration transcript analysis revealed HPV-cellular fusion transcripts, intact cellular transcripts, and several genomic rearrangements, indicating genomic instability in the cell.

We propose that viral integration is an early carcinogenic event, associated with disruption of the E1/E2 region and alternate E6*I transcription, leading to increased viral oncogene expression as the carcinogenic driver in responsive tumors. Further, we hypothesize that HPV integration into cellular genes may result in secondary alterations in cellular gene expression or dysfunction, resulting in a more aggressive malignant phenotype resistant to current therapy.

CHAPTER I

Introduction

Human Papillomavirus

History: Human papillomaviruses (HPVs) belong to a family of small DNA viruses, all having a circular double-strand DNA genome of approximately 8kb. Papillomaviruses are species-specific and infect cutaneous or mucosal keratinocytes in mammals as well as several amniotes. More than 100 unique HPV types have been identified; clinical manifestations include benign warts, papillomas, condylomas, and less frequently, mucocutaneous malignancies.

Animal papillomaviruses have been studied as models of HPV infection and disease etiology. Bovine papillomaviruses, BPV1-10, are members of the delta-, epsilon-, and xi-papillomaviruses, transmitted by direct and indirect routes including flies, fence posts, and halters[1,2]. BPVs produce mucosal and cutaneous lesions in cattle, which are typically benign, but in some cases become malignant, particularly in bladder and upper gastrointestinal lesions in association with exposure to bracken fern, an environmental carcinogenic cofactor[2,3]. Early molecular examination of BPV supported subsequent characterization of human papillomavirus types[4].

Cottontail rabbit papillomaviruses (CRPVs) are members of the kappa genus of papillomaviruses, and cause warty cutaneous growths on animals infected with the virus.

CRPV transmission is both direct between animals and indirect through arthropod vectors[5]. CRPV was discovered as the first DNA tumor virus by Richard Shope in 1933[6], and has been widely studied since then as a model of carcinogenesis. CRPV in rabbits was used to study virus-mediated carcinogenesis in association with host immune response[7], exposure to chemical carcinogens[8,9], and host major histocompatibility complex (MHC) class II haplotype[10].

The infectious nature of human skin and genital warts was recognized in the 19th century, and wart contagion was reported by Payne in 1891[11]. The causative "human wart virus" was confirmed in the early 1900s, and the double-strand DNA (dsDNA) circular genome was characterized in 1965[12,13]. Throughout the 1970s, studies were performed examining viral DNA and RNA isolated from different types of warts. Hybridization and endonuclease restriction digest experiments demonstrated the heterogeneity and diversity of human papillomavirus types. Much earlier, in 1842, Rigoni-Stern published his observation that cervical cancer rates were much higher in sexually active women than in virgins and nuns, and suggested that cervical cancer was associated with sexual contact[4]. Many studies to establish a link between cervical cancer and sexually transmitted infections (particularly Herpes simplex type 2) followed, but none were successful. It was not until the 1970s that researchers began studying the possible association between human papillomaviruses and cancer, based in part on sporadic instances of genital wart transformation to squamous cell carcinoma[4]. In 1976, Harald zur Hausen published his hypothesis implicating human papillomavirus as the causative agent in cervical cancer, and several years later he and others identified the novel types HPV6 in genital warts and HPV11 in laryngeal papillomas, followed by studies reporting HPV in invasive condylomata

acuminata[14,15]. Using portions of known HPV types as probes, researchers were able to detect known types and isolate novel HPVs in studies of cervical cancer, and in 1983 and 1984, zur Hausen and his collaborators specifically identified HPV16 and HPV18 in cervical tumors[16,17]. The first epidemiological study on the association between cervical cancer and HPV was published in 1987, comparing HPV positivity (HPV 6, HPV11, HPV16 and HPV18) with cytological findings in nearly 10,000 cervical specimens[18].

Biology: The human papillomavirus life cycle begins with infectious virion particles gaining access to epithelial basal lamina, where the virus infects basal cells. The host cell machinery is used to replicate viral genes along with the cellular genome, increasing the number of infected calls and producing multiple episome copies that are maintained in the basal layer. As the HPV-infected keratinocytes differentiate and move toward the epithelial surface, different viral genes are expressed, allowing high viral genome amplification and the expression of the late region genes that encode the viral capsid proteins. As the cells reach the surface, the HPV episomes are packaged within the capsids for final viral assembly and release.

When HPV infects oropharyngeal squamous epithelium, the virus typically enters basal cells in the crypt epithelium overlying the lymphoid tissue of the tonsils (Figure I.1). Initially, the virus induces replication of the infected cells and along with it, replication of the viral genome, resulting in release of infectious virus. Eventually, the infection becomes latent and no infectious virus is produced, although HPV DNA can be detected in desquamated cells. In some individuals, rare malignant transformation occurs,

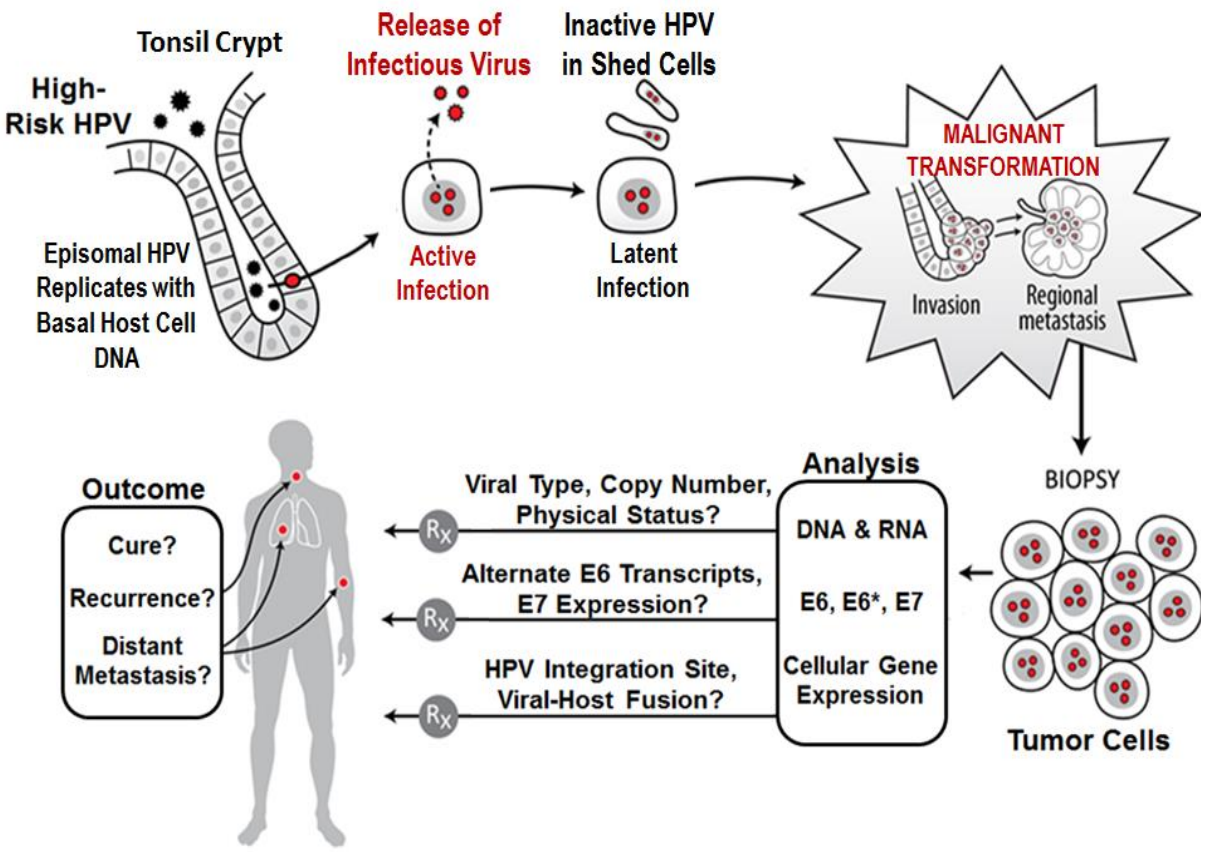

Figure I.1. Schematic Representation of hrHPV Oncogenesis in the Oropharynx.

with HPV E6 and E7 oncogenes driving carcinogenesis. HPV-induced tumor behavior may be influenced by viral type, copy number, or physical status, as well as splicing of the E6 oncogene or where HPV integration occurs in the host cell genome (Figure I.1).

Viral genome: Human papillomaviruses have a circular dsDNA genome of approximately 8kb, with an upstream regulatory region (URR) and eight open reading frames (ORFs) (Figure I.2). The ORFs are divided into early (E1, E2, E4, E5, E6 and E7) and late regions (L1 and L2). In a productive high-risk HPV (hrHPV) infection, a polycistronic messenger RNA is produced, resulting in early region protein expression. E6 and E7 proteins subvert cell cycle control primarily by respectively inhibiting p53 and Retinoblastoma (Rb) protein function. HPV E7 binds to Rb, sequestering it and allowing

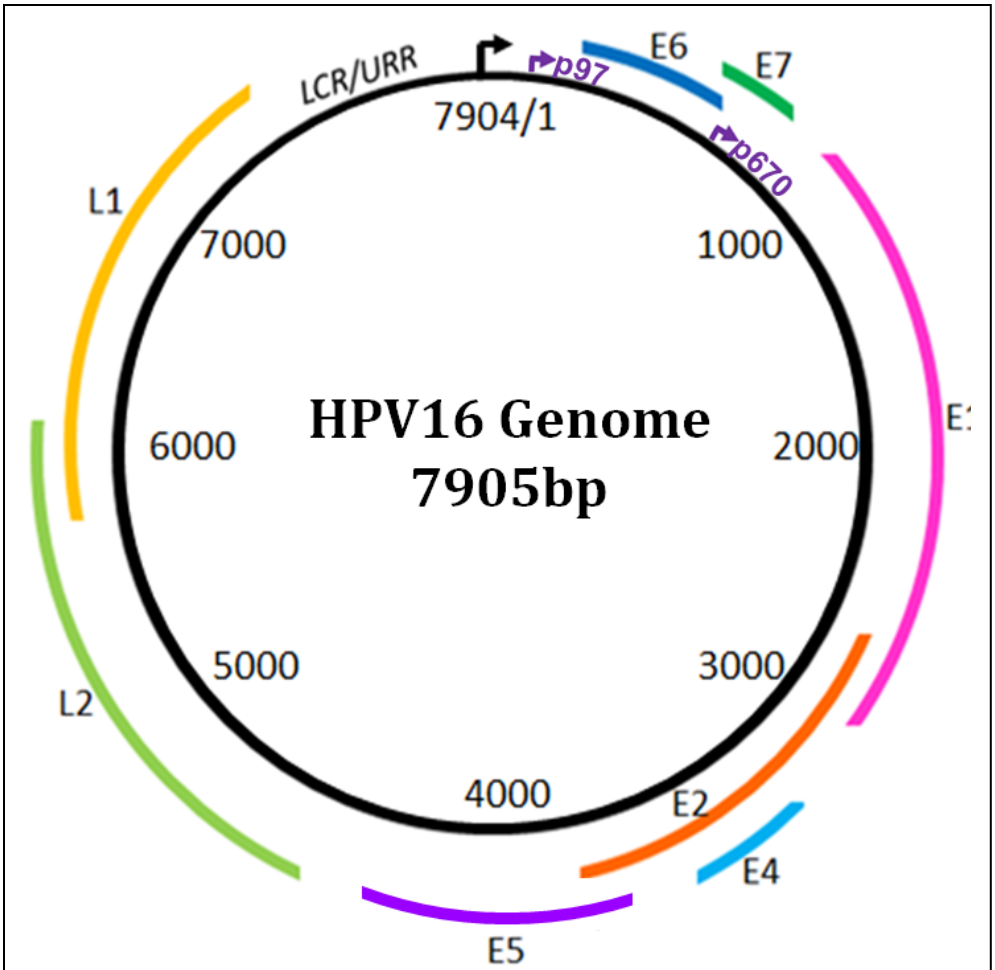

Figure I.2. Genome Organization of HPV16.

the E2F family of transcription factors to induce expression of genes that drive the cell into the cell cycle. In high-risk HPVs, E7 also targets Rb for ubiquitin-mediated degradation, resulting in sustained cell cycle progression. HPV E6 recruits the ubiquitin ligase E6-Associated Protein (E6AP)[19] to p53, thereby removing p53-mediated control of the cell cycle and p53-mediated gene expression. In high-risk HPVs, the E6 interaction with E6AP and p53 results in polyubiquitination, export from the nucleus, and proteasomal degradation of p53. HPV E1 and E2 proteins complex with the host cell polymerase and the viral DNA to drive replication of the viral genome in the infected cells, resulting in production of multiple episomal copies of virus. The HPV E2 protein is also a

transcriptional repressor of E6 and E7[20], which allows the infected cells containing multiple copies of the viral genome to differentiate, express L1 and L2 capsid proteins, and package the viral episomes, creating infectious virus that are released to infect another cell or another host (Figure I.1).

***Oncogenesis*:** The E6 and E7 proteins of the hrHPV types differ from those of the low-risk, non-oncogenic HPV (lrHPV) types and more effectively disrupt the critical cellular growth control mechanisms maintained by Rb and p53, such that infection with hrHPV can lead to malignant transformation[19,21-24]. E7 binding to Rb allows E2F-driven expression of S-phase genes. E7 also inhibits the cyclin-dependent kinase (CDK) inhibitors p27 and p21, deregulating cyclin/CDK complexes that drive progression through the cell cycle and unrestricted cellular proliferation. Considering the large proportion of the population that is infected with hrHPV[25], malignant transformation is comparatively rare, and hrHPV infections are usually cleared within 1-2 years[26]. However, genomic instability from impaired p53 function combined with the unrestricted proliferation induced by the viral oncogenes leads to malignant transformation in a subset of cases.

Most hrHPV-induced cancers are driven by the viral oncogenes and HPV-positive tumor cell lines are dependent on E6 and E7 expression for proliferation and survival[27-29]. Most HPV-positive tumors respond well to therapy, but some do not[30-40]. The factors that differentiate such tumors are poorly understood. Viral integration into the host cell genome is common and may increase risk of cancer by disruption of E2 and higher expression of E6 and E7[41,42]. The low-risk HPV viral oncogenes lack transforming ability and typically cause benign tumors such as vaginal condylomas and laryngeal papillomas, but in rare cases low-risk viruses can lead to malignant transformation[43,44]. In some cases

integration of low-risk types into a host gene results in gene fusion or disruption of the cellular gene and invasive cancer[43,45-49]

Viral copy number: Pre-invasive cervical lesions with high viral copy number are reported to be more likely to progress to invasive cancer[50]. Perhaps multiple copies lead to higher expression of the viral oncoproteins, reduced control of cell replication machinery, more frequent cell division, more replication errors, and a greater chance for integration, disruption of E2, and the development of tumors. Viral integration is implicated in progression from cervical intraepithelial neoplasia (CIN) to invasive cancer in cervical lesions [51]. Integration is thought to increase the risk of malignant transformation through disruption of E2 and upregulated expression of E6 and E7 oncogenes[52] as has been observed in HPV-positive cervical cancer cell lines[50,53] and tumors[41,54]. Nevertheless, Gray et al. report progression to cancer in HPV16 episome-only containing epithelial cells[55]. Our preliminary data[34], and that of Hafkamp et al[56], and Holzinger, et al.[57] suggest that high numbers of viral episomes in oropharyngeal cancer are associated with better response to treatment[40]. It is not known if high episomal viral copy number is sufficient for malignant transformation in oropharyngeal cancer. Hafkamp et al. reported that only 41% of HPV-positive oropharyngeal carcinomas have integrated HPV[56,58]. Thus, high copy episomal number, as occurs in a productive infection, may be associated with increased risk of transformation. We postulate that HPV-positive tumors with high episomal copy numbers[40,57] likely represent the early evolution of the cancer, and consequently better response rates.

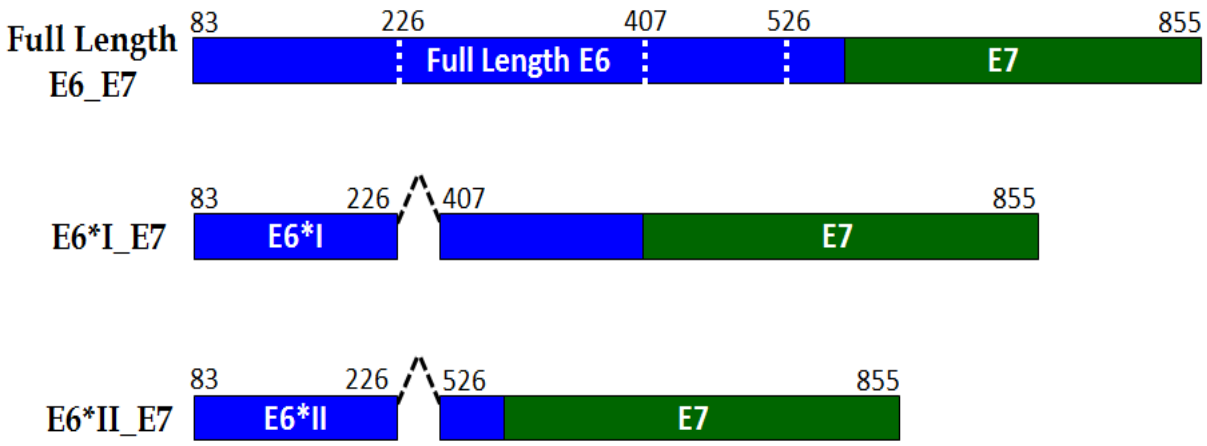

Figure I.3. Illustration of HPV16 E6 Splicing.

Alternate transcription: The HPV genome is transcribed as a polycistronic pre-mRNA with overlapping ORFs. High-risk HPV E6 and E7 oncogenic proteins are drivers of malignant transformation. The E6-E7 transcript has two potential introns, each with three possible 3' splice sites (Figures I.3 and I.4), resulting in multiple coding possibilities[59]. HPV transcript splicing is only seen in high-risk HPV types, suggesting that the splice variants are involved in HPV oncogenesis[60]. The E6 oncogene is expressed as full length or as alternate transcripts designated E6*I and E6*II. Alternate E6* expression has been implicated in oncogenesis [42,52,61] and may be related to viral integration and/or loss of regulation by E2 [41,61,62]. In cervical specimens, high expression of virus[63] has been linked to high-grade CIN and cervix cancer, respectively[61]. HPV E6 alternate splice forms may be a surrogate for the transition from episomal only to integrated virus in the oncogenic pathway. The alternate E6 splice forms may also alter p53 degradation and influence response to therapy in a dichotomous manner. The solution structure of full length E6 dimers was recently elucidated and dimerization was shown to be required for E6-mediated p53 degradation initiated by ubiquitination by E6AP[64]. The amino acids of the E6 oncoprotein that are involved in dimerization (I23, H24, R39, R40, E41, Y43, D44, A46 and

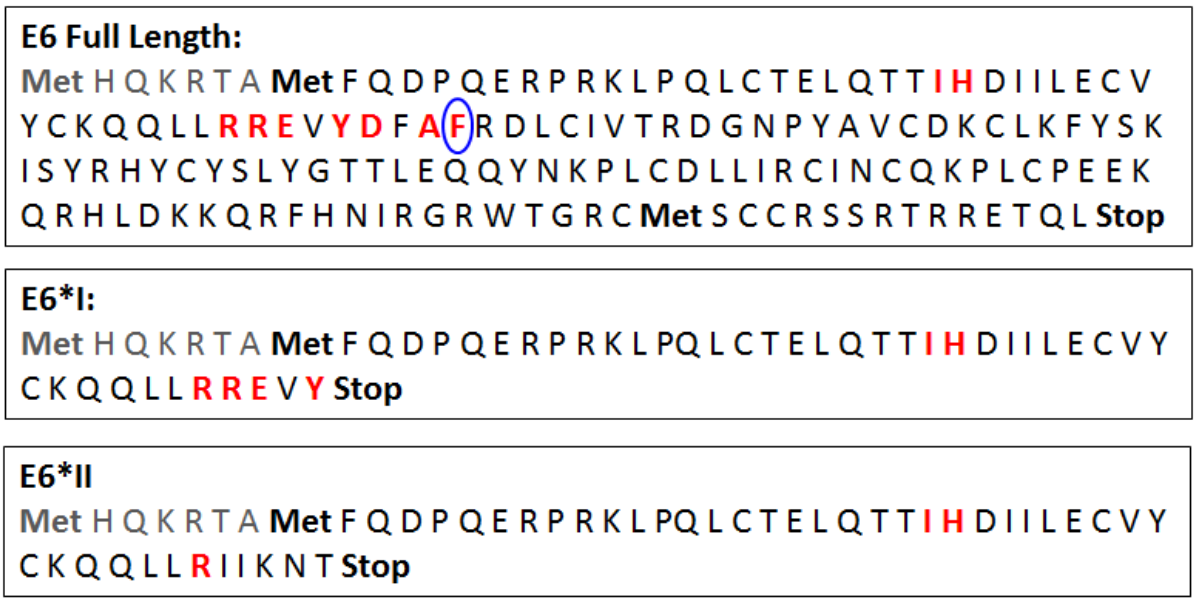

Figure I.4. Amino Acid Sequence of Alternate E6 Oncoproteins. Dimerization amino acids are shown in red. E6*I and E6*II lose the F47 residue (circled in blue) that is essential for E6 dimerization.

F47) are shown in red in Figure I.4. Phenylalanine at amino acid 47 (F47) is essential for E6 dimerization and p53 degradation[65-67]. We compared the amino acid sequences of full length E6 to E6*I and E6*II (Figure I.4). Critical amino acids necessary for dimerization including D44, A46, and F47 are absent in E6*I and E6*II, suggesting that the E6 splice variants are less able to dimerize and less able to induce p53 degradation. The effect of the alternate E6 variants on p53 expression and degradation has not been characterized. In a previous study we found that many HPV-positive that oropharyngeal squamous cell carcinoma (OPSCC) cells overexpress wild type p53[34]. E6 alternate transcripts unable to dimerize and recruit E6AP for ubiquitination of p53 may allow retention of p53 functions such as induction of apoptosis or cell cycle arrest and DNA repair. If p53 is functional in HPV-positive tumors that express the alternate transcripts, p53-induced apoptosis could explain the high response rate of HPV-positive oropharynx tumors to chemotherapy and radiation[30,31,33-40]. However, if the apoptotic pathway is blocked, functional p53 can induce

cell cycle arrest and DNA repair resulting in resistance to chemoradiation (ChemoRT)[68]. We reported that high EGFR expression and the combination of wild-type p53 and high Bcl-xL expression are linked to poorer survival in HPV-positive OPSCC[34]. EGFR up-regulates Bcl-xL which blocks apoptosis[69-71]. Together, wild type p53 and elevated Bcl-xL are associated with resistance to chemotherapy in vivo and in vitro[33,34,68,72,73]. Accordingly, if E6*I and E6*II fail to degrade p53, wild type p53 and anti-apoptotic Bcl-xL can result in ChemoRT resistance via p53-mediated arrest and repair[34,68]. The HPV E7 oncogene is considered to be the dominant transforming gene in HPV-induced tumors[74,75]. Some studies suggest that E7 is expressed exclusively from spliced E6*, and never from full length E6, which is the transcript that produces the functional E6 oncoprotein[24,60,76-81]. Other studies describe E7 oncoprotein translation from full length and spliced E6 in cervical cancer cell lines, as well as detection of the different splice profiles in cervical pre-cancerous lesions, tumor specimens and cell lines[82,83]. The ratio of E6*I to full-length E6 transcript was reported to correlate with increased levels of E7 transcript and E7 protein in HPV-positive cervix cancer cell lines[76], which are derived from tumors that fail to respond to therapy. The relationship between E6* expression, E7 expression, and response to treatment in head and neck cancers has not been studied.

Integration: HPV-positive OPSCC tumors are driven primarily by the powerful viral oncogenes E6 and E7[74], yet these tumors generally respond well to therapy[32], suggesting that patients with HPV-positive tumors that do not respond may have additional factors that lead to their worse outcome, but these factors are unknown. Two studies have shown that viral integration can lead to viral host fusion transcripts[46,84]. The low-risk HPV viral oncogenes lack transforming ability, yet there are examples of cancer developing in

patients with HPV6-[46] or HPV11-induced[45,85] laryngeal papillomas. Integrated HPV6a has been identified in tonsillar carcinomas[44,47] and the carcinogenic mechanism linked to viral integration into the cellular genome. Viral-cellular fusion transcripts and disruption of cellular genes are also reported for hrHPV types[84]. There is a controversy in the HPV literature about the role of cellular gene disruption by integration. Studies in early cervix lesions report that HPV integration is a stochastic event with random sites of integration, often in fragile sites or regions of active chromatin and highly transcribed genes[57,86]. In such early lesions there are few examples of HPV integration affecting cellular genes[86]. However, in more advanced tumors, such events might be important. In the HPV68-containing ME180 cervical cancer line, viral integration resulted in inactivation of *APM-1*, a putative tumor suppressor gene. Re-expression of *APM-1* inhibited growth of HPV-positive cell lines HeLa and CaSki[28]. HPV insertion near *c-myc* has been observed in several cases[29]. HPV-positive anogenital lesions with integration in transcribed genomic regions also had HPV-oncogene-cellular gene fusions in 15 of 19 carcinomas[87]. This suggests that viral integration can interrupt expression of genes that control cellular behavior, or viral-host gene fusion can result in elevated expression of a gene that drives a cancer phenotype. Recent observations from TCGA (The Cancer Genome Atlas) consortium support this hypothesis. Of 30 HPV-positive head and neck squamous cell carcinoma (HNSCC) tumors, three (10%) that have been analyzed exhibit cellular gene disruption. In one tumor, HPV integrated in *RAD51B*, an essential gene for DNA repair, suggesting that the disruption of *RAD51B* contributes to the progression of this tumor. Two other HPV-positive cancers examined by exome analysis had HPV-*ETS* gene fusion transcripts. This is intriguing because *ETS* transcription factor family fusions are drivers and prognostic markers of poor

outcome in prostate cancer[43,48,49] and Ewing's sarcoma[55,88]. These are also targets of therapies being used to interfere with *ETS* family fusions[43,89]. Therefore, it is important to examine HPV integration in HNSCC.

Classification of types: Human papillomaviruses were originally grouped together with closely-related polyomaviruses in the family *Papovaviridae* based on common characteristics, including a double-strand circular DNA genome and non-enveloped capsid. However, these viruses have since been classified into separate families, *Polyomaviridae* and *Papillomaviridae*, due to dissimilar genome size and organization and lack of sequence homology. The taxonomy of the *Papillomaviridae* family is based on sequence comparisons of the L1 ORF, which is the most conserved among papillomaviruses[90-94] (Figure I.5). The human papillomaviruses include alpha, beta, gamma, delta, mu, and nu genera; those with at least 40% homology in the L1 ORF are classified in the same genus (α, β, ɣ, μ & ν). Within genera, HPV species are distinguished by number, share common biological and pathological properties, and demonstrate 60-70% L1 sequence homology. Perhaps best recognized as they relate to human disease are specific HPV types; there are currently over 170 sequenced and classified human papillomavirus types, as well as many additional candidate types yet to be validated. An HPV type is considered unique if the L1 ORF differs at least 10% from the closest known type, HPV subtypes are 2-10% different, and variants are <2% different from the closest known type.

Alpha-papillomaviruses infect mucosal and cutaneous epithelium in humans and primates, include high- and low-risk viruses, and share a conserved genome containing the E5 ORF, which is absent in some of the other genera. Alpha-papillomaviruses contain 15 species and 66 viral types, the most relevant to human disease are species 4 through 10.

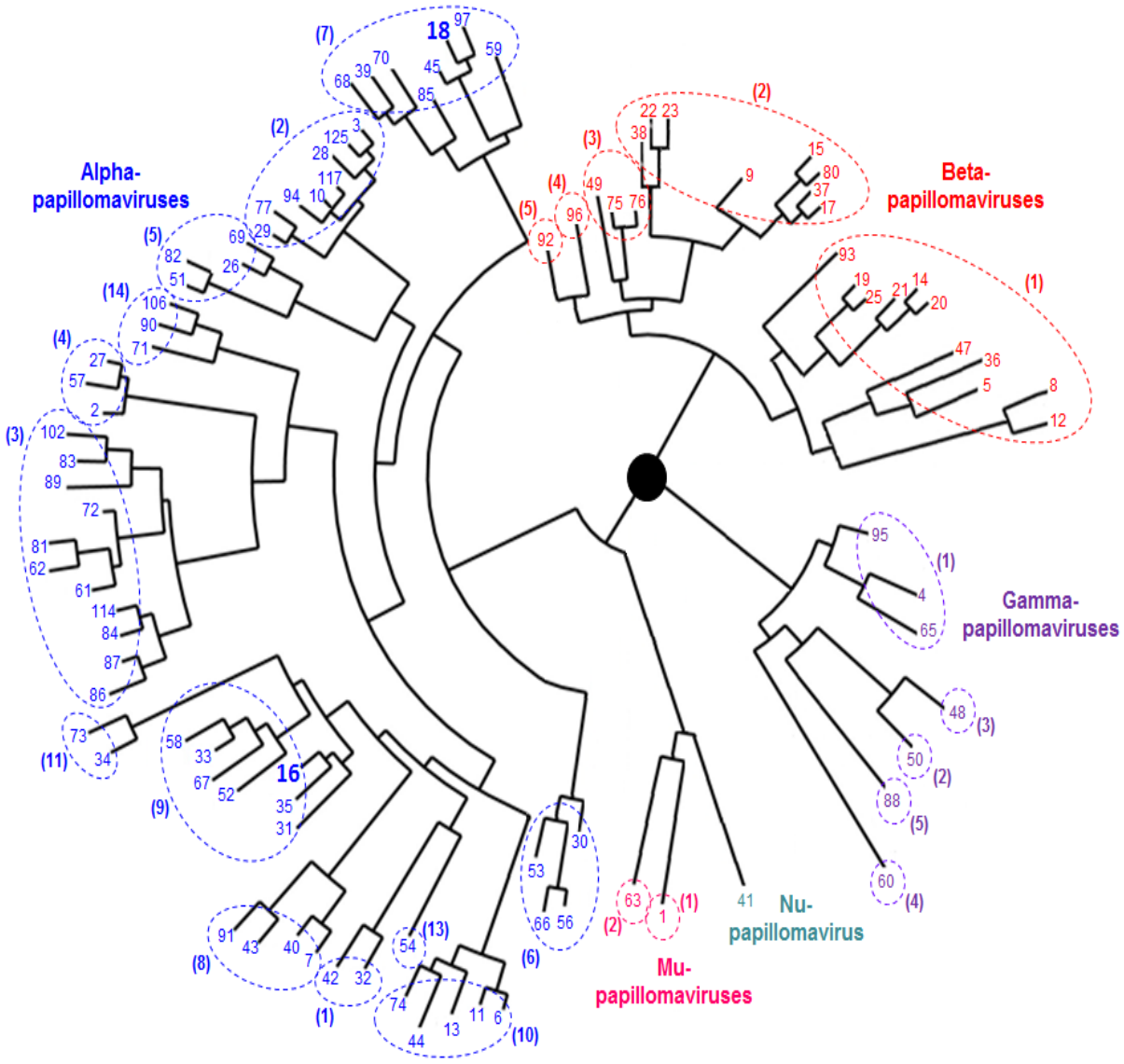

Figure I.5. HPV Phylogenic Tree Based on L1 Sequence Homology. Adapted from de Villiers, E. M. et al. Virology (2004), and White, E. A. and Howley, P. M. Virology (2013).

Species 4 includes HPV2, HPV27, and HPV57, and infection generates common skin warts. HPV26, HPV51, HPV69, and HPV82 are high- and low-risk mucosal types and belong to species 5. Similarly, species 6 contains high- and low-risk mucosal types HPV53, HPV30, HPV56, and HPV66. Species 7 is comprised of high-risk mucosal HPV18, HPV39, HPV45, HPV59, HPV68, and HPV70. Species 8 includes low-risk mucosal and cutaneous types

HPV7, HPV40, and HPV43. The most frequent causal factor in cervical cancer, HPV16 belongs to species 9, along with other high-risk mucosal types HPV31, HPV33, HPV35, HPV52, HPV58, and HPV67. Finally, species 10 is comprised of low-risk mucocutaneous HPV6, HPV11, HPV13, HPV44, and HPV74; these types cause benign laryngeal papillomas, genital warts, and condylomata acuminata, and may become malignant in rare instances.

Beta-papillomaviruses infect cutaneous epithelium in humans, generating mostly benign, but occasionally malignant lesions. These viruses are frequently latent, but become activated in immunosuppressed individuals. The E5 ORF is lacking in this genera. There are 45 HPV types among the 5 β-papillomavirus species. Species 1 and 2 are together referred to as EV HPV types, due to their association with Epidermodysplasia Verruciformis (EV); these types include HPV5, HPV8, HPV9, HPV12, HPV14, HPV15, HPV17, HPV19, and others.

Gamma-papillomaviruses infect human cutaneous epithelium, producing benign lesions that are distinguishable by histological detection of intracytoplasmic inclusion bodies. The viruses in this genus also lack the E5 ORF, and are grouped into 5 species. The most common of the 54 ɣ-papillomavirus types are HPV4, HPV48, HPV50, HPV60, HPV65, HPV88, HPV95, and HPV99.

The mu-papillomaviruses are specific for cutaneous human epithelium and cause benign foot warts, which contain viral type-specific intracytoplasmic inclusion bodies. Genomes of viruses in this genus have relatively large URRs. Each of the two μ-papillomavirus species has only a single HPV type, discernible by the length of the URR; HPV1 (982bp URR) is a member of species 1, and HPV63 (558bp URR) is a member of species 2.

The nu-papillomavirus genus is comprised of single species and type, HPV41, originally isolated from a facial wart[95]. The nu-papillomavirus causes benign and malignant cutaneous lesions in humans, as evidenced by the detection of HPV41 in warts, skin carcinomas, and premalignant keratoses[96]. The HPV41 genome is unique in that it contains several large, uncharacterized ORFs, and has modified E2 binding sites in the URR.

Evolution: Papillomaviruses are highly host-restrictive, suggesting "host-linked" evolution. However, exclusive co-evolution is precluded by 1) the phylogenetic separation of the 5 different genera of HPV (α, β, ɣ, μ & ν), 2) the phylogenetic position of non-human primate papillomaviruses within groups of human papillomaviruses, as opposed to basal, and 3) the phylogenetic incongruence between papillomavirus early and late gene sequences[97,98].

Recent phylogenic analysis has suggested that papillomavirus evolution is driven initially by host niche-specificity, followed by virus-host co-speciation. There are multiple mechanisms thought to influence this process, including gain and possible subsequent loss of early gene sequences or complete open reading frames and recombination[98]. In addition, viral speciation and generation of papillomavirus type variants are caused by lineage fixation, when sequential single-nucleotide polymorphisms (SNPs), insertions or deletions (INDELs) accumulate and become fixed in the genome[99].

High- and low-risk: In early experiments, the genome of hrHPV was shown to transform primary rodent cells (with activated *ras*), immortalize primary human keratinocytes, produce tumorigenic primary keratinocytes with activated *ras*, and alter differentiation of an organotypic raft culture, whereas lrHPV lacked these abilities[100]. The oncogenic potential of high- and low-risk HPV types is based on the E6 and E7 oncoprotein

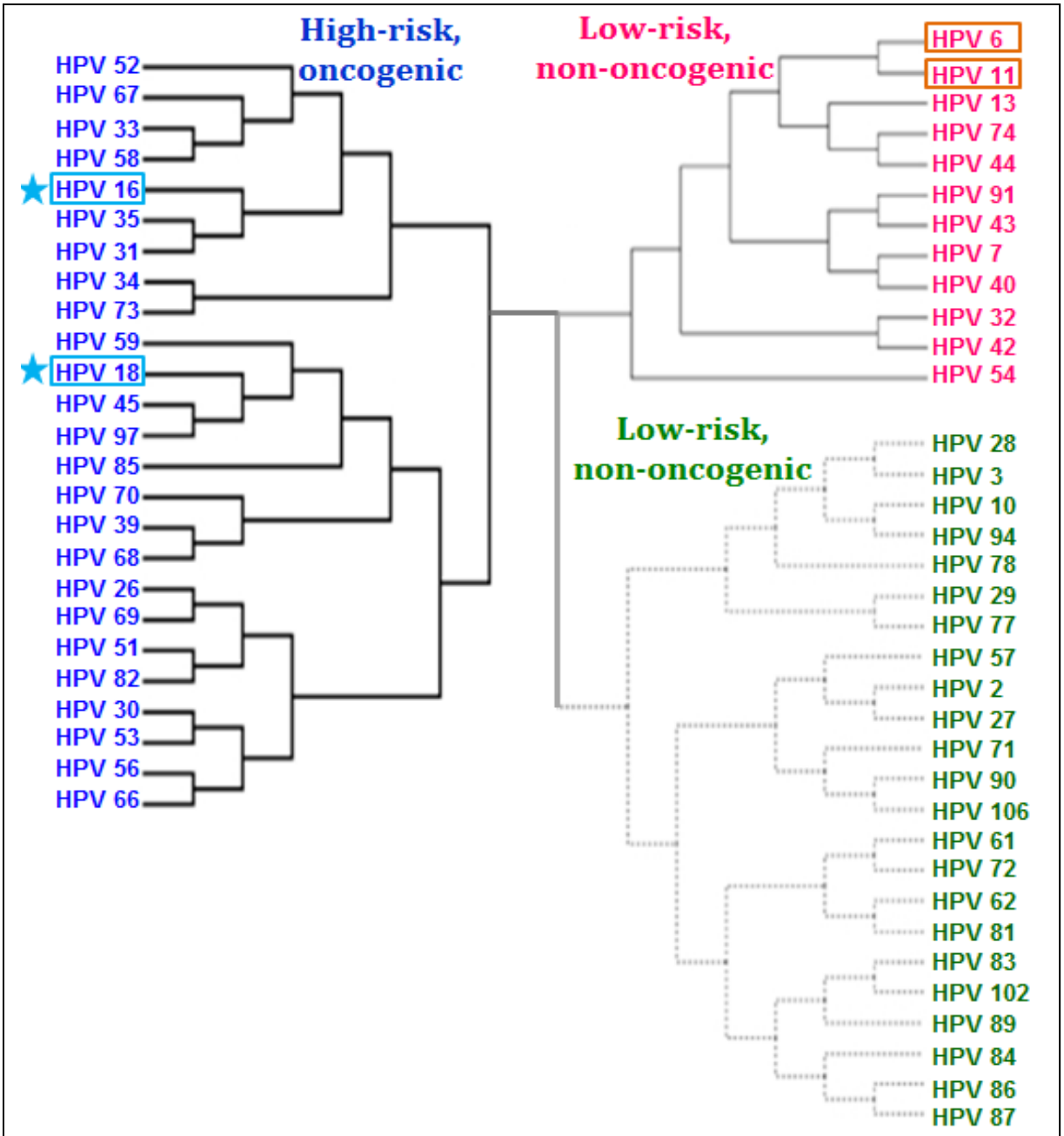

Figure I.6. Phylogenic Tree of Mucosal/Genital Alpha-Human Papillomaviruses. Organization based on early gene sequence homology. Most prevalent high-risk HPV16 and HPV18 outlined in blue and starred, most prevalent low-risk HPV6 and HPV11 outlined in orange. Adapted from Burk, R. D. et al. Public Health Genomics (2009).

activity and cellular interaction, demonstrated in multiple studies by in vitro transfection of E6 and E7[100]. Early gene sequence analysis separates high-risk HPV types from two distinct groups of low-risk HPVs (Figure I.6). It is important to note the difference in

regulation of E6 and E7 transcription and translation between hrHPV and lrHPV that may affect expression levels of the oncoproteins. High-risk HPV has a single promoter region upstream of E6, and produces a polycistronic mRNA, while lrHPV has promoter regions upstream of both E6 and E7. However, when early regions of both lrHPV and hrHPV were expressed from an identical strong promoter, only the hrHPV immortalized keratinocytes, and when high and low-risk E6 and E7 were separately expressed from the same promoter, the resulting lrHPV E6 and E7 protein products were determined to be non-oncogenic[100].

The major functional differences in high- and low-risk HPV oncogenes are summarized in Table I.1[101,102]. High-risk HPV types have E6 splice variants that are not found in low-risk types; spliced transcripts are reported to produce higher levels of E7[24,60,76-81]. Studies comparing lrHPV and hrHPV E6 function demonstrate that both types inhibit p53 transactivation and acetylation, all high-risk but not all low-risk types bind to p53 and E6AP, and only high-risk has been shown to degrade p53[102]. Other oncogenic E6 functions that are exclusive to hrHPV include immortalization of Rb-inactivated human cells, inhibition of keratinocyte differentiation, telomerase activation, c-myc activation, and induction of genetic instability[100]. The hrHPV E6 oncoprotein contains a C-terminal PDZ binding domain, which functions to bind and degrade multiple tumor suppressor proteins such as DLG1, MAGI-1, and Scribble, while all but a few lrHPV types lack this motif[103]. There are similarities in hrHPV and lrHPV E6 function; both bind and degrade pro-apoptotic BAK, both abolish cellular senescence induced by PML (promyelocytic leukemia), and both lrHPV and hrHPV E6 participate in mediating the transition from G1 to S in the cell cycle[100]. The conserved functions of E6 in both lrHPV and hrHPV may be related to viral life cycle and favorable for viral replication.

High-Risk HPV E6	Low-Risk HPV E6
Alternate Transcription *E6*I, E6*II, E6*III* *Higher E7 Expression*	No Splicing *Full Length E6* *Lower E7 Expression*
Strong p53 Binding & Degradation *DNA Damage= Evasion of Growth Arrest* *Inhibition of Apoptosis*	Weaker p53 Binding, No Degradation *DNA Damage= Growth Arrest*
Degradation of PDZ-Domain Proteins *Reduction in Tumor Suppressor Activity*	No Interaction with PDZ Proteins
Telomerase Activation *Immortalization*	No Telomerase Activation
c-myc Activation *Dysregulated Proliferation*	No c-myc Activation
Life-Cycle Associated E6 Similarities	
Interaction with E6-Associated Protein Inhibition of p53 Transactivation and Acetylation	

High-Risk HPV E7	Low-Risk HPV E7
Binding & Degradation of Rb *Continuous Cell Cycle Progression* *Cell Survival*	Weaker Rb Binding, No Degradation
Binding & Degradation of p107 *Continuous Cell Cycle Progression*	Weaker p107 Binding, No Degradation
p21 Binding *DNA Damage= Evasion of Growth Arrest*	Weaker p21 Binding *DNA Damage= Growth Arrest*
Production of Genomic Instability *Cellular Transformation*	No Genomic Instability
Life-Cycle Associated E7 Similarities	
Targeting & Degradation of p130 Binding of p600 Activation of Cell Cycle and DNA Synthesis Binding of Rb for Cellular Proliferation	

Table I.1. Primary Functional Differences and Similarities in High- and Low-Risk Human Papillomavirus E6 and E7 Oncoproteins. Adapted from Doorbar, J. et al. Vaccine (2012) and Pim, D. and Banks, L. APMIS (2010).

The E7 oncoprotein produces genomic instability through a variety of mechanisms that are exclusive to hrHPV. High-risk HPV E7 induces cellular DNA synthesis at the G1 to S transition by binding and targeting Rb for ubiquitin-mediated degradation, disrupting the Rb-E2F complex and releasing the E2F transcription factor. This activity has been shown to be much greater in hrHPV E7 than lrHPV E7, which binds but cannot degrade Rb. The LXCXE protein domain of hrHPV E7 allows specific and high-affinity binding of additional members of the Rb family, p107 and p130, which are subsequently degraded, while lrHPV E7 binds these proteins with much lower affinity, and only degrades p130. The hrHPV E7 disruption of Rb-family complexes causes de-repression of many additional S-phase genes. Additional E7 functions that are present in hrHPV but lacking in lrHPV include activation of the c-fos and p73 promoters and STAT-1 suppression. Furthermore, hrHPV E7 but not lrHPV E7 is able to bypass DNA damage- or differentiation-mediated growth arrest. This difference can be attributed to the inability of lrHPV E7 to degrade Rb and its lower affinity for p21 abrogation, both required for growth arrest evasion. Functions of E7 shared between lrHPV and hrHPV are associated with the viral life cycle, including targeting and degradation of p130, binding p600, activation of cellular pathways for viral genome amplification, and low-level disruption of Rb for cellular proliferation[100].

Disease: Human papillomaviruses have specific tropisms for infection of cutaneous or mucosal epithelium. The type of epithelium infected, together with the oncogenic potential of the HPV, determines the clinical presentation of the associated lesions. The four major categories of HPV involved in human disease (cutaneous benign, cutaneous malignant, mucosal benign, and mucosal malignant), are summarized in Table I.2.

Tissue Tropism	Oncogenic Potential	Associated Human Disease	HPV Types
Cutaneous	Benign	Skin Warts	1, 2, 3, 4, 7, 8, 10, 11
		Epidermodysplasia Verruciformis	3, 5, 8, 10
	Malignant	Extra Genital Bowen's Disease	6, 11, 27, 76 & other hrHPV
		Skin Cancer	EV-related, 16, 31
Mucosal	Benign	Focal Epithelial Hyperplasia	13, 32
		Condyloma Acuminata	6, 11
		Laryngeal Papillomatosis	6, 11
		Bowenoid Papulosis	16, 18 & other hrHPV
	Malignant	Genital Bowen's Disease	16, 31
		Anogenital Cancers	16, 18 & other hrHPV
		Head and Neck Cancers	16, 18 & other hrHPV

Table I.2. Summary of HPV-Induced Human Disease.

Cutaneous benign: Benign cutaneous HPV conditions include skin warts caused by HPV1, HPV2, HPV3, HPV4, HPV7, HPV8, HPV10, and HPV63. Juvenile epidermodysplasia verruciformis, an autosomal recessive immune disorder associated with HPV infection is classified as benign cutaneous, causing plane warts (HPV3 and HPV10) and verrucous lesions (HPV5 and HPV8).

Cutaneous malignant: In adults, EV transitions to a malignant cutaneous condition, with squamous cell carcinoma developing in 30-60% EV patients by the age of 40[104,105]. This malignant transformation occurs in association with UV sun exposure and the primary HPV types involved are HPV5, HPV8, HPV14, HPV17, HPV20 and HPV47[106]. HPV-induced

cutaneous squamous cell carcinomas also occur independent of EV. These tumors are most frequently associated with UV sun exposure and caused by beta-HPV types[107] and mucosal types HPV16 & 31[108]. HPV-induced skin tumors are seen with much higher frequency in immunocompromised patients; a 200-fold increased incidence of squamous cell carcinoma (SCC) is reported in renal transplant recipients[105], and the proportion of HPV-positive SCC in immunocompromised patients is 88-100%[104,109,110]. Cutaneous SCCs are far less frequent in immunocompetant patients, and fewer (30-78%) are HPV-positive[105,107].

Extra Genital Bowen's Disease (EGBD) is a cutaneous squamous cell carcinoma in situ, and up to 5% of cases progress to invasive carcinoma[104]. Both high- and low-risk HPV types have been associated with EGBD[111].

Mucosal benign: Focal epithelial hyperplasia (Heck's disease) is a benign mucosal condition caused by HPV13 or HPV 32, and presents with multiple pink papules on the oral mucosa[104]. Condylomata acuminata (anogenital warts) are most commonly caused by HPV6 or HPV11, although other low-risk types, and occasionally high-risk HPV types have been detected, and 10-15% of lesions contain multiple HPV types[104].

Laryngeal papillomatosis (recurrent respiratory papillomatosis) is associated primarily with HPV6 and HPV11, with fewer than 5% of cases attributed to other types[112]. This condition is characterized by persistent growth of papillomas in the larynx; juvenile prevalence is linked to maternal genital warts during pregnancy and delivery[113]. In approximately 16% of cases (primarily associated with HPV11), recurrent respiratory papillomatosis can spread to the bronchi and lungs, and of these, 1-7% develop pulmonary malignant transformation[85,114,115]. Rare cases (2%) of malignant conversion outside of

pulmonary involvement have been reported, and have been associated with either lrHPV6 viral integration[46] or lrHPV11 infection[45,85].

Bowenoid papulosis is characterized by anogenital papular lesions classified as undifferentiated intraepithelial neoplasia. HPV16, HPV18 and other hrHPV types are detected in 66.7% of cases[108], and malignant transformation is rare (2-3%)[104].

Mucosal malignant: The most prevalent malignant HPV-related disease involves the mucosal epithelium of the anogenital region. Genital Bowen's disease is a condition of genital carcinoma in situ, most commonly associated with HPV16[116], but HPV31 has also been detected[108]. Vulvar cancer is fairly uncommon, representing only 3% of worldwide gynecological cancers[104]. High-risk HPV was detected in 97.1% of vulvar intraepithelial neoplasia 3 (VIN3) cases, and in 68.8% of invasive vulvar cancer (IVC), with the majority of HPV-positive cases identified as HPV16[117,118]. Vaginal and penile cancers were 60-90% and 30-70% HPV-positive, respectively, with HPV16 the prominent type detected[118-120]. The HPV type distribution of anal cancer, 80-96% of which are HPV-positive, has been reported to be 70% HPV16, 5% HPV18, and remaining 25% HPV6, HPV31, or HPV33[104,118]. High-risk HPV is a necessary cause in 100% of cervical cancers, and 61% are induced by HPV16, with the remaining HPV-positive cases identified as non-HPV16 high-risk types or multiple infections[121-124]. No significant difference in odds ratio was seen for infection with multiple HPV types compared to infection with a single type, although the highest odds ratio reported was for multiple-type infections that include HPV16[121]. Smoking is a significantly associated carcinogenic risk factor for HPV-positive, but not HPV-negative, vulvar and vaginal squamous cell carcinomas[125]. Additionally, smoking is a cofactor in the progression

of HPV-positive, low-grade squamous intraepithelial lesion (LSIL) to high-grade squamous intraepithelial lesion (HSIL) and cervical cancer[126].

Head and neck cancers are also associated with mucosal HPV. Among head and neck tumors, OPSCC is most frequently associated with hrHPV. The worldwide proportion of HPV-positive oropharyngeal tumors is increasing; prior to 2000, 40.5% of OPSCC were HPV-positive, and this has increased to 72.2% between 2005 and 2009[127], and in our recent study, we found 83% of OPSCC to be HPV-positive[128]. Among the HPV-positive oropharynx tumors, 80-90% contain HPV16[124,128]. Although the basis for this disparity is not understood, HPV-positive oropharynx tumors respond better to therapy than HPV-negative tumors, and patients with HPV-positive tumors have better outcome than patients with HPV-negative OPSCC[30,32-40].

The predictive value of HPV in non-oropharynx HNSCC is largely unstudied, likely due to the far lower proportion of HPV-positive tumors at these sites[128]. A recent study by Isayeva et al. reported the weighted prevalence of HPV-positive tumors in non-oropharynx HNSCC as 20.2% in oral cavity tumors, with HPV16 the most common type detected; 23.6% in larynx tumors, again HPV16 was most frequently detected, but larynx tumors contained a much greater diversity of hrHPV types than oral cavity or oropharyngeal tumors; 29.6% in sinonasal cancers; 20.5% in nasopharyngeal cancers; and 47% in salivary neoplasias (mucoepidermoid carcinomas), which all contained HPV16, HPV18, or both[129].

Patients with HPV positive head and neck tumors are far more likely than patients with HPV-negative tumors to be non-smokers[34,130]. However, in our own data we observed that over two thirds (68%) of patients with HPV positive tumors were tobacco users and that current tobacco users were significantly more likely to suffer recurrence than never-

users (Hazard Ratio= 5.2, Confidence Interval 1.1-24.4; significance, p=0.038)[36]. Tobacco use (synergistic with alcohol use) persists as the predominant etiologic factor in HNSCC tumors that are negative for HPV[131-133].

Head and Neck Squamous Cell Carcinoma

Epidemiology: In a recent comprehensive examination of oral HPV infection in the United States[25], the prevalence of oral HPV infection in adults was 6.9%, hrHPV was 3.7%, and HPV16 was 1.0% (men and women, 14-69 years old). Men were more likely to have oral HPV infection than women (10.1% and 3.6% respectively), and prevalence by age distribution showed bimodal peaks at 30-34 and 60-64 years old, with the highest prevalence at 55-64 years old. The transmission of HPV through sexual contact is demonstrated by the significant association of HPV infection and reported sexual activity, categorized and reported as any sex, vaginal sex, oral sex, and anal sex. Prevalence of oral HPV was more than 8-fold greater in those reporting any sexual history than those reporting no sexual history. The prevalence of oral HPV infection increased in association with number of past or recent sexual partners, reaching a maximum oral HPV prevalence of 20% among those reporting greater than 20 lifetime sexual partners[25]. The direct relationship between oral HPV infection and lifetime number of oral sex partners has been demonstrated in multiple studies, and an analysis of concurrent oral and genital HPV infection and HPV-type concordance in women together demonstrate the multidirectional transmission corridors between anogenital and oral HPV infections[25,134,135].

HPV detection methods: The incidence of cervical cancer is in a state of steady decline, due to routine cytological screening, histological biopsy assessment, and hrHPV

testing, all leading to diagnosis and treatment of early lesions[124]. Unfortunately, there are currently no equivalent methods for early detection in HNSCC. With the increasing prevalence of hrHPV in HNSCC and the predictive value of hrHPV in tumor response to therapy, a gold-standard HPV test is required for quick, robust, and reliable assessment of each tumor biopsy. There are two primary categories of HPV tests that are available for use on tumor specimens: signal hybridization and target amplification assays.

The signal hybridization group includes 3 methods for detection of HPV DNA 1) In situ hybridization (ISH), such as the Inform HPVIII test, which detects 16 hrHPV types with pooled DNA probes, 2) the Digene Hybrid Capture 2 test, which employs RNA probes (13 hrHPV and 5 lrHPV types in separate reactions), followed by labeled antibodies to DNA/RNA hybrids, and 3) the Cervista Invader test, which detects 14 hrHPV types through adjacent hybridization of probe and invader oligonucleotides (overlapping by one nucleotide base), forming a triplex structure. The triplex is enzymatically removed, generating a reporter signal in the subsequent reaction.

Additional signal hybridization tests involve protein detection by immunohistochemistry (IHC). Immunohistochemical staining for $p16^{INK4a}$ provides a surrogate marker for HPV E7 activity, and IHC for HPV E6 and E7 oncoproteins is also used for visualization of oncoprotein expression.

Application of HPV target amplification tests is broader than that of the hybridization tests; the first is consensus PCR of HPV DNA, which theoretically detects any HPV types present, using pooled primer sets to amplify HPV DNA. Consensus primer assays include degenerate L1 primers to amplify 450bp amplicons (MY09/MY11), overlapping L1 primers to amplify 450bp amplicons (PGMY), a single pair of L1 primers

under very low stringency PCR conditions to generate 150bp amplicons (GP5+/6+), L1 primers to amplify short 65bp amplicons (SPF10), and the RealTime HR HPV assay, which uses real-time PCR for HPV16- and HPV18-specific detection and pooled detection for other hrHPV types.

The Aptima HPV test uses consensus reverse-transcription PCR of RNA to detect E6-E7 transcripts of 14 hrHPV types. The other HPV RNA detection assay is the HPV Proofer test, which combines RNA Nucleic Acid Sequence Based Amplification (NASBA) with type-specific molecular beacons for 5 hrHPV types.

HPV genotyping commonly combines target amplification with signal hybridization. Midrange genotyping is achieved with the BD Viper Assay, using multiplex real-time PCR to detect HPV (consensus primers) plus individual typing 6 hrHPVs. Consensus PCR is routinely the first step in full genotyping methods. GP5+/6+-PCR-EIA amplifies a region of L1 with GP5+/6+ consensus primers and identifies specific HPV types by probe hybridization and enzyme immunoassay. Consensus PCR followed by hybridization of amplicons to type-specific probes immobilized to nylon strips is the method employed by the Linear Array (PGMY consensus primers) and InnoLiPA (SPF10 consensus primers) tests.

HPV genotyping can also be performed by microarray methods. The Papillocheck HPV test combines PCR amplification of the E1 region with hybridization to 24 lrHPV and hrHPV type-specific probes on a low-density microarray, which generates a fluorescent signal for any of the types present in the sample. Similarly, the CLART HPV2 test amplifies and biotinylates a portion of the L1 region, followed by hybridization to type-specific probes in a low-density microarray; an enzymatic color reaction indicates the presence of

up to 35 HPV types. The high-risk HPV multiplex PCR-MassArray method that is used in our group detects and identifies 16 hrHPV and 2 lrHPV subtypes using type-specific, multiplex, competitive PCR and single base extension followed by MALDI-TOF (Matrix-Assisted, Laser Desorption/Ionization- Time of Flight) Mass Spectrometry analysis. Finally, the Multimetrix HPV genotyping test uses Luminex technology for identification of up to 100 different HPV targets; color-coded microsphere beads are covalently attached to type-specific HPV probes and are identified by flow cytometry when the target is present.

There are benefits and challenges associated with each HPV detection method used. There is no one best method; determination of the most appropriate test is often dependent on sample availability and state (formalin-fixed, paraffin-embedded (FFPE) or fresh-frozen). Among signal hybridization methods, none of the assays identify specific HPV types in the sample. HPV ISH is optimized for FFPE tissues and provides a visual demonstration of viral distribution, but lacks specificity and interpretation may be subjective. The Hybrid Capture II and Cervista tests are both solution hybridization assays approved for cervical screening, but may not be suitable for tissue analysis. IHC for $p16^{INK4a}$ is optimized for FFPE tissues and frequently represents active virus, but has low specificity. HPV E6 and E7 IHC allows a visual correlate to viral oncoprotein expression, but performance of the current assay is poor. A common difficulty encountered with target amplification methods is the need for tissue processing to obtain the DNA or RNA required for the assay. All of the consensus PCR methods have high sensitivity, but are time- and labor- intensive and are unable to identify HPV types present. RNA-based target amplification methods are informative in that they exclude latent or inactive HPV that would be detected by DNA methods, but are severely limited by the requisite fresh-frozen

tumor specimen for suitable RNA isolation. The more complex genotyping methods provide the greatest information, and are highly sensitive and specific, but typically require expensive specialized instrumentation or assay platforms that may not be available or obtainable.

Oropharynx: The incidence of OPSCC is increasing as smoking-related cancers decrease. The increase is due to hrHPV-induced disease[136,137], which has emerged as the primary etiologic factor in OPSCC, surpassing tobacco and alcohol in this type of head and neck squamous cell carcinoma[31,34,36,40,136-140]. The incidence of oropharyngeal cancer is predicted to reach 13,000 cases in the US in 2013; 65-85% HPV-positive with expected 3-year failure rates of 30-36%[30]. There is growing controversy over the optimal strategy for oropharynx cancer treatment, given the evolving epidemiology. Early lymph node metastasis is common in HPV-driven head and neck cancers and most patients present with advanced disease. Response to therapy and survival is dramatically better in patients with HPV-positive tumors than in those with HPV-negative oropharynx cancer[30-40]. Because of this, many clinicians advocate for a reduction in treatment intensity for patients with HPV-induced cancers to minimize post-treatment morbidity[141,142]. However, a reduction in treatment intensity risks the possibility of under-treatment, tumor progression, and metastasis in patients who might have been cured with the current intensity of treatment. Under current treatment regimens, a subset of 20-30% of patients with HPV-induced tumors fail to respond to therapy and develop distant metastasis[143]; this subset would likely increase with a reduction in treatment intensity.

Nasopharynx: The primary etiologic agent of non-keratinizing type II and III nasopharyngeal carcinoma (NPC) is the Epstein-Barr herpesvirus (EBV); however,

keratinizing type I NPC is not associated with EBV, indicating an alternate pathogenic mechanism[144,145]. The thin epithelium in the crypts of the palatine and lingual tonsils in the oropharynx is very similar to the epithelium of the adenoidal tonsil in the nasopharynx. Both pharyngeal sites contain abundant lymphoid cells and both are part of Waldeyer's ring of lymphocytic tissues. These tissue similarities have recently prompted interest in the possibility of HPV as an alternate etiologic agent of NPC. Several studies have reported HPV-positive NPC, most frequently as HPV-positive/EBV-negative tumors[146-151]. A recent study at our institution (unpublished data, Stenmark et al., 2013) has demonstrated mutually exclusive EBV and HPV in NPC tumors, and that both EBV-positive and HPV-positive tumors were predominantly non-keratinizing type II and III, while EBV-negative/HPV-negative tumors were largely keratinizing type I NPC. The results of this study also demonstrate that among nasopharynx cancer patients treated at the University of Michigan, those with HPV-positive nasopharyngeal tumors had poorer outcome than EBV-positive patients (unpublished data, Stenmark et al., 2013).

Oral cavity: The possible role for hrHPV in the etiology of oral cavity squamous cell carcinoma (OCSCC) is currently undefined. The role of hrHPV in carcinogenesis of the oral cavity is further obscured by discordant findings between $p16^{INK4a}$ IHC and HPV testing in these tumors. While HPV is detected in relatively few OCSCC tumors, the majority of these tumors exhibit overexpression of p16. It was recently reported that the majority of p16-positive OCSCC tumors were negative for hrHPV, making $p16^{INK4a}$ IHC an unsuitable surrogate for HPV detection[152,153]. Reports that patients with OCSCC have poorer outcome than patients with HNSCC at other sites, regardless of HPV status[152], suggest that HPV may not be a primary carcinogenic driver in these tumors.

Larynx: Tobacco and alcohol remain the primary etiologic agents in laryngeal carcinogenesis[154]. While hrHPV has been detected in a subset of larynx cancers, it has also been reported to be present in benign and normal larynx tissue, likely representing latent infections[155]. A recent study by Halec et al. assessed laryngeal carcinoma specimens for active HPV involvement, examining hrHPV DNA, HPV E6*I transcripts, and p16 expression[154]. They found that 32 of 92 (35%) samples were positive for HPV16 DNA, 6 of 30 (20%) samples had HPV16 E6*I transcripts, and p16 expression was seen in 4/75 (5%) of cases, with correlation between analyses. The authors conclude that the agreement between HPV16 DNA, HPV16 E6*I transcripts and p16 expression provides evidence for a causal role of HPV16 in this subset of laryngeal tumors[154]. Involvement of HPV in laryngeal carcinogenesis is further supported by detection of integrated hrHPV in laryngeal tumors[156].

Data Chapter Overview

In this dissertation, we examined high-risk human papillomavirus-driven carcinogenesis in HNSCC. In Chapter II we present evidence that the HPV PCR-MassArray assay, together with $p16^{INK4a}$ immunohistochemical staining, is the optimal assessment for HPV detection, typing, and viral oncogene activity in formalin-fixed, paraffin-embedded HNSCC tissue biopsies. In chapter III, we evaluated seven HPV16-positive HNSCC cell lines. Each cell line expressed HPV E6 and E7 oncogenes and demonstrated alternate splicing, indicating active viral oncogenesis. Additionally, each cell line exhibited viral integration into known cellular genes, including cancer related genes *TP63*, *DCC*, *JAK1*, *TERT*, *ATR*, *ETV6*, *PGR*, *PTPRN2*, and *TMEM237*. This assessment was extended into HNSCC tumors in

chapter IV, where we examined HPV oncogene expression and viral integration in five tumors that were responsive to treatment and five that did not respond to treatment and recurred. All of the tumors demonstrated active viral oncogenesis, indicated by expression of HPV E6 and E7 oncogenes and alternate E6 splicing. In the responsive tumors, HPV integration was found in numerous extragenic chromosome regions, as well as one integration event into a known cancer-related gene, *TP63*. In the recurrent tumors, two HPV integration events were found in extragenic regions on chromosome 10, and each recurrent tumor exhibited HPV integration into known cellular genes, including cancer-associated genes *TNFRSF13B, SCN2A, SH2B1, UBE2V2, SMOC1, NFIA,* and *SEMA6D.*

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CHAPTER II

High-risk Human Papillomavirus Detection in Oropharyngeal, Nasopharyngeal, and Oral Cavity Cancers: Comparison of Multiple Methods

Abstract

Human papillomaviruses are now recognized as an etiologic factor in a growing subset of head and neck cancers and have critical prognostic importance that affects therapeutic decision making. There is no universally accepted gold standard for high-risk HPV (hrHPV) assessment in formalin-fixed, paraffin-embedded (FFPE) tissue specimens, nor is there a clear understanding of the frequency or role of hrHPV in sites other than oropharynx.

The objective of this study was to determine the optimal assessment of hrHPV in FFPE head and neck tumors. We tested 338 FFPE head and neck squamous cell carcinoma (HNSCC) specimens from the oropharynx (OPSCC), nasopharynx (NPC), and oral cavity (OCSCC) for hrHPV, using $p16^{INK4a}$ immunohistochemical (IHC) staining, HPV in situ hybridization (ISH), and PCR-MassArray (PCR-MA). We used PCR with HPV L1 PGMY consensus primers (L1 PGMY-PCR) and sequencing to resolve method discordance. Relative sensitivity and specificity were compared to develop a standard optimal test protocol.

Using combined PCR-MA with L1 PGMY-PCR and sequencing for conclusive results, we found PCR-MA to have 99.5% sensitivity and 100% specificity, p16 IHC to have 94.2% sensitivity and 85.5% specificity, and ISH to have 82.9% sensitivity and 81% specificity.

Among HPV-positive tumors, HPV16 was most frequently detected, but 10 non-HPV16 types accounted for 6-50% of tumors, depending on site. Overall, 86% of oropharynx, 50% of nasopharynx and 26% of oral cavity tumors were positive for hrHPV.

This work demonstrates highlights the advantages of the HPV PCR-MA assay, including low DNA (5ng) requirement, efficacy for small tissue sample testing, high throughput, and rapid identification of HPV types. The PCR-MA assay also has the highest sensitivity and specificity of the methods tested. PCR-MA together with $p16^{INK4a}$ provided accurate assessment of HPV presence, type, and activity, and was determined to be the best approach for HPV testing in FFPE head and neck tumors.

Introduction

The role of carcinogenic hrHPV in the etiology of head and neck cancer has been increasing in significance over the past 20 years[1-5]. In our institution, 80 to 90 percent of oropharyngeal cancers are HPV-positive[6], and evidence for hrHPV in head and neck squamous cell carcinoma (HNSCC) of other sites is also increasing[5,7-9]. Generally, HPV-positive oropharyngeal cancers exhibit better responses to treatment than do HPV-negative tumors[6,10-18]. A recent trial conducted in our institution using concurrent platinum-taxol based chemotherapy and intensity modulated radiation therapy resulted in 88% three year progression-free survival among oropharynx cancer patients with stage 3 and 4 disease[19]. However, a recent report from Belgium reported that survival among HPV-positive patients with oral cavity cancer was worse than their HPV-negative counterparts[9]. Similarly, among nasopharynx cancer patients treated at our institution,

those with HPV-positive nasopharyngeal tumors had poorer outcome than those with EBV-positive tumors (unpublished data, Stenmark et al., 2013).

Many reports indicate that HPV-positive tumors with transcriptionally-active viral oncogenes are those most likely to respond well to treatment[20-22]. In contrast to low-risk HPV types such as HPV6 and HPV11 which also infect mucosal epithelia but rarely cause cancer, the high risk HPV types HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, have all been implicated in oncogenesis[23-27]. This difference between low and high-risk HPV types is due in part to the nature of the E6 and E7 viral oncogenes that exhibit alternate splicing in high-risk HPV, resulting in transforming capacity. Thus, for precision medicine[28] it is important to assess not only the presence of HPV16 but also other hrHPV types. This will be essential to accurately determine the most effective treatment option for each patient based on their individual tumor characteristics. Optimally, viral oncogene activity is determined using high quality tumor RNA[20,21] to identify alternate transcripts linked to transformation[20] or assessing HPV E6 and E7 indirectly by detection of patient antibodies to E6 and E7[22]. However, availability of fresh frozen tumor tissue or access to serologic assays is rare, whereas fixed tumor from the diagnostic biopsy is more readily accessible. Therefore, it is essential to have robust and accurate testing methods using FFPE materials to complement the histopathologic and clinical staging data to arrive at the optimal therapeutic plan.

Multiple methods of HPV detection and assessment are widely used but the optimal testing method has yet to be clearly defined. Immunohistochemical detection of highly expressed $p16^{INK4a}$ is a widely used surrogate method for the presence of HPV in a tumor. This biomarker is indicative of hrHPV E7 oncogene expression, which upregulates p16

through disruption of the Rb/E2F transcription factor complex. However, p16 can be upregulated by mechanisms other than HPV, leading to false positive assessment of HPV in the sample[9,29,30]. In head and neck cancers, p16 is also one of the most frequently lost genes, and as such could provide a false negative evaluation of the presence of HPV[31-36]. Direct detection of HPV DNA in tumor cells by ISH is also widely used in pathology departments, and has the advantage of identifying the presence and location of the viral DNA within tumor cells, but this method lacks sensitivity[37]. Polymerase chain reaction (PCR) based methods are highly advantageous because they require little DNA, but these often lack the ability to detect multiple high-risk types or to identify the high-risk type present, a shortcoming shared by both p16 assessment and in situ hybridization. Several years ago, Yang et al. [38] developed a multiplex HPV PCR-MA assay that allows highly sensitive detection of multiple HPV types and allows specific HPV type identification in a single assay. We have used this assay extensively in our studies on hrHPV in head and neck cancers[6,7,11,12,14].

Because many HPV-positive tumors respond well to therapy, there are national and local efforts to significantly decrease treatment intensity for patients with these tumors to spare them from unnecessary treatment morbidity. However, even with very aggressive concurrent chemoradiation therapy, a subset of 20-30% of patients with HPV-positive oropharyngeal cancer progress either locally or with distant metastases. It has been suggested that the HPV-positive tumors that are driven primarily by the HPV viral oncogenes are the most likely to have a good response to treatment. Thus, there is a growing need for reliable and rapid tests for detection of transcriptionally active HPV in head and neck cancers to select patients for the most appropriate treatment based on their

own tumor characteristics. In this study we compared three commonly used HPV assessment tools: HPV PCR-MassArray for 15 high-risk HPV types, HPV in situ hybridization for 12 high-risk types, and p16 IHC staining. We then used consensus L1 PGMY-PCR[39,40] and sequencing to resolve discordant results for tumors that were HPV-negative by PCR-MassArray but p16-positive or ISH-positive. From our results we propose an optimal HPV detection/identification algorithm.

Methods

Patient specimens: All patients provided written informed consent to study their tissue under a study approved by the Institutional Review Board for the medical school. Tumor specimens from 338 advanced stage head and neck cancer patients enrolled in the Head and Neck SPORE were obtained and evaluated, including 212 oropharyngeal, 18 nasopharyngeal, and 108 oral cavity cancers. FFPE tumor cores from pretreatment biopsies and/or post-treatment recurrences (when available) were used to construct tissue microarrays for in situ hybridization and immunohistochemical staining. FFPE tumor cores were also taken from each tumor block at the time of array construction for genomic DNA extraction.

$p16^{INK4a}$ immunohistochemical staining: Assessment of $p16^{INK4a}$ was performed per supplier protocol (CINtec $p16^{INK4a}$ Histology Kit; mtm Laboratories). Antibody binding was scored by an experienced head and neck pathologist (J.B.M.), using a continuous scale for the proportion of tumor cells demonstrating nuclear and cytoplasmic p16 staining. Percentage scored was divided into a quartile scale of 1 to 4 (1 was less than 5%; 2, 5%-20%; 3, 21%-50%; and 4, 51%-100% tumor staining). Intensity was also scored on a scale

of 1 to 4 (1 equal to no staining; 2, low intensity; 3, moderate; and 4, high intensity). Staining for p16 was considered positive when the proportion score was equal to 4 and the intensity score was 3 or 4. Examples of $p16^{INK4a}$ staining are shown in Figure II.A, II.C, and II.E.

HPV in situ hybridization (ISH): HPV in situ hybridization was performed per supplier protocol (INFORM HPVIII ISH assay; Ventana) in our pathology laboratory and scored by a pathologist (J.B.M.). The assay includes probes for 12 hrHPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66. Examples of HPV ISH are shown in Figure II.B, II.D, and II.F.

HPV PCR-MassArray (PCR-MA): Genomic DNA was extracted from FFPE tumor cores using the DNeasy Blood and Tissue Kit (Qiagen). Samples were assayed in quadruplicate using a validated, ultra-sensitive method of real-time competitive polymerase chain reaction, followed by probe-specific single base extension. Multiplex PCR amplification of the E6 region of 15 discrete high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 73), as well as a human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control were processed to saturation, followed by a shrimp alkaline phosphatase quenching. Amplification reactions included synthetic competitor oligonucleotides identical to each natural amplicon except for a single nucleotide difference. Multiplex single base extension reactions used probes to identify unique sequences in the amplified E6 region of each hrHPV type. Single terminal base extension was designed to create a 40 to 80 Da difference in mass between each competitor and wild type extension product. These were then analyzed by mass separation in assay-defined

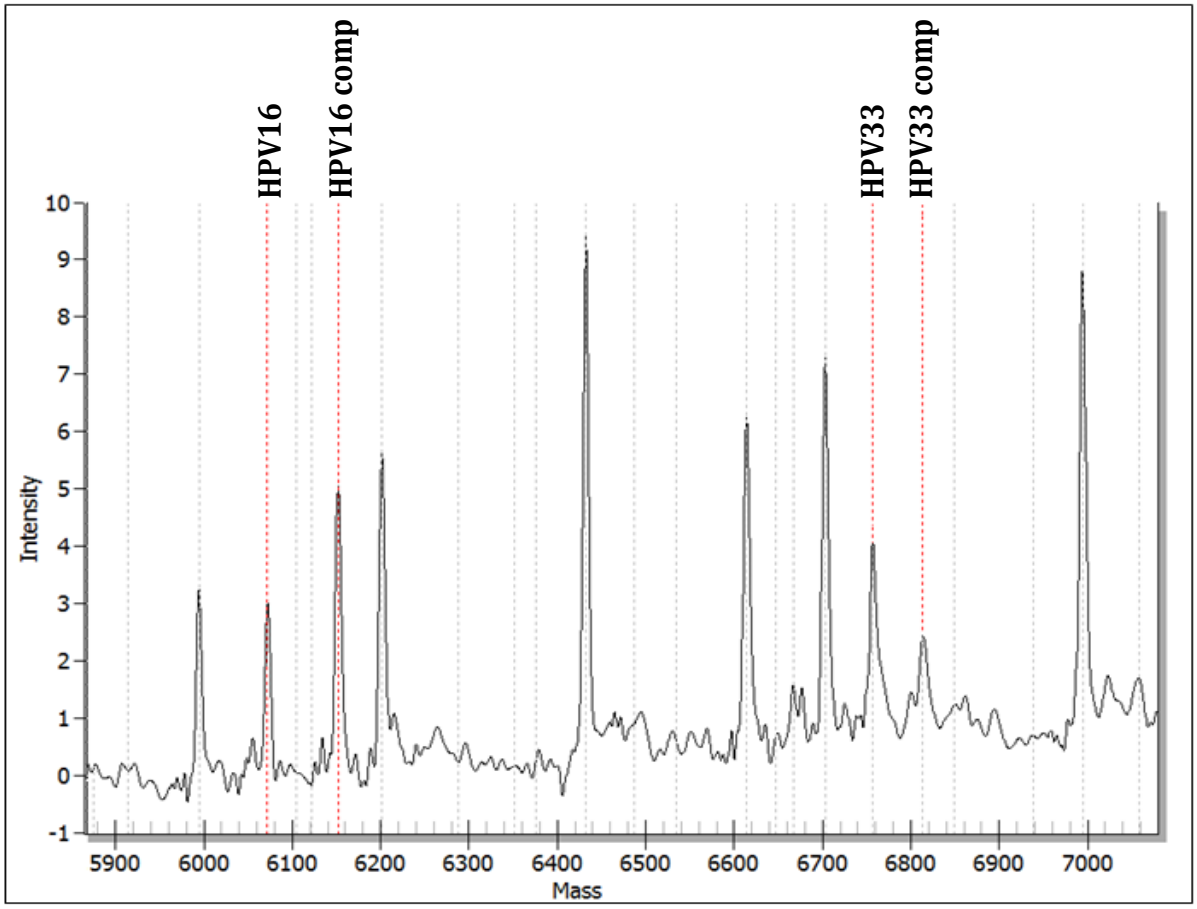

Figure II.1. PCR-MassArray Detection of hrHPV. Expanded mass spectrum region from 5900 to 7100 Daltons, showing HPV16 (at 6071) and HPV33 (at 6757) peaks in this oropharyngeal cancer sample.

profiles by matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) mass spectrometer[6,7,11,12,14] (Figure II.1).

HPV L1 PGMY-PCR and sequencing: Consensus PCR using L1 PGMY09/PGMY11 primers was carried out on tumor DNA from p16-positive or ISH-positive cases that were negative by HPV PCR-MassArray. The conditions of PCR were as previously described[39,40]. Amplicon products from L1 PGMY-PCR-positive cases were submitted for Sanger sequencing in the University of Michigan DNA sequencing core.

Results

The 338 tumors were tested and compared by at least 2 of the 3 methods as summarized by tumor site in Table II.1. As expected, the most frequently HPV-positive tumors were from oropharynx; with 173/208 (83%) positive by PCR-MA, 170/205 (83%) positive by p16, and 120/164 (73%) positive by ISH. Taken together, 183/212 (86%) of the oropharynx tumors were HPV-positive by one or more method, and 29/212 (14%) of the oropharynx tumors were HPV-negative by all tests performed on those samples (minimum of two tests each). The predominant HPV type determined by PCR-MA in

A.

OROPHARYNX*								
PCR-MassArray			p16			ISH		
POS	173	83%	POS	170	83%	POS	120	73%
NEG	35	17%	NEG	35	17%	NEG	44	27%
total	208		total	205		total	164	

* Of 212 oropharynx cancers, 183 were positive by at least one test.
** 6 HPV16-positive oropharynx tumors lacked p16 data

	HPV Type N (%)	p16-POS N (%)
HPV16**	162 (93.6)	151 (96.8)
HPV16, 35, 66	1 (0.6)	1 (100)
HPV16, 33	1 (0.6)	1 (100)
HPV18	2 (1.2)	2 (100)
HPV33	3 (1.7)	3 (100)
HPV35	3 (1.7)	3 (100)
HPV39	1 (0.6)	0
total	173	161 (96.4)

B.

NASOPHARYNX*								
PCR-MassArray			p16			ISH		
POS	8	44%	POS	9	50%	POS	0	0%
NEG	10	56%	NEG	9	50%	NEG	1	100%
total	18		total	18		total	1	

* Of 18 nasopharynx cancers, 9 were positive by at least one test.

	HPV Type N (%)	p16-POS N (%)
HPV16	3 (37.5)	3 (100)
HPV18	2 (25.0)	2 (100)
HPV39	1 (12.5)	1 (100)
HPV59	2 (25.0)	2 (100)
total	8	8 (100)

C.

ORAL CAVITY*								
PCR-MassArray			p16			ISH		
POS	10	10%	POS	20	19%	POS	5	26%
NEG	94	90%	NEG	86	81%	NEG	14	74%
total	104		total	106		total	19	

* Of 108 oral cavity cancers, 28 were positive by at least one test.

	HPV Type N (%)	p16-POS N (%)
HPV16	4 (40.0)	3 (75.0)
HPV16, 35	1 (10.0)	0
HPV31	1 (10.0)	1 (100)
HPV33	1 (10.0)	1 (100)
HPV35	1 (10.0)	0
HPV39, 58	1 (10.0)	0
HPV66	1 (10.0)	0
total	10	5 (50.0)

Table II.1. HPV Detection Test Results by Tumor Site and Method. PCR-MassArray hrHPV type determination, and p16 status. POS- positive, NEG- negative

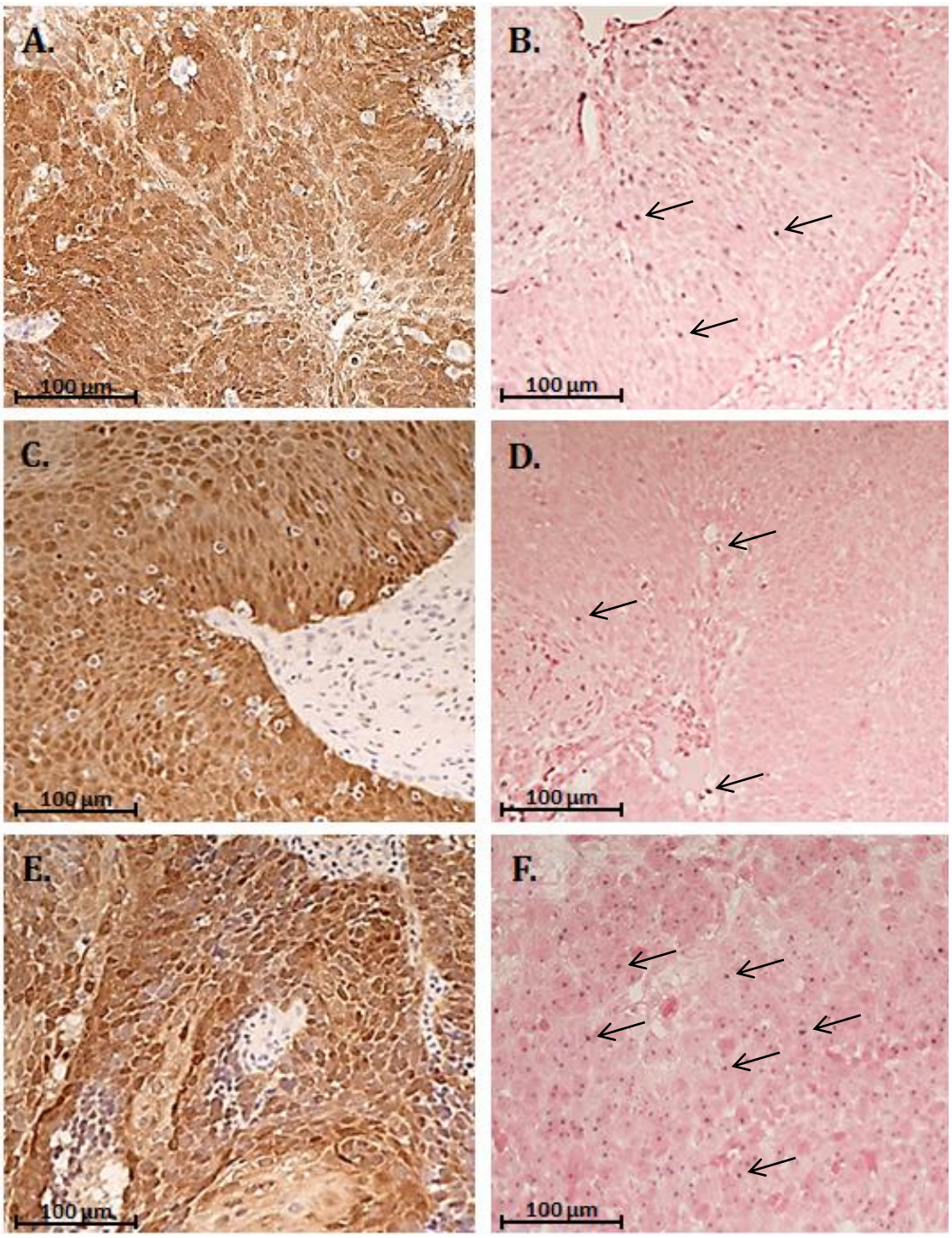

Figure II.2. Representative p16^{INK4a} Immunohistochemistry and HPV In Situ Hybridization in Oropharyngeal Tumors. Panels A, C and D show p16 and panels B, D and F show ISH. Panels A and B show p16 and ISH (respectively) in a tumor with 1.5 copies HPV/cell; Panels C and D show p16 and ISH (respectively) in a tumor with less than 1 copy HPV/cell; and Panels E and F show p16 and ISH (respectively) in a tumor with 4.3 copies HPV/cell. Arrows point to representative HPV ISH signals.

oropharynx was HPV16 alone (162/173, 94%). However, six percent of oropharynx tumors contained other high-risk types including: HPV18 (2), HPV33 (3), HPV35 (3), HPV39 (1), and 2 cases that contained multiple HPV types, one positive for HPV16, 35 and 66, and one positive for HPV16 and 33 (Table II.1A). The mass spectrum for the oropharyngeal tumor that contains both HPV16 and HPV 33 is illustrated in Figure II.1, and Figure II.2 shows representative p16 and HPV ISH results for 3 HPV-positive oropharynx tumors.

Of the 18 nasopharynx tumors, 9/18 (50%) were p16-positive and 8/18 (44%) were PCR-MA-positive, only 1/18 was tested by ISH and it was negative by ISH, but positive by both other assays. The hrHPV types identified in the 8 PCR-MA-positive nasopharynx tumors were more heterogeneous than in the oropharynx tumors with HPV16 (3), HPV18 (2), HPV39 (1) and HPV59 (2) (Table II.1B). The oral cavity tumors were less frequently HPV-positive, with only 28/108 (26%) tumors HPV-positive by one or more methods, and 80 (74%) tumors HPV-negative by all tests (minimum of two tests). The hrHPV types identified in the oral cavity tumors were HPV16 (4), HPV31 (1), HPV33 (1), HPV35 (1), HPV66 (1), and two cases with multiple infections, one containing HPV16 and 35, and one with HPV39 and 58 (Table II.1C). Of the 330 tumors from all sites tested by HPV PCR-MassArray, 191 (58%) were HPV-positive and 140 (42%) were HPV-negative. Of the 329 tumors tested by p16 staining, 199 (60%) were p16-positive and 130 (40%) were p16-negative. Of the 184 tumors tested by HPV in situ hybridization, 125 (68%) were HPV-positive and 59 (32%) were HPV-negative (Table II.2).

PCR-MA: 330 Tumors	PCR-MA Positive	191/330 (58%)
Concordant Positive-All Methods	POS by PCR-MA, p16 and ISH	105
	POS by PCR-MA and p16	49
	POS by PCR-MA and ISH	5
PCR-MA Positive, Discordant Other	POS by p16, NEG by ISH, POS by PCR-MA	20
	NEG by p16, NEG by ISH, POS by PCR-MA	3
	NEG by p16, POS by ISH, POS by PCR-MA	2
	NEG by p16, POS by PCR-MA	6
	NEG by ISH, POS by PCR-MA	1
p16: 329 Tumors	**p16 Positive**	**199/329 (60%)**
Concordant Positive-All Methods	POS by p16, PCR-MA and ISH	105
	POS by p16 and PCR-MA	49
	POS by p16 and ISH	3
p16 Positive, Discordant Other	POS by ISH, NEG by PCR-MA, POS by p16	5
	NEG by ISH, NEG by PCR-MA, POS by p16	3
	NEG by ISH, POS by PCR-MA, POS by p16	20
	NEG by PCR-MA, POS by p16	13
	NEG by ISH, POS by p16	1
ISH: 184 Tumors	**ISH Positive**	**125/184 (68%)**
Concordant Positive-All Methods	POS by ISH, p16 and PCR-MA	105
	POS by ISH and p16	3
	POS by ISH and PCR-MA	5
ISH Positive, Discordant Other	POS by p16, NEG by PCR-MA, POS by ISH	5
	NEG by PCR-MA, NEG by p16, POS by ISH	1
	NEG by p16, POS by PCR-MA, POS by ISH	2
	NEG by PCR-MA, POS by ISH	3
	NEG by p16, POS by ISH	1
L1 PGMY-PCR: 25 Tumors	**PCR-MA Negative, L1 PGMY-PCR Positive**	**1/25 (4%)**
PCR-MA Negative, Discordant Other	POS by p16, NEG by ISH, NEG by PCR-MA	0/3
	POS by p16, POS by ISH, NEG by PCR-MA	1/5
	NEG by p16, POS by ISH, NEG by PCR-MA	0/1
	POS by p16, NEG by PCR-MA	0/13
	POS by ISH, NEG by PCR-MA	0/3

Table II.2. Summary Table of All HPV Test Results and Method Discordance.

There were 167/338 tumor specimens tested by all three methods, 105 were HPV-positive by all methods, 28 were negative by all methods, and 34 samples were discordant by at least one method (Figure II.3, Panel A and Table II.2). The remaining 171/338 samples

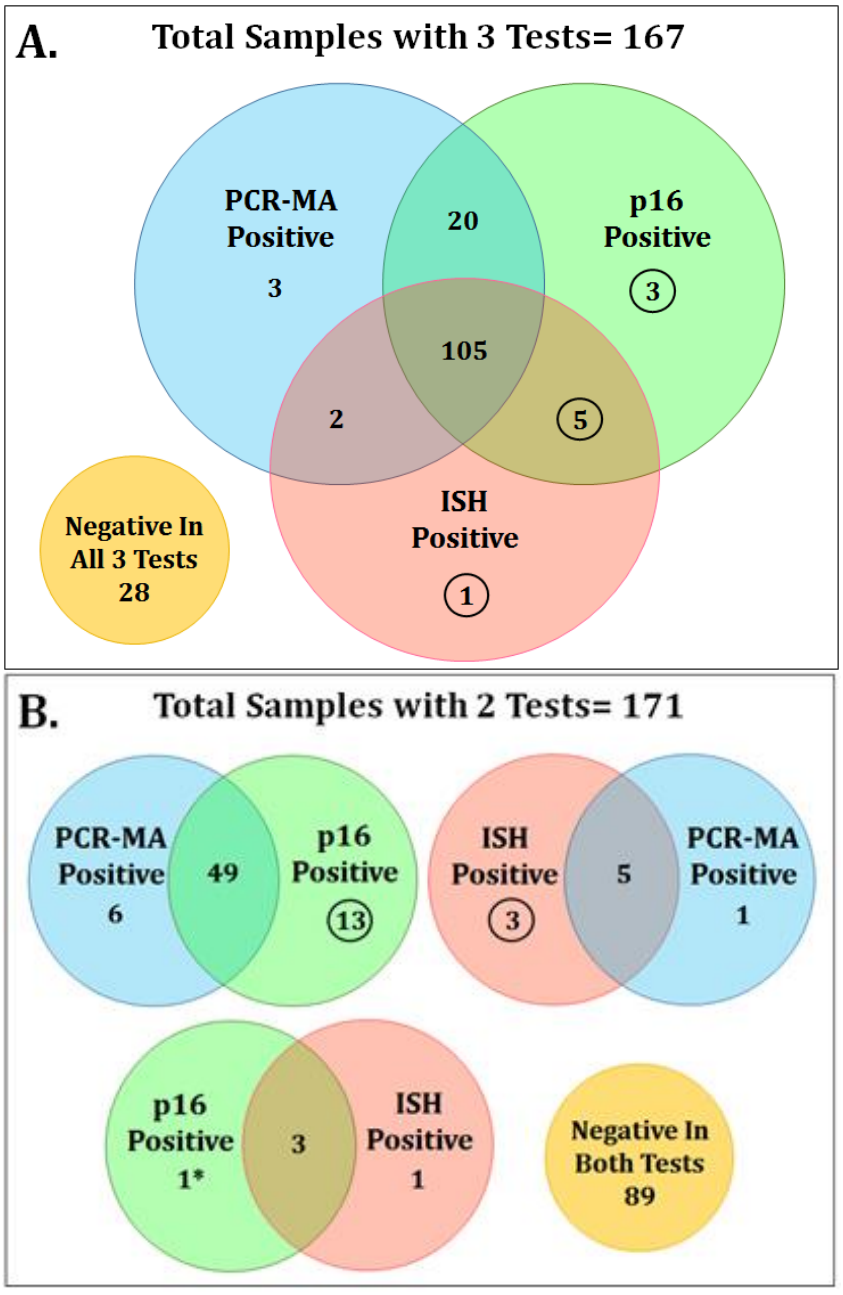

Figure II.3. Assay Agreement Between Detection Methods Tested. Panel A: Assay agreement among 167 samples tested with all 3 methods. Panel B: Assay agreement among 171 samples tested with 2/3 methods. Circled numbers represent samples retested by L1 HPV consensus PGMY-PCR.

were tested by only 2 of the 3 methods. Of these, 57 (49 by PCR-MA and p16, 5 by ISH and PCR-MA, and 3 by p16 and ISH) were HPV-positive by the 2 methods used, 89 were HPV negative by the 2 methods used, and 25 samples were discordant (Figure II.3, Panel B and Table II.2). All 25 tumors that were HPV-negative by HPV PCR-MA but p16-positive or ISH-positive (9 cases shown circled in Figure II.3, Panel A and 16 cases shown circled in Figure II.3, Panel B) were further analyzed by consensus PCR using optimized L1 PGMY09/PGMY11 primers[39,40]. Of these, all 17 of the oral cavity tumors that were negative PCR-MassArray but p16-positive or ISH-positive remained HPV-negative when tested with L1 PGMY-PCR. Of the 7 oropharynx cancers that were negative by HPV PCR-MassArray but p16-positive or ISH-positive, 1 was found to contain HPV DNA using L1 PGMY-PCR. That tumor was p16-positive and ISH-positive, and was found to harbor HPV16 as determined by Sanger sequencing of the L1 PGMY-PCR product. The single nasopharynx cancer that was p16-positive but HPV-negative by PCR-MassArray was also HPV-negative by HPV L1 PGMY-PCR. Of note, all of the EBV-positive nasopharynx tumors were HPV-negative and p16-negative. Across the three sites, 169 tumors contained only HPV16, of these 163 had p16 data: 157 (96%) were p16-positive and 6 (4%) were p16-negative. Twenty-two tumors contained other high risk HPV types, three in combination with HPV16. Of these, 5 of the 22 (23%) failed to express p16: one tumor with HPV16 and HPV35, one with HPV35, one with HPV39, one with HPV39 and HPV58, and one with HPV68 (Table II.1).

HPV PCR-MA

	PCR/SEQ +	PCR/SEQ -	
PCR-MA+	191 (TP)	0 (FP)	191
PCR-MA-	1 (FN)	138 (TN)	139
	192	138	330

Sensitivity= TP/(TP+FN)= 191/192= 99.5%
Specificity= TN/(FP+TN)= 138/138= 100%

p16 IHC

	PCR/SEQ +	PCR/SEQ -	
p16+	178 (TP)	20 (FP)	198
p16-	11 (FN)	118 (TN)	129
	189	138	327

Sensitivity= TP/(TP+FN)= 178/189= 94.2%
Specificity= TN/(FP+TN)= 118/138= 85.5%

HPV ISH

	PCR/SEQ +	PCR/SEQ -	
In Situ+	116 (TP)	8 (FP)	124
In Situ-	24 (FN)	34 (TN)	58
	140	42	182

Sensitivity= TP/(TP+FN)= 116/140= 82.9%
Specificity= TN/(FP+TN)= 34/42= 81%

Table II.3. Assay Performance Using PCR-MA/L1 PGMY- PCR and Sequencing (PCR/SEQ) as the Definitive Testing Assay.

When evaluating the performance of the assays using combined PCR-MA with L1 PGMY-PCR and sequencing as the definitive assay, the HPV PCR-MassArray had a sensitivity of 99.5% and a specificity of 100%, p16 assay had a sensitivity of 94.2% and a specificity of 85.5%, and the in situ hybridization assay had lower sensitivity of 82.9% and specificity of 81% (Table II.3).

Discussion

The association of high-risk HPV with oropharynx cancer is now well established[1,3,6,10-12,14,20,41-43]. Recently, hrHPV has also been implicated in a subset of nasopharyngeal carcinomas in white North Americans [7,44-47], and in other head and neck cancer sites, including oral cavity cancers[48,49] The goal of this study was to carefully assess a large number of head and neck squamous cancers from three different sites (oropharynx, oral cavity and nasopharynx) using $p16^{INK4a}$ staining, HPV in situ hybridization, and HPV PCR-MassArray on the same samples, to determine the true incidence of high-risk HPV involvement in the tumors, and to assess the relative sensitivity and specificity of each detection method. This study revealed that among these tumor sites at a large Midwestern referral center, more than 80% of oropharynx cancers, approximately half of nasopharyngeal cancers, and 10% of oral cavity cancers contain high-risk HPV.

In the oropharynx, 95% of the HPV-positive tumors contained HPV16, including 2 tumors that also contained one or two additional high-risk HPV types. Five percent of the HPV-positive oropharynx cancers contained other high-risk types (HPV18, HPV33, HPV35, and HPV39). However, in nasopharynx and oral cavity, more than half of the HPV-positive tumors contained non-HPV16 high-risk types. The presence of both HPV16 and other high risk HPV types in the absence of EBV in NPC is a strong indictment of HPV as a causal factor in a subset of nasopharynx cancers. This is reinforced by the strong concordance of p16 positivity with HPV and with the observation that neither EBV-positive nor HPV and EBV double negative tumors express p16.

In oropharynx cancer, HPV-positive tumors have a more favorable outcome than HPV-negative tumors. However, little is known about the effect of HPV on outcome in

other sites in the head and neck. Studies from Belgium have reported both a high incidence (44%) of hrHPV-positive oral cancers and a very poor prognosis for these tumors when compared with the HPV-negative oral cavity tumors[49]. Similar results were reported from Taiwan[48]. We found a much lower proportion of high-risk HPV-positive oral cavity tumors (10%) and among these we observed a higher rate of HPV heterogeneity with 40% containing only HPV16 and 60% that had other hrHPV types. Our proportion of HPV-positive oral cavity cases is relatively small, and whether these represent a separate prognostic group in oral cavity cancer is unknown. We also found a higher rate of HPV type heterogeneity in nasopharynx cases with 38% HPV16 and 62% other high-risk types. We have recently completed a survey of a much larger set of nasopharyngeal cancer cases that confirms the HPV type heterogeneity as well as demonstrates that HPV-positive nasopharyngeal cancer has a poorer prognosis than EBV-positive/HPV-negative nasopharyngeal cancer (unpublished data, Stemark et al., 2013).

While HPV16 is the most common cause of cervical cancer, only 59% of cervical cancers are driven by only HPV16; the remaining 41% contain other hrHPV types[23,50]. These types other than HPV16 constitute a significant subset of cancers in the oropharynx and other sites, and exhibit evidence that the virus is driving the cancer by high levels of p16 expression. Therefore, these tumors cannot be excluded from consideration. Furthermore, a significant subset of p16 and HPV-positive oropharynx tumors (20-30%) recur or progress with distant metastatic spread, yet the reason for this is unknown. It may be that non-HPV16 types are responsible for the outlier tumors, or perhaps these tumors driven by other hrHPV types are responding well to therapy. We already know that many of the recurrent and/or metastatic cancers in our set contain HPV16, suggesting that

tumors driven by non HPV16 types are as likely to respond to current therapies as the HPV16-driven tumors. Accordingly, it is important to include the other types, and to carry out larger studies to determine if tumors driven by non-HPV16 types can be assigned to reduced intensity treatments.

In this study, p16 expression status was determined in the majority of tumors evaluated, and there was a significant correlation between p16-positive and HPV-positive results (correlation coefficient= 0.999, $p<0.001$). This indicates that the virus is transcriptionally active in nearly all HPV-containing tumors, suggesting that it is unlikely that the HPV is strictly an incidental passenger. Nevertheless, of 186 tumors for which an HPV type was identified and p16 staining was carried out, 175 (94%) were p16-positive and 11 (6%) were p16-negative. Whether this represents a subset of 6% in which HPV is not a driving mechanism or whether this subset has incurred a mutation, deletion or methylation event affecting CDKN2A is a subject for further investigation.

Many groups have surveyed oropharyngeal tumors for HPV, and some have examined multiple head and neck tumor sites[43], and have used a variety of detection methods[51]. The present study represents one of the largest series of head and neck tumors from different sites evaluated by multiple assay methods for the presence of hrHPV. The PCR-MassArray assay has features that make it the optimal test in our hands. The minimum requirement for input DNA is very low; 5ng is adequate for evaluation. The assay has high sensitivity and specificity, and identifies each hrHPV type using specific primers, probes and competitors, and it focuses on the E6 region to confirm that this transforming oncogene is present in the sample. Evaluation of the cyclin-dependent kinase inhibitor protein, $p16^{INK4a}$, is a valuable diagnostic addition to the PCR-MA assay, because it typically

represents the transcriptional activity of the E7 oncogene. In our series, there was one oropharynx tumor that was negative by HPV PCR-MassArray but p16-positive and ISH-positive that was later confirmed to contain HPV16 by L1 PGMY-PCR and sequencing. We speculate that the PCR-MA assay missed this single case owing to rearrangement of the viral genome that affected part of the E6 oncogene. More commonly, p16 is overexpressed in a subset of tumors in the absence of hrHPV. Other mechanisms of p16 overexpression include mutation of Rb, amplification of cyclinD1, and overexpression of E2F family members. In this series of tumors, reanalysis of discordant cases revealed that 24/25 tumors that were negative by HPV PCR-MassArray but p16-positive remained HPV-negative by consensus L1 PGMY-PCR.

In situ hybridization for hrHPV has high specificity among known HPV-positive tumors, but has comparatively low sensitivity and can miss HPV-containing tumors as assessed by other methods. Reanalysis of ISH/PCR-MA discordant cases revealed that all 4 tumors that were negative by HPV PCR-MassArray but ISH-positive remained HPV-negative by consensus L1 PGMY-PCR.

Our findings support the use of a combination of p16 immunohistochemical staining and HPV PCR-Mass Array analysis as the optimal assessment for HPV detection, typing, and viral oncogene activity in formalin-fixed, paraffin-embedded tissue biopsies. For discordant p16-positive/PCR-MA-negative tumors we recommend L1 PGMY-PCR and sequencing.

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CHAPTER III

Integration of High-Risk Human Papillomavirus into Cellular Cancer-Related Genes in Head and Neck Cancer Cell Lines

Abstract

High-risk HPV-driven carcinogenesis has become the predominant etiologic factor in oropharyngeal cancer. Most HPV-positive oropharynx tumors respond well to therapy, prompting interest in establishing reduced-intensity treatment protocols, but approximately 20% of these tumors fail to respond to therapy or recur within 5 years for yet unknown reasons. This study evaluates viral oncogene expression, copy number, and integration sites in HPV-positive head and neck squamous cell carcinoma cell lines from patients who progressed.

Viral oncogene alternate transcripts and copy number were assessed. Detection of integrated papillomavirus sequences-PCR (DIPS-PCR) and sequencing was used to identify insertion sites and the host genes affected by viral integration. RNA expression analysis across viral integration sites within cellular genes was also assessed.

Seven HPV16-positive HNSCC cell lines were evaluated. All expressed HPV E6 and E7 oncogenes and exhibited alternate splicing, indicating active viral oncogenesis. In addition, HPV integration was found within known cellular genes, including cancer related genes *TP63*, *DCC*, *JAK1*, *TERT*, *ATR*, *ETV6*, *PGR*, *PTPRN2*, and *TMEM237*.

The HPV-positive cells lines represent virally-induced tumors that failed to respond to therapy and likely represent the non-responsive outlier tumors that must be identified before development of reduced-intensity clinical trials. These results strongly implicate viral integration into known cancer related genes as a secondary carcinogenic driver that may distinguish non-responsive cancers that will require increased or alternate treatment.

Introduction

High-risk human papillomaviruses are frequently identified as etiologic factors in the increasing incidence of head and neck cancer, particularly hrHPV-positive oropharynx cancers. In contrast, rates of HPV-negative oropharyngeal tumors, which are more often smoking and alcohol related, are declining in frequency. Among patients studied at the University of Michigan, over 80% of oropharyngeal cancers, 33% of nasopharynx cancers, 14% of larynx cancers and 10% of oral cavity cancers are positive for hrHPV[1]. In the oropharynx, hrHPV is generally considered to be associated with better prognosis[2-11].

Clinical trial data from the University of Michigan shows that patients with stage 3 and 4 oropharynx cancer had 88% three year progression-free survival after treatment with concurrent platinum-taxol based chemotherapy and intensity modulated radiation therapy (chemo-RT)[12]. However, HPV involvement at other head and neck sites is not as predictive of better response. In fact, others have suggested that patients with HPV-positive oral cavity tumors have worse outcomes than those with HPV-negative tumors[13], and our work has similarly demonstrated that patients with HPV-positive/EBV-negative nasopharynx tumors have poorer outcome than those with HPV-negative/EBV-positive tumors (Stenmark, et al., unpublished). Furthermore, we have yet to determine why a

subset of HPV-positive oropharynx tumors fails to respond to highly intensive concurrent therapies.

There are relatively few cell lines established from HPV-positive head and neck tumors. We have collaborated with our colleagues around the world to collect and study the HPV copy number, oncogene transcription, physical status, integration sites, and identification of the cellular genes affected by integration, of all seven HPV16-positive HNSCC cell lines available (UD-SCC-2, UM-SCC-47, UM-SCC-104, UPCI:SCC90, UPCI:SCC152, UPCI:SCC154, and VU-SCC-147). All of these cell lines are derived from tumors that failed to respond to therapy. Our findings indicate that these tumors have transcriptionally active HPV as demonstrated by strong expression of E6 and E7 oncogenes, including expression of the E6 alternate transcripts (E6*I and E6*II) associated with viral oncogenesis. Also, each cell line demonstrated viral integration into genes known to be associated with cancer development. As the overwhelming majority of cervical clinical tumor specimens that have been reported in the literature exhibit integration into either intragenic regions of the cellular genome or integration into fragile sites[14,15], we postulate that integration into important cellular genes may be a secondary driver of more highly malignant HPV-positive head and neck tumors. If so, this could provide a way to distinguish those HPV-positive tumors unlikely to respond to conventional therapies.

Methods

Cell lines: Seven HPV16-positive HNSCC tumor cell lines were studied. Two were developed in our lab: UM-SCC-47[16,17], and UM-SCC-104[16]. UD-SCC-2[18], was obtained from H. Bier and T. Hoffmann, University of Düsseldorf[18]; VU-SCC-147 (previously called 93-VU-

147T)[19], from R. Brakenhoff, Vrije Universiteit, Amsterdam; and UPCI:SCC90, UPCI:SCC152, and UPCI:SCC154, from S. Gollin and R. Ferris, University of Pittsburgh Cancer Institute[20-22] (Table III.1). The external cell lines were obtained directly from the originators in 2010. All lines were genotyped in the University of Michigan Genomics Core using ProfilerPlus, which interrogates 10 tetranucleotide short tandem repeats (STR), and were confirmed to have unique genotypes. UPCI:SCC90 and UPCI:SCC152 share the same genotype, as they are derived from separate tumors in the same patient. All lines were tested upon receipt from the donors and repeat confirmatory tests were performed immediately prior to the integration experiments carried out between 2012 and 2013. Genomic DNA was extracted from cells using the DNeasy Spin Column kit (Qiagen). RNA was isolated from cells using the RNeasy Mini Kit (Qiagen), followed by on-column DNase treatment.

HPV detection, identification, and copy number analysis: All cell lines were grown on glass slides and examined for HPV in situ hybridization (ISH) using the Ventana INFORM HPVIII assay (detects 12 hrHPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66) per supplier protocol. Single color fluorescence in situ hybridization (FISH) was performed on UM-SCC-47 using a fluorescein labeled HPV16 bacterial artificial chromosome. Metaphase spreads were harvested from UM-SCC-47 cells in their 34th to 36th passages using trypsinization and 0.075M potassium chloride hypotonic solution. Spectral karyotyping was performed at the Van Andel Institute. All cell lines were tested for the presence and type of HPV using the HPV PCR-MassArray assay[1,4,16,23,24] as described in detail in Tang et al[16]. HPV16 copy number analysis was carried out using two methods that measure copies of HPV DNA: a modified quantitative PCR-MassArray(qPCR-MA) assay which examines a specific region of HPV16 E6, and a TaqMan quantitative PCR assay, which

queries both E6 and E7. Both methods use GAPDH as an endogenous two copy/cell endogenous reference control.

HPV E6 and E7 transcript analysis: Two complementary reverse transcription-PCR (RT-PCR) methods were used to evaluate and quantify the relative expression of the viral transcripts. The E6*I and E6*II alternate transcripts result from a single donor site at nucleotide (nt) 226 of the viral genome and two acceptor sites at nt 407 (E6*I) and at nt 526 (E6*II). To examine the expression of HPV16 E6 and E7 transcripts, primer sets were designed that specifically and discretely amplify the intact, non-spliced, full-length E6-E7 transcript, the spliced E6*I-E7 transcript, and the spliced E6*II-7 transcript, as illustrated in Figure III.1 The full-length E6-E7 transcript was generated using a forward primer located within the region that is eliminated by splicing, while the transcripts for the alternate splice forms were generated using unique forward primers that span the respective splice junctions. (Primer sets are listed in Table SIII.1). As a negative control, primers for GAPDH were used to confirm the absence of contaminating genomic DNA, indicated by a 158bp GAPDH amplicon product. Quantitative RT-PCR (qRT-PCR) was

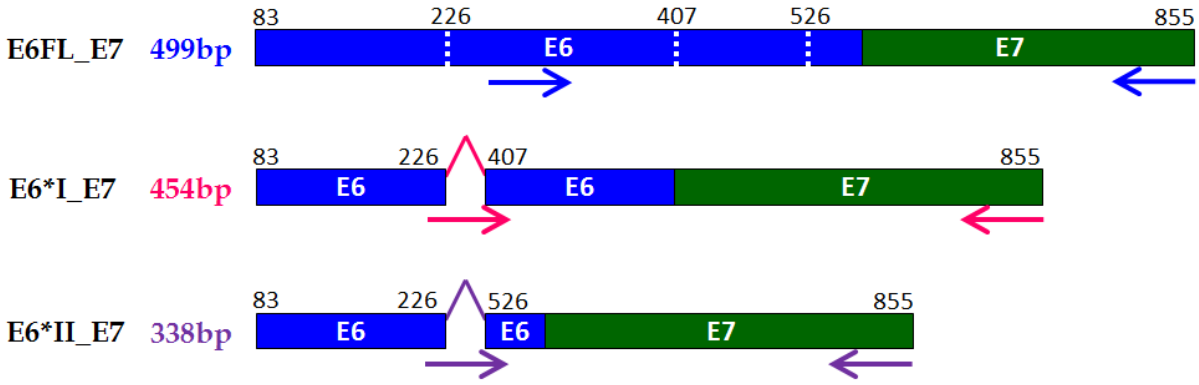

Figure III.1. RT-PCR Strategy for Transcript-Specific E6-E7 Oncogene Evaluation in HPV16-Positive HNSCC Cell Lines. Primers within the spice region or across splice junctions allow for exclusive amplification of full length E6 or alternate E6 transcripts.

subsequently performed using TaqMan assays designed to exclusively amplify each HPV early gene transcript: E1, E2, E5, non-spliced, full-length E6, spliced E6*I, spliced E6*II and E7. TaqMan chemistry works using forward and reverse primers together with a probe labeled with a fluorescent dye on the 5' end and a signal quencher on the 3' end. In the reaction, the primers and probe anneal to the denatured cDNA template, and *Taq* polymerase synthesizes the new DNA strand 5' to 3' along the template. When the polymerase enzyme reaches the probe, its 5' nuclease activity cleaves it, releasing the

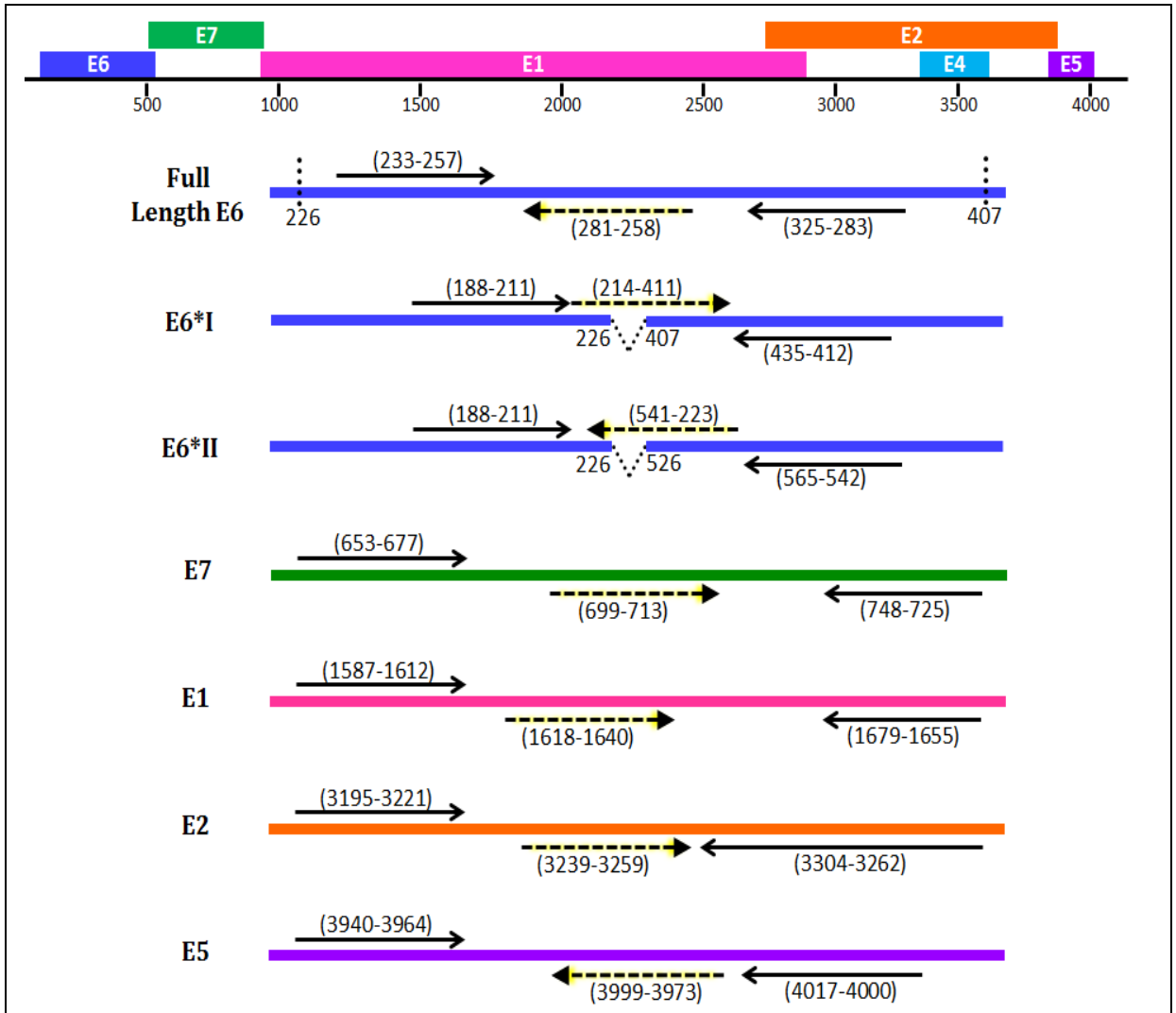

Figure III.2. Illustration of TaqMan Primers and Probes for Quantitative RT-PCR in HPV16-Positive HNSCC Cell Lines. Dotted lines indicate splice sites, solid arrows represent primers and dashed arrows represent TaqMan probes.

fluorescent dye from the quencher and resulting in generation of a measurable signal. The primers and probe for the full-length E6 TaqMan assay anneal to the region between splice sites, and the TaqMan probes for the E6*I and E6*II alternate transcripts anneal to the particular splice junction for each, allowing for absolute specificity in the reactions. All of the TaqMan assays are illustrated in Figure III.2, and primer sequences are listed in Table SIII.2. A prepared GAPDH endogenous control primer/probe assay was used to quantify relative viral gene expression.

Detection of Integrated Papillomavirus Sequences-Polymerase Chain Reaction (DIPS-PCR): Integration analysis was performed using a modified technique that is based on a previously published method[25,26] and is illustrated in Figure III.3. Briefly, genomic DNA was isolated from each cell line, and digested with the restriction enzyme, Taq$^{\alpha}$1, which cuts the primary HPV16 viral genome only once at position 505 within E6 (additional Taq$^{\alpha}$1 restriction sites have been described in HPV16 variants at positions 311 and 2608) and cuts the cellular genomic DNA at approximately 1.5 million sites. After ligating a double-strand DNA adapter (5'-CGCAACGTGTAAGTCTG-NH2-3' annealed to 5'-GGGCCATCAGTCAGCAGTCGTAGCCGGAT CCAGACTTACACGTTG-3') to the overhanging ends of each fragment, linear PCR amplification with 11 viral-specific primers was followed by a second logarithmic PCR using 11 nested viral primers and a reverse adapter-specific primer (Table SIII.3). Thermocycling conditions used for both rounds of PCR included 3 minute extension cycles that limited amplification of large (>3kb), episome-only fragments. PCR products were separated by agarose gel electrophoresis. To search for a previously reported HPV insertion into 9q31[14] that was not detected by DIPS-PCR in UPCI:SCC90, we used primers from multiple regions of HPV16 and within 9q31 for direct PCR using DNA

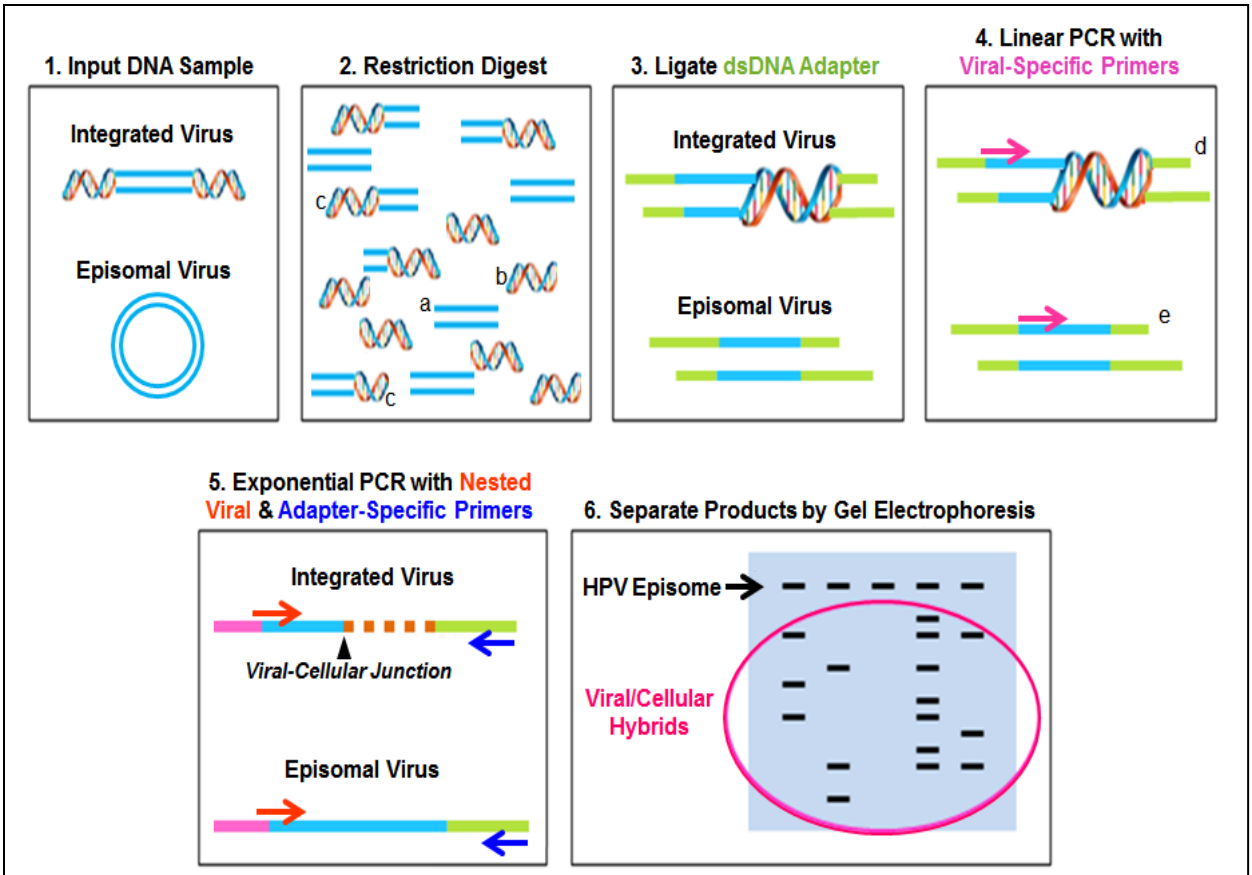

Figure III.3. Illustration of the HPV DIPS-PCR Method. 1. Genomic DNA is isolated from the sample, containing HPV in integrated, episomal, or both physical state; 2. Taqα1 restriction enzyme digests the DNA, resulting in linearized episomal virus (a), cellular-only (b), or viral-cellular hybrid (c) fragments; 3. A dsDNA adapter is ligated to ends of the fragments; 4. Unidirectional, linear PCR amplifies from viral-specific primers (pink arrows) into cellular regions (d) or within the episomal virus (e); 5. A second round of PCR exponentially amplifies the products of the linear PCR with nested viral-specific primers (orange arrows) and a reverse primer that is specific to the adapter sequence (blue arrows); 6. Amplicons are separated and visualized by agarose gel electrophoresis, where episome-only viral products are distinguished from viral-cellular fusions based on known episome-only sizes.

from UPCI:SCC90 and the second cell line from the same patient, UPCI:SCC152 (Primers listed in Table SIII.4). PCR products were separated by gel electrophoresis; bands were purified and sequenced with the appropriate primer sets.

Sequence analysis of cellular genes with integrated virus: Fragments generated exclusively from non-integrated virus were excluded based on amplicon sizes predicted for episome-only bands, which were based on viral-specific primer locations in relation to the

Taqα1 restriction site in the viral genome. Viral-cellular amplicons were identified, excised from the gels, purified, and sequenced. Viral integrations into known genes were verified by direct PCR and sequencing of the otherwise unmodified cell line genomic DNA, using primers specific to each viral and cellular region.

Integration site transcript analysis: Cell line RNA was evaluated for viral-cellular fusion transcripts and cellular gene transcripts affected by confirmed viral integrations. RT-PCR assays were used that amplified virus-cellular fusion transcripts from HPV ORFs into cellular gene exons, cellular gene exon-exon transcripts across the integration site, and distant cellular gene transcripts. All amplified transcripts were separated by agarose gel electrophoresis sequenced for confirmation.

Results

HPV detection, identification, and copy number analysis: All seven cell lines were verified to contain HPV16 by PCR-MassArray. HPV16 copy number was estimated by PCR-MassArray and TaqMan assays (Table III.1) using the HPV16 cervical carcinoma cell lines CaSki (200-600 copies/cell) and SiHa (2 copies/cell) as reference cell lines. HPV copy number ranged from a low 1-2 copies/cell in UM-SCC-104 and UPCI:SCC154 to hundreds of copies per cell in UPCI: SCC90, which has been previously reported to contain between 100 and 150 copies of HPV16[20]. The average for all cell lines was 100.6 HPV16 copies/cell.

Metaphase chromosome spreads of UM-SCC-47 were examined by HPV16 FISH which revealed a strong signal, likely representing multiple copies of the viral genome, integrated into the distal long arm of a single autosomal chromosome (Figure III.4A). All of

Cell Line	Tumor Site	HPV copies per cell			
		Reported	qPCR-MA	TaqMan qPCR	Average
CaSki	Cervix	200-600	182.8	423	302.9
SiHa	Cervix	2	2	2	2
UD-SCC-2	Hypopharynx		47.1	38.1	42.6
UM-SCC-47	Lateral Tongue		9.4	0.3	4.9
UM-SCC-104	Floor of mouth		0.3	2.9	1.6
UPCI:SCC90	Base of tongue	100-150	423.1	91.4	257.2
UPCI:SCC152*	Hypopharynx		46.8	309.5	178.2
UPCI:SCC154	Base of tongue		1.8	0.8	1.3
VU-SCC-147	Floor of mouth		230.5	206.1	218.3

Table III.1. Cell Line HPV16 Copy Number as Determined by Quantitative HPV PCR-MassArray (qPCR-MA) and TaqMan Quantitative PCR. * UPCI:SCC90 Recurrence

the cell lines were examined for nuclear viral DNA by ISH (Figure III.4B-H) with deep blue hybridization signals that varied in intensity corresponding to the HPV copy number (Table III.1). UM-SCC-104 (Figure III.4D) and UPCI:SCC154 (Figure III.4G) have very faint hybridization signals, consistent with low viral copy number.

All seven HPV16-positive HNSCC cell lines express viral oncogene transcripts (Figure III.5). The HPV16 E6 gene contains two introns that can be spliced out, generating alternate E6*I-E7 and E6*II-E7 transcripts that have been linked to increased expression of E7 at the expense of full length E6[27]. As shown in Figure III.5 A-G, all of the cell lines strongly express the viral oncogene transcripts and all express the alternate E6-E7 transcripts, primarily E6*I, and to a lesser extent E6*II, with relatively low levels of full length E6 (qRT-PCR, bar graphs) or full length E6-E7 (RT-PCR, gel images). These findings are consistent with the viral oncogenes as drivers of tumor development. In all of the cell lines, the expression of E1 and E2 is reduced compared to the E6-E7 transcripts, as

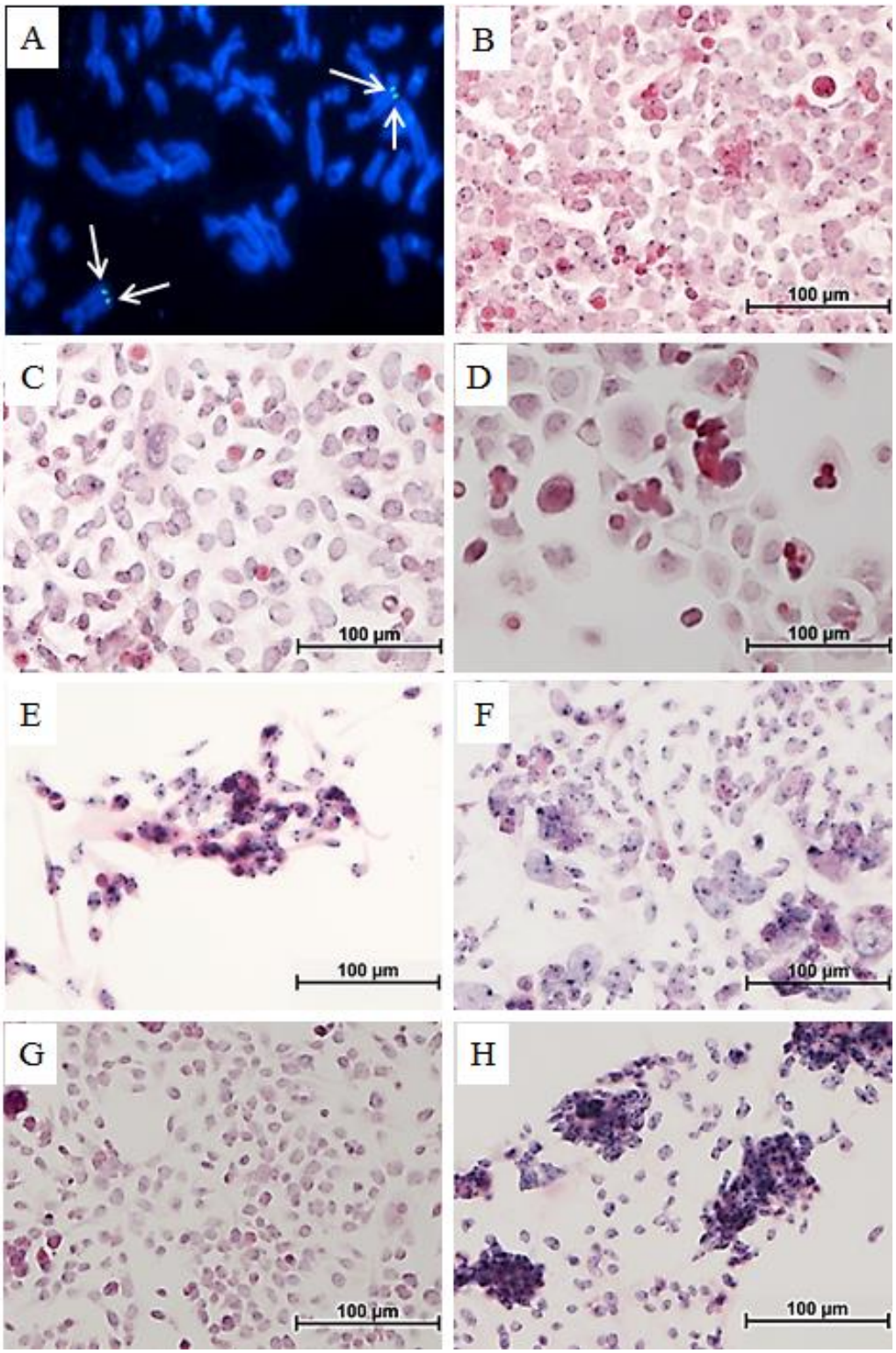

Figure III.4. HPV In Situ Hybridization in HNSCC Cell Lines. Panel A: UM-SCC-47 fluorescence in situ hybridization for HPV16 in 2 cells, white arrows point to HPV signals, Panels B-H show hrHPV in situ hybridization indicated by dark blue signals. Panel B. UD-SCC-2, Panel C. UM-SCC-47, Panel D. UM-SCC-104, Panel E. UPCI:SCC90, Panel F. UPCI:SCC152, Panel G. UPCI:SCC154, Panel H. VU-SCC-147.

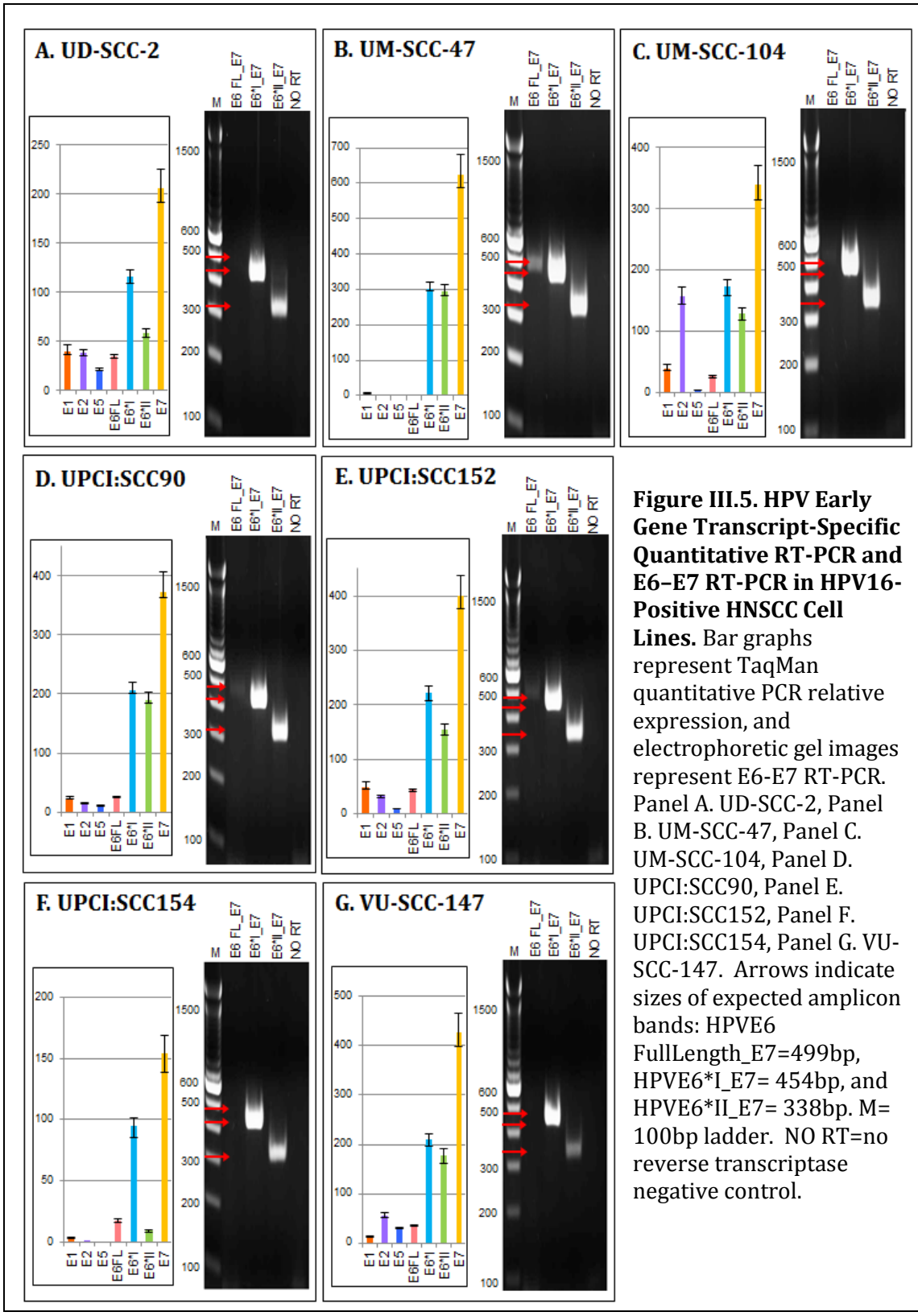

Figure III.5. HPV Early Gene Transcript-Specific Quantitative RT-PCR and E6–E7 RT-PCR in HPV16-Positive HNSCC Cell Lines. Bar graphs represent TaqMan quantitative PCR relative expression, and electrophoretic gel images represent E6-E7 RT-PCR. Panel A. UD-SCC-2, Panel B. UM-SCC-47, Panel C. UM-SCC-104, Panel D. UPCI:SCC90, Panel E. UPCI:SCC152, Panel F. UPCI:SCC154, Panel G. VU-SCC-147. Arrows indicate sizes of expected amplicon bands: HPVE6 FullLength_E7=499bp, HPVE6*I_E7= 454bp, and HPVE6*II_E7= 338bp. M= 100bp ladder. NO RT=no reverse transcriptase negative control.

measured by TaqMan qRT-PCR relative to GAPDH. Only UM-SCC-104 showed moderate levels of E2, but still had low levels of E1 expression. These findings are consistent with disruption of the viral E1-E2 region.

Detection of Integrated Papillomavirus Sequences-Polymerase Chain Reaction (DIPS-PCR): Separated amplicon DIPS-PCR bands are shown in the representative example gels in Figure III.6 A-F. A total of 87 hybrid viral-cellular amplicons were isolated and sequenced, ranging from 5 to 16 amplicons for each cell line.

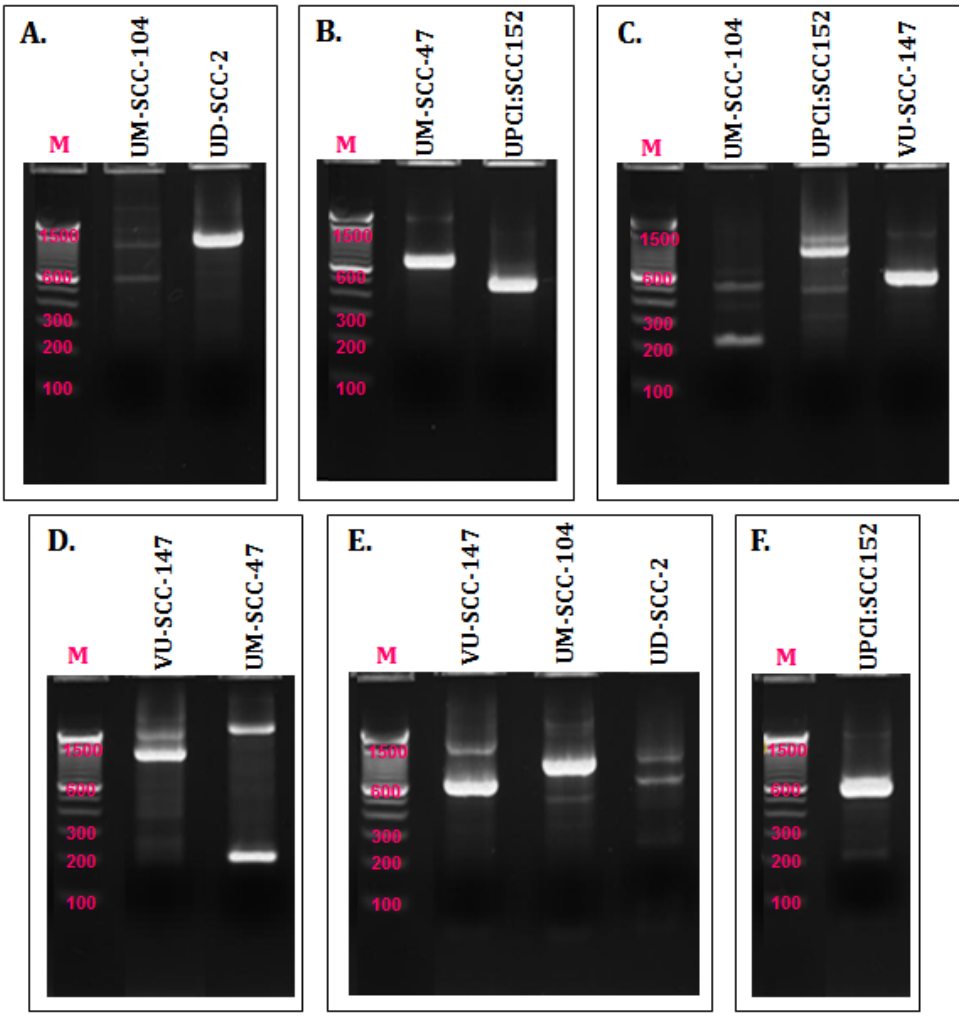

Figure III.6. Representative Cell Line DIPS PCR Gels. Panel A. HPV-E1a/Adapter primers, Panel B. HPV-E1c/Adapter primers, Panel C. HPV-E5a/Adapter primers, Panel D. HPV-L2a/Adapter primers, Panel E. HPV-E2b/Adapter primers, Panel F HPV-E6a/Adapter primers, M=100 bp ladder.

Sequence analysis of cellular genes with integrated virus: Viral-host DNA fusions were identified by sequence analysis. The sequence reads mapped to viral-only sequence, viral-cellular hybrids as described below, or were unmapped due to poor sequence resolution. Diagrammatic representations of the viral rearrangements and insertion sites determined by this method are shown in Figure III.7A-H, and Table III.2 summarizes the integration results for all seven cell lines, indicating the chromosome locus, known genes, and the regions of integration into the cellular gene. Two rearrangements and two fusion events were detected in UD-SCC-2. The HPV16 internal rearrangements involved reverse HPVE6 joining forward LCR into a second copy of E6, and the second was HPV E1 directly joining the LCR. The first UD-SCC-2 integration was from HPV E2 into an intergenic region of chromosome 17q12, and a second fusing HPV E1 to intron 14 of *JAK1*. *JAK1* is a large protein tyrosine kinase involved in the proper function of the interferon receptor complexes and signaling through the STAT1-4 pathway.

UM-SCC-47 exhibited two HPV integration events with breakpoints within E2 each extending into *TP63;* one into *TP63* reverse intron 10 and the second into *TP63* exon 14. As *TP63* is located at chromosome 3q28, this is finding is consistent with the FISH result (Figure III.4A) showing a strong signal on the distal arm of an aberrant chromosome that is likely a t(3;7) chromosome rearrangement identified by Spectral Karyotyping (SKY) (Figure III.8). Integration into *TP63* has been observed in cervical cancers and Schmitz et al[28] reported a region of homology between the HPV16 E1 region and a segment of chromosome 3q28 within *TP63* that may facilitate this integration. *TP63* is a homolog to *TP53* and *TP73*, and is a tumor suppressor gene, functioning as both a sequence-specific DNA binding transcriptional

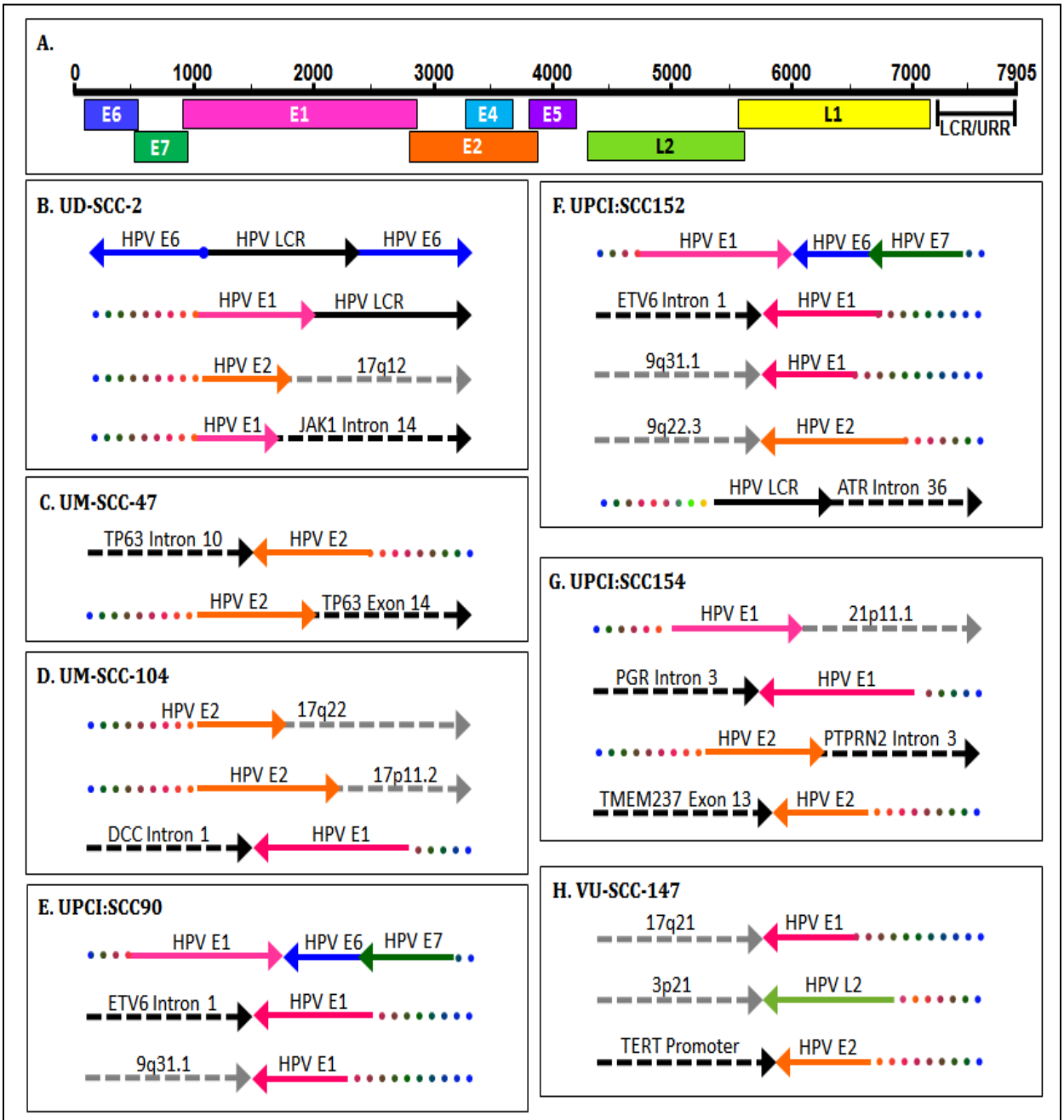

Figure III.7. Diagrammatic Representation of Viral Rearrangements and Integration Events in HPV16-Positive HNSCC Cell Lines. Panel A. Linear organization of the HPV genome, Panel B. UD-SCC-2, Panel C. UM-SCC-47, Panel D. UM-SCC-104, Panel E. UPCI:SCC90, Panel F. UPCI:SCC152, Panel G. UPCI:SCC154, Panel H. VU-SCC-147. Arrow direction indicates orientation of genes. Solid colored arrows represent HPV, Dotted colored arrows indicate HPV sequence outside of mapped region, Dashed grey arrows are cellular intragenic regions, Dashed black arrows are cellular genes. The colors in the sequenced amplicons correspond to the color coded viral genome at the top of the figure.

Cell line	HPV Site	Cellular Locus	Gene	Gene Name	Region	Domain
UD-SCC-2	(F)E2	17q12	Intergenic			
	(F)E1	1p32.3	***JAK1***	Janus kinase 1	Intron 14	Within protein kinase domain
UM-SCC-47	(R)E2	3q28	***TP63***	Tumor protein p63	Intron 10	Between DNA binding and SAM domains
	(F)E2	3q28	***TP63***	Tumor protein p63	Exon 14	SAM domain
UM-SCC-104	(F)E2	17q22	Intergenic			
	(F)E2	17p11.2	Intergenic			
	(R)E1	18q21.3	***DCC***	Deleted in colorectal carcinoma	Intron 1	Within extracellular domain
UPCI:SCC 90	(R)E1	9q31.1	Intergenic			
	(R)E1	12p13	***ETV6***	Ets variant 6	Intron 1	Upstream of PNT domain
UPCI:SCC 152	(R)E2	9q22.3	Intergenic			
	(R)E1	9q31.1	Intergenic			
	(F)LCR	3q23	***ATR***	Ataxia telangiectasia and Rad3 related	Intron 36	Within PIK-related kinase domain
	(R)E1	12p13	***ETV6***	Ets variant 6	Intron 1	Upstream of PNT domain
UPCI:SCC 154	(F)E1	21p11.1	Intergenic			
	(R)E1	11q22-23	***PGR***	Progesterone receptor	Intron 3	Within nuclear localization signal domain
	(F)E2	7q36	***PTPRN2***	Protein tyrosine phosphatase, receptor N polypeptide 2	Intron 3	Within extracellular domain
	(R)E2	2q33.2	***TMEM237***	Transmembrane protein 237	Exon 14	After transmembrane domains
VU-SCC-147	(R)E1	17q21	Intergenic			
	(R)L2	3p21	Intergenic			
	(R)E2	5p15.33	***TERT***	Telomerase catalytic subunit	Promoter	Promoter region

Table III.2. Summary of Integration Events in HPV16-Positive HNSCC Cell Lines. (F) and (R)= Forward or Reverse viral orientation in relation to the cellular gene.

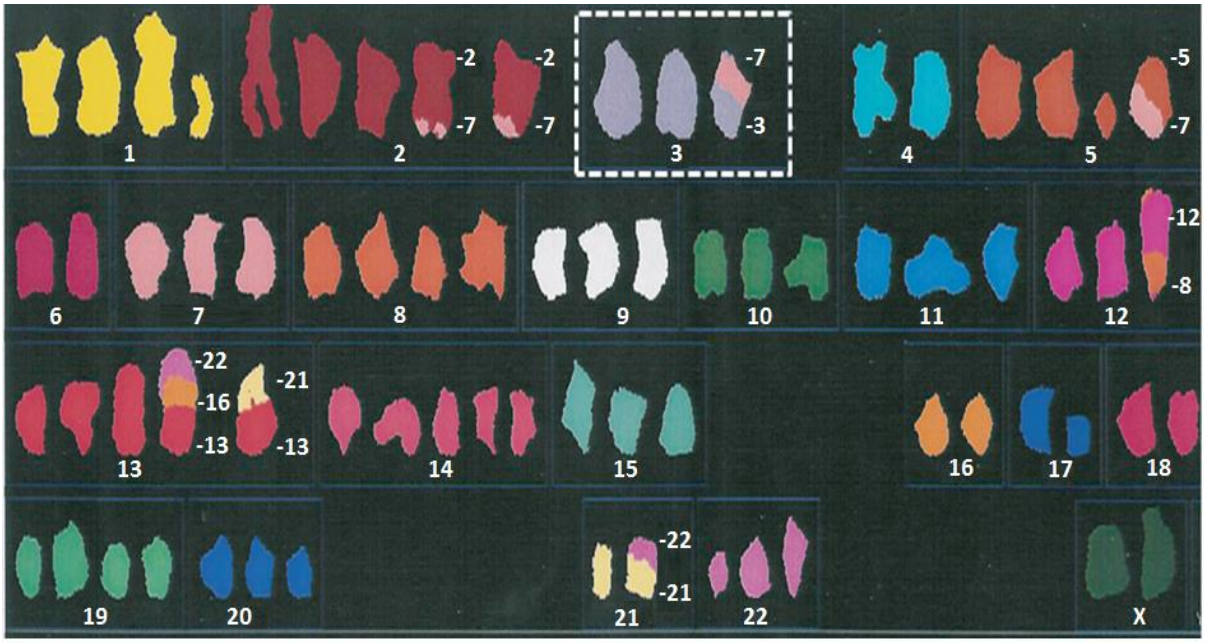

Figure III.8. Spectral Karyotyping (SKY) Chromosome Analysis of UM-SCC-47, Showing the t(3;7) Rearrangement. UM-SCC-47 is a pseudotetraploid cell line from a male donor with several characteristic chromosomal rearrangements. 72,XX,+1,+del(1q),+2,+der(2)t(2;7), +der(2)t(2;7),+der(3)t(3;7),+i(5p),+der(5)t(5;7),+7,+8,+8,9, +10,+11,+der(12)t(8;12),+i(13q), der(13)t(13;16;22),der(13)t(13;21),+14,+14,+14,+15,i(17p),i(17q),+19,+19,+20,der(21) t(21;22),+22. (Y chromosome is lost in most cells including the one shown).

repressor and activator. The p63 protein product of *TP63* is involved in differentiation and cell-cycle regulation, as well as TGFβ and WNT signaling[29].

UM-SCC-104 exhibited multiple integration events including two HPV E2 integration events into intergenic regions of 17q22 and 17p11.2 (Figure III.7D). Additionally, in UM-SCC-104, HPV E1 integrated into reverse *DCC* intron 1. *DCC* is a receptor for netrin-1, and when not bound, functions as a tumor suppressor in the caspase-9 dependent apoptosis pathway. *DCC* is located in a region of chromosome 18q that is frequently lost in squamous cell carcinomas[30,31].

UPCI:SCC90 and UPCI:SCC152 (tumors from the same patient) share the identical HPV rearrangement of HPV E6 connecting to reverse E6 into E7, as well as the same

integration from HPV E1 into intron 1 of *ETV6*. These similarities are consistent with these being early events occurring before the primary tumor and recurrent populations diverged. *ETV6* is a transcription factor involved primarily in development and hematopoiesis. Gene fusions involving *ETV6* have been discovered in multiple hematological malignancies[32], and there is evidence suggesting mutational inactivation of *ETV6* in prostate carcinoma[33]. Interestingly, an *ETV6* fusion oncogene was recently identified in a subset of salivary gland tumors[34]. A previous study of UPCI:SCC90 reported a complex rearrangement of HPV that resulted in a rearranged chromosome 9 with fusions between HPV16 and 9q31.1 and 9p24[14]. Because we did not find this by integration by DIPS-PCR, we confirmed its presence by targeted PCR. Sequence analysis revealed HPV E1 integrated into the same sequence as reported by Ragin et al.[14], which was confirmed by BLAST analysis to map to 9q31.1.

In addition to the HPV E1- *ETV6* integration, analysis of UPCI:SCC152 identified a viral rearrangement resulting in fusion of HPV E2 into an intergenic region of reverse chromosome 9q22.33, and a second integration from HPV LCR into *ATR* intron 36 on chromosome 3q23. *ATR* codes for a cell-cycle checkpoint protein kinase required for arrest and repair in response to DNA damage. UPCI:SCC152 was also evaluated for the 9q31.1 integration that was previously reported, and was detected by direct PCR exactly as in UPCI:SCC90. The multiple viral integrations into chromosome 9 in UPCI:SCC90 and UPCI:SCC152 appear to be complex, involving both the 9p and 9q arms. Thus far, our indications are that this integration involves exclusively intergenic regions of the chromosome.

UPCI:SCC154 exhibited four integration events detected by DIPS-PCR, including HPV E1 into an intergenic region of chromosome 21p11.1, HPV E1 into reverse *PGR*, and two involving HPV E2; one into *PTPRN2* intron 3 and the second into reverse *TMEM237* exon 14. *PGR* is a steroid receptor for progesterone, and participates in estrogen and glucocorticoid receptor pathways as well as signaling by binding to transcription factors such as NF-κB, AP-1 or STAT. Overexpression of *PGR* has been associated with disease-related mortality and recurrence in breast and gastric cancers[35,36]. *PTPRN2* (protein tyrosine phosphatase, receptor type, N2) belongs to the transmembrane protein tyrosine phosphatase family, and is reported to be a tumor suppressor involved in the regulation of the cell cycle, as well as growth, differentiation, and oncogenic transformation. It has been demonstrated that *PTPRN2* is hypermethylated and subsequently inactivated in squamous cell lung cancer[37]. *TMEM237* is a tetraspanin membrane protein that is thought to participate in the WNT signaling pathway.

Three integration sites were identified in VU-SCC-147, one from HPV E1 into reverse chromosome 17q21, a second from HPV L2 into reverse chromosome 3p21, and a third from HPV E2 into reverse *TERT* (telomerase reverse transcriptase) in the promoter region. In a study that evaluated the frequency of *TERT* promoter mutations in 60 tumor types, squamous cells carcinomas of the head and neck were among the highest, with 17% of tumors having mutations in the promoter region of the gene[38].

These results show that in every cell line, viral integration into one or more cancer related genes was identified. Table III.2 summarizes the integration results for all seven cell lines, indicating the chromosome locus, known genes, and the regions of integration

into the cellular gene. Each of the viral integrations was confirmed by direct sequencing of the cell line genomic DNA.

Integration site transcript analysis: Based on the DIPS-PCR integration results, RT-PCR assays were designed to assess virus-cellular fusion transcripts from HPV ORFs into cellular gene exons, cellular gene exon-exon transcripts across the integration site, and distant cellular gene transcripts. Transcripts targeted for evaluation are represented in Figure III.9. HPV fusion transcript and cellular gene transcript RT-PCR amplicon products are shown in Figure III.10 and the results of transcript RT-PCR and sequence analysis are summarized in Table III.3.

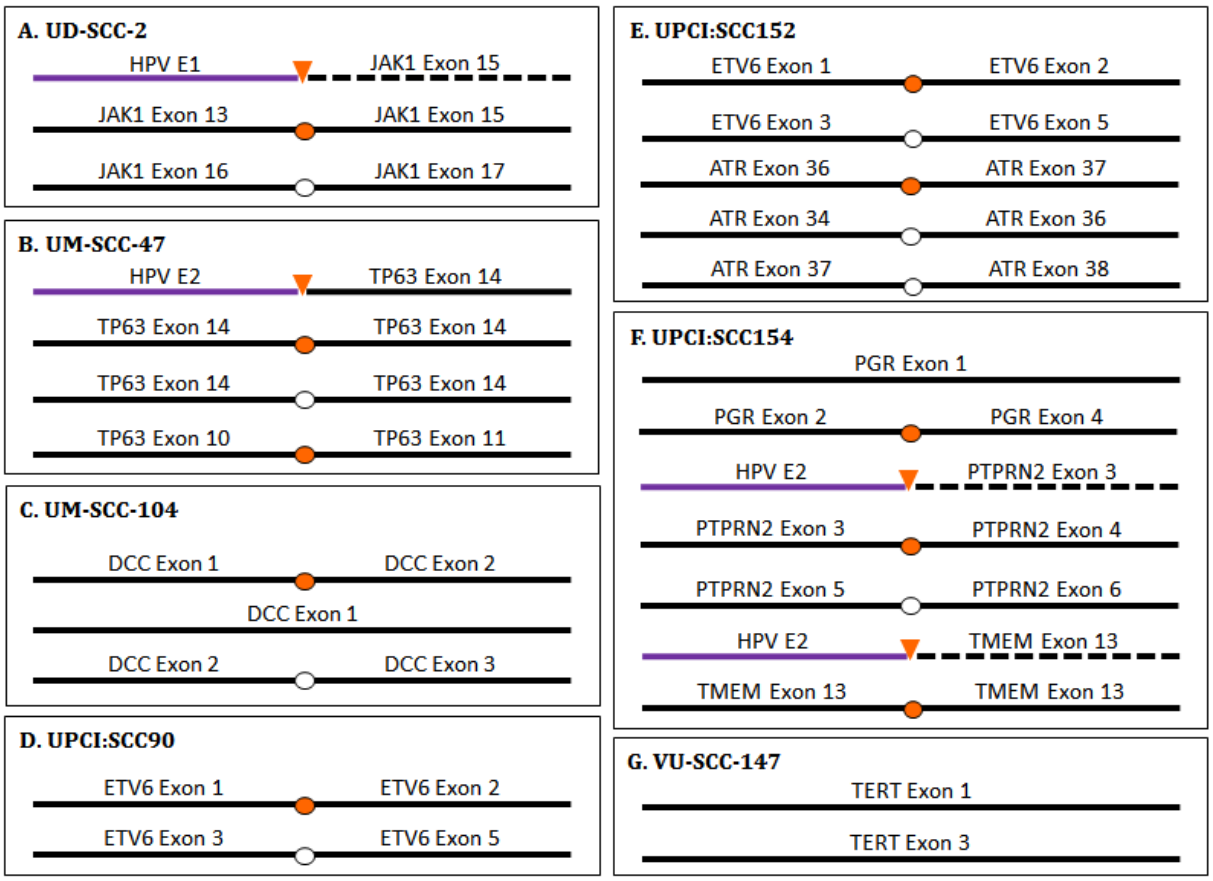

Figure III.9. Diagram of Assays for Cell Line HPV Integration Site Transcript Analysis. Purple line= HPV sequence, Black dashed line= cellular exon following intron of integration, Black solid line= cellular exon, Orange triangle= viral-cellular junction, Orange filled circle= spanning of integration site, White filled circle= exon-exon boundary outside integration site.

In the UM-SCC-104 cell line, *DCC* transcripts within exon 1, across exons 1 and 2 that spanned the HPV integration site in intron 1, and across exons 2 and 3 were interrogated, but no *DCC* transcripts were detected. This suggests that one copy may have been disrupted by HPV integration and the other lost or silenced by methylation[31].

In both UPCI:SCC90 and UPCI:SCC152 cell lines, *ETV6* transcripts were found outside of the intron 1 integration site, across exons 3 and 5. Interestingly, the *ETV6* transcript across exons 1 and 2, spanning the integration site in intron 1 was produced in UPCI:SCC152, but not in UPCI:SCC90. The transcripts that were generated in UPCI:SCC90 and UPCI:SCC152 were all correct and in-frame. Evaluation of the second integration event in UPCI:SCC152 revealed the correct, in-frame, *ATR* transcript present upstream of the integration site in intron 36 (across exons 34 to 36). However, the transcript across exons 36 and 37, spanning the integration site, was generated but was not spliced in-frame. Furthermore, the *ATR* transcript across exons 37 and 38, downstream from the intron 36 integration site, was not generated at all.

In the UPCI:SCC154 cell line, neither the *PGR* transcript across exons 2 to 4, spanning the integration site in intron 3, nor the exon 1 transcript (outside of the integration region) was generated. There was no HPV/*PTPRN2* fusion transcript produced, but the *PTPRN2* transcript across exons 3 and 4, spanning the integration junction, was produced, as was the *PTPRN2* transcript across exons 5 and 6, located downstream of the viral integration site. Both *PTPRN2* exon-exon transcripts were in-frame. Similarly, there was no HPV/*TMEM237* fusion transcript, but the *TMEM237* transcript generated within exon 13 that spanned the integration site was the correct, in-frame sequence. In VU-SCC-147, the correct, in-frame, TERT exon 1 and exon 3 transcripts were produced.

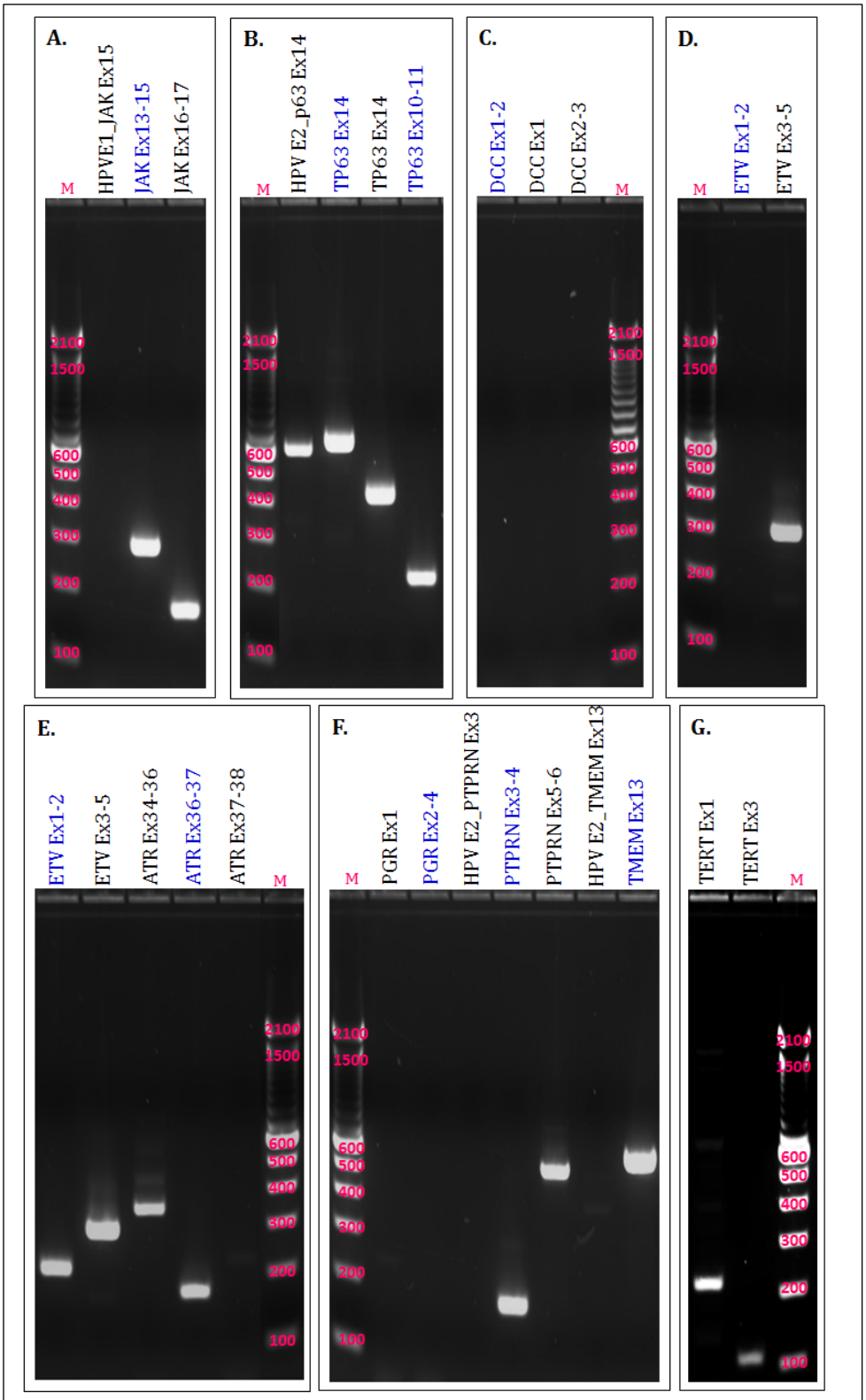

Figure III.10. Gel Analysis of Cell Line Integration Transcripts. Blue text= transcript spanning integration site. Panel A. UD-SCC-2, Panel B. UM-SCC-47, Panel C. UM-SCC-104, Panel D. UPCI:SCC90, Panel E. UPCI:SCC152, Panel F. UPCI:SCC154, Panel G. VU-SCC-147. M= 100bp ladder.

Cell Line	HPV Site	Gene	Region	Transcript Analysis		
UD-SCC-2	(F)E1	*JAK1*	Intron 14	HPVE1_Ex15	JAK1 Ex13-15 ★	JAK1 Ex16-17 ★
UM-SCC-47	(F)E2	*TP63*	Exon 14	HPVE2_ p63Ex14 ✕	p63 Ex14 ★	p63 Ex14 ★
	(R)E2	*TP63*	Intron 10	p63 Ex10-11 ★		
UM-SCC-104	(R)E1	*DCC*	Intron 1	DCC Ex1	DCC Ex1-2	DCC Ex2-3
UPCI:SCC 90	(R)E1	*ETV6*	Intron 1	ETV6 Ex1-2	ETV6 Ex3-5 ★	
UPCI:SCC 152	(R)E1	*ETV6*	Intron 1	ETV6 Ex1-2 ★	ETV6 Ex3-5 ★	
	(F)LCR	*ATR*	Intron 36	ATR Ex34-36 ★	ATR Ex36-37 ✕	ATR Ex37-38
UPCI:SCC 154	(R)E1	*PGR*	Intron 3	PGR Ex1	PGR Ex2-4	
	(F)E2	*PTPRN2*	Intron 3	HPVE2_ PTPRN2 Ex3	PTPRN2 Ex3-4 ★	PTPRN2 Ex5-6 ★
	(R)E2	*TMEM237*	Exon 13	HPV E2_ TMEM237 Ex13	TMEM237 Ex13 ★	
VU-SCC-147	(R)E2	*TERT*	Promoter	TERT Ex1 ★	TERT Ex3 ★	

Table III.3. Summary of Integration Transcription Analysis in HPV16-Positive HNSCC Cell Lines. (F) and (R)= Forward or Reverse viral orientation in relation to the cellular gene. Green text= Viral/cellular fusion transcript, Blue text=Transcript spans integration site, Grey shade=No transcript produced, ★= Sequence spliced in-frame, ✕= Spliced sequence out of frame.

Discussion

The incidence of HPV-positive oropharyngeal cancer is increasing[4,39,40]. Unlike cervical cancers that are detected early by Pap smear screening programs and often cured by colposcopy, there is no method for early detection of HPV-related head and neck cancer, and most such tumors present in an advanced state. The incidence of invasive cervical cancer is declining in western countries secondary to early detection and intervention. In contrast, largely due to high-risk HPV, the incidence of oral, oropharyngeal, and laryngeal

cancers is expected to exceed that of cervix cancer in 2013[41]. Nevertheless, HPV-related oropharyngeal cancers are significantly more responsive to current therapeutic regimens than are HPV-negative cancers arising at the same anatomic sites[2,42-45], prompting interest to reduce the intensity of treatment for this disease. However, even with rigorous therapeutic approaches combining concurrent chemotherapy with radiation[4,43,46], 20-30 percent of HPV-positive cancers progress and become unresponsive to further treatment efforts. Thus, it is important to understand why some tumors respond and others progress.

Only a small number of HPV-positive head and neck cancer cell lines have been developed. All were derived from tumors that failed to respond to therapy, and therefore may be representative of an aggressive subset of such tumors with features consistent with tumor progression. All seven of the HPV16-positive head and neck cancer cell lines express $p16^{INK4a}$ strongly, and exhibit HPV E6-E7 viral oncogene expression, with dominant expression of the E6-E7 alternate transcripts. In addition, all exhibit viral integration into the host cellular genome. As shown in this study, the integration is often complex, with rearrangements and multiple cellular sites of integration involving different segments of the viral genome. A somewhat surprising finding in our study was that in each cell line, the virus had integrated into cellular genes involved in cancer-related pathways. These findings suggest that assessment of cellular sites affected by viral integration in HNSCC may provide a second mechanism of oncogenesis through cellular gene disruption. Such a mechanism has been reported for oncogenesis by low-risk HPV types[47] which lack the transforming ability of the high-risk E6 and E7 genes[48].

High-risk HPV integration has been widely examined in uterine cervix samples, and is strongly associated with high-grade cervical intraepithelial neoplasia and cancer

development[49]. HPV E2, a transcriptional repressor of E6 and E7, is frequently reported to be disrupted upon integration, resulting in proliferative expression of E6 and E7[33, 34]. In cervical cancer studies, as well as a small number of studies on HNSCC, viral integration has been found primarily in intragenic sites (~90% of the genome is intragenic), and in chromosome fragile sites[14,15], although integration into cellular genes has also been reported in a minority of cases [26,28,50,51].

In this study, we detected integration sites that differed from other investigators studying the same cell lines[14]. Studies using DIPS-PCR may detect different sites of integration depending on the restriction enzymes used for DNA digestion, the amplification primers used in the PCR steps, the thermocycling conditions, and amplicon bands selected for sequence analysis. The DNA digest is typically performed with Taq$^{\alpha}$1, which has a single restriction site within the HPV genome, or Sau3A1, with 10 restriction sites in the HPV genome. Both enzymes cut at numerous sites in the host cellular genome, but since the sites occur at different locations in the genome, the enzyme used will determine the cellular regions amplified in the assay. Subsequent PCR steps include viral-specific primers intended to amplify from the virus into the adjacent cellular sequence. The number and location of these primers direct generation of viral-cellular amplicon products; when few primers are used, or the primers are exclusive to the E2 region, integration events will be missed, particularly if the viral disruption occurs outside of the E2 region, or the viral-specific primers are too far from the viral-cellular junction for efficient amplification and sequencing, or viral rearrangements preclude primer annealing. Furthermore, failure to detect integration events that involve multiple concatenated viral genomes may occur if amplicon separation by gel electrophoresis and sequencing are not adequate to

discriminate within-viral from viral-cellular amplicon products. In this study we selected and sequenced all bands less than 2kb to reduce detection of virus-only amplicons. In spite of finding integrations sites previously unreported, our DIPS-PCR approach did not find the previously reported chromosome 9 intergenic insertion in UPCI:SCC90. However, using direct PCR we confirmed the presence of this insertion in the UPCI:SCC90 cells we studied. Another common method used to detect HPV integration, Amplification of Papillomavirus Oncogene Transcripts (APOT)[52], which detects fusion transcripts from integrated HPV, has similar challenges in that this method will detect some but not all events due to limitations of viral primer location, possible gene rearrangement, absence of fusion transcripts, or insufficient assay sensitivity. We confirmed the integration events that were discovered through DIPS-PCR by direct PCR and sequencing of each HPV-cellular fusion from cell line genomic DNA. This confirmation eliminated false-positive integration events that could have been induced through the substantial DNA manipulation of the DIPS-PCR method.

Disruption of a cellular gene due to viral integration may or may not eliminate expression of the gene, depending on whether the second copy (or multiple copies, in the case of aneuploid tumor cells) is affected. The affected cellular gene may be upregulated, disrupted, or unaffected, contingent on strand orientation, as well as the precise viral-cellular junction relative to sequence elements such as promoters and splice sites.

Our assessment of cellular transcripts affected by viral integration provides important but limited information on the consequence of HPV integration on cellular gene expression. In the most straightforward cases, viral integration into *DCC* in UM-SCC-104 and PGR in UPCI:SCC154, our analysis indicates that there are no transcripts generated for either of these cellular genes. The DCC protein can function as a tumor suppressor, so it is

feasible that disruption of this gene through HPV integration could provide a growth advantage for tumor cells. Similarly, the clinical relevance of PGR deficiency in these tumors is yet uncertain.

The HPV integration into *ATR* is of special interest. In this case the integration into intron 36 did not abrogate transcription across exons 34 and 36, but was associated with out of frame splicing in exons 36-37 and absence of transcription across exons 37 and 38. It will be necessary to expand the evaluation of each integration event to fully examine the effects on the complete cellular gene transcript.

In the remaining cases, further investigation is needed to fully understand the effect HPV integration has on cellular gene expression. No in-frame HPV-cellular fusion transcripts were identified, and in nearly all cases, in-frame sequence of transcripts across viral-cellular integration junctions suggests the existence of at least one intact copy of the genes evaluated. In the majority of these cases, the viral integration occurs in an intron (UD-SCC-2 JAK1 Exon 13-15, UM-SCC-47 p63 Exon 10-11, UPCI:SCC90 ETV6 Exon 3-5, UPCI:SCC152 ETV6 Exon 3-5 and ATR Exon 36-37, and UPCI:SCC154 PTPRN2 Exon 3-4), and we speculate that perhaps the virus is contained within the intron, and is spliced out upon cellular RNA processing. A probable explanation for retained exon-exon transcription of genes with integrated HPV is the presence of additional unaffected gene copies that can generate the intact transcripts. Another possibility in cases with viral integration into either cellular introns or exons may be unanticipated splicing from upstream viral regions into cellular exons, such that the transcripts generated do not contain viral regions proximal to the DNA integration sites. In addition to further analysis of the cellular transcripts and protein expression, it may be useful to examine the HPV genome distal to

the integration site in order to determine whether the virus has integrated into 2 different sites in possibly rearranged chromosomes.

The discovery of hrHPV integration into cancer-related genes in all seven of the HNSCC cell lines examined is remarkable, and provides a basis for further investigation of this finding as a possible mechanism of tumor progression and response to therapy. However, ascertaining the true impact of viral integration on the expression or activity of cellular genes is complicated by both irregular patterns of viral integration (multiple concatenated copies, alternating forward/reverse copies, and rearrangements within integrated viral copies) and atypical, disordered, and likely aneuploid cellular genomes.

Comprehensive investigation to understand the specific cellular alterations caused by HPV integration may provide insight for development of alternate therapies for non-responsive tumors. Implementation of viral integration analysis to differentiate responsive from non-responsive HPV-positive head and neck tumors may provide further insight into the factors that distinguish responsive and non-responsive oropharyngeal cancers. This understanding will be necessary to avoid under-treatment of patients selected to receive reduced-intensity therapy and to improve treatment of those with more aggressive tumors who fail to respond to intensive treatment.

We postulate that integration into gene poor or chromosome fragile sites probably occurs in the majority of HPV-driven cancers, but that secondary integration events into cellular genes, such as tumor suppressor genes or genes involved in cancer pathways may be linked to more aggressive malignant behavior. Design of a model to distinguish responsive from non-responsive HPV-positive head and neck tumors assumes viral integration as a primary carcinogenic event, associated with disruption of the E1/E2

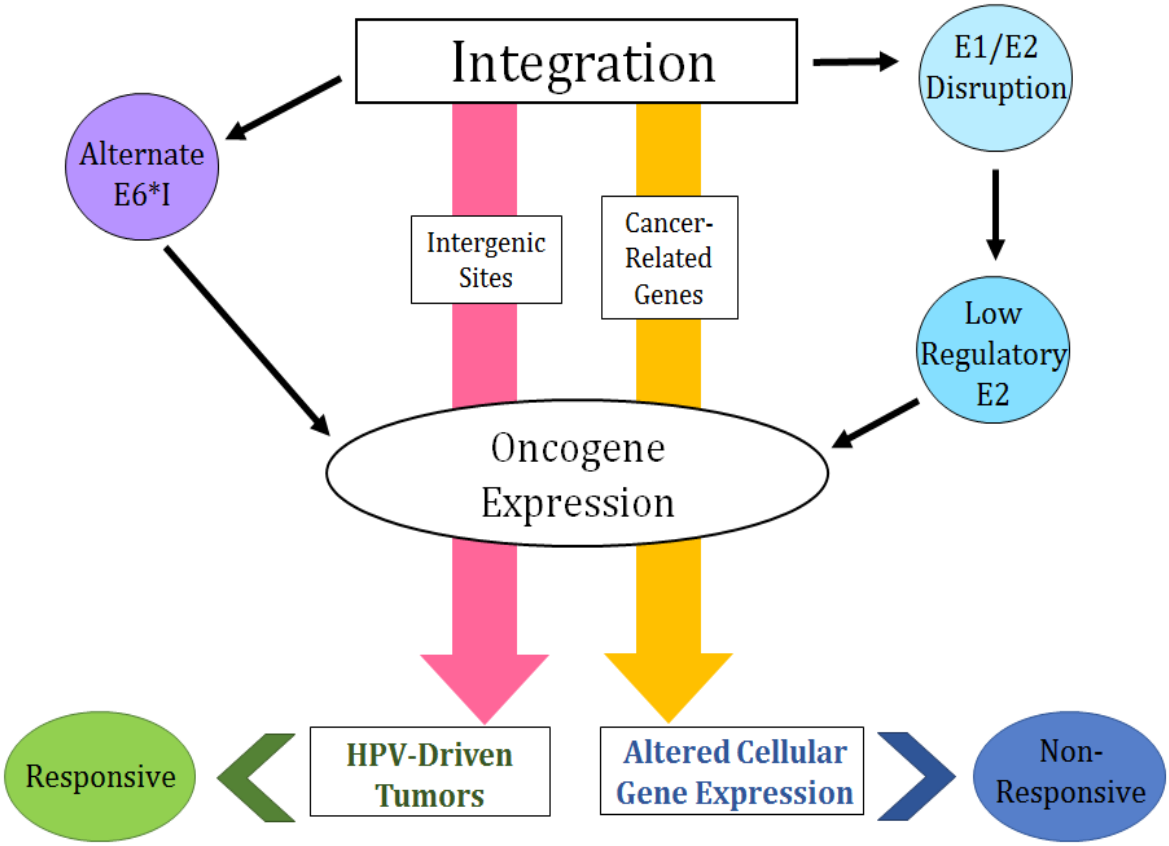

Figure III.11. Proposed Model for Differentiation of Responsive and Non-Responsive HPV-Positive Head and Neck Squamous Cell Carcinoma Tumors.

region, and alternate E6*I, E6*II transcription, which lead to increased viral oncogene expression[53-55]. In such a model, tumors with HPV integration into intergenic chromosome sites or fragile sites are maintained as primarily HPV-driven tumors and are likely to respond to current or reduced-intensity treatment, but tumors with HPV integration into cancer-related genes may acquire secondary alterations in cellular gene expression or dysfunction, resulting in a more aggressive malignant phenotype resistant to current therapies (Figure III.11).

Supplemental Tables

Transcript	Forward Primer	Reverse Primer	Product
Full Length E6-E7	5'-GAACAGCAATAC AACAAACCGTTGTG-3'	5'-TCTGAGAACAG ATGGGGCACACA-3'	499bp
Spliced E6*I-E7	5'-ACTGCGACGTGAG GTGTATTAACTGTC-3'	5'-TCTGAGAACAGA TGGGGCACACA-3'	454bp
Spliced E6*II-E7	5'-ACTGCGACGTGA GATCATCAAGAAC-3'	5'-TCTGAGAACAGA TGGGGCACACA-3'	338bp
GAPDH	5'-CAAGAAGGTGGT GAAGCAG-3'	5'-TGAGCTTGAC AAAGTGGTCG-3'	158bp

Table SIII.1. RT-PCR Assay Primer Sequences and Corresponding Amplicon Lengths for Viral Oncogene Transcript Analysis.

Transcript	Forward Primer	Reverse Primer	TaqMan Probe
E1	5'-GGACTTACACCCAG TATAGCTGACA-3'	5'-TCCCCATGAACATG CTAAACTTTGA-3'	5'-AAAAACACTATT ACAACAATATTG-3'
E2	5'-GGTAGAGGGTCAAG TTGACTATTATGG-3'	5'-CATACTTTATTTTT ACTATATTTTTCTGCAT CATCTTTAAACTG-3'	5'-AAGGAATACG AACATATTTTG-3'
E5	5'-GCTGCTTTTGTCTG TGTCTACATAC-3'	5'-ACGCAGAGGCTGCTGTT-3'	5'-ATCCACAATAGT AATACCAATATT-3'
Full Length E6	5'-GACTTTGCTTTTC GGGATTTATGCA-3'	5'-ACTAATTTTAGAA TAAAACTTTAAACATT TATCACATACAGCA-3'	5'-ATGGATTCCCAT CTCTATATACTA-3'
Spliced E6*I	5'-GAATGTGTGTACT GCAAGCAACAG-3'	5'-GACACAGTGGCTTTT GACAGTTAA-3'	5'-CTGCGACGT GAGGTGTA-3'
Spliced E6*II	5'-GAATGTGTGTACT GCAAGCAACAG-3'	5'-GCATGATTACAGCT GGGTTTCTCT-3'	5'-ACGTGTTCTT GATGATCTC-3'
E7	5'-GCTCAGAGGAGG AGGATGAAATAGA-3'	5'-GAGTCACACTTGCA ACAAAAGGTT-3'	5'-ACCGGACAG AGCCCAT-3'

Table SIII.2. TaqMan Quantitative RT-PCR Assay Primer and Probe Sequences for Viral Oncogene Transcript Analysis.

Primer Set	Forward Primer 1 Sequence	Forward Primer 2 Sequence	Reverse Primer Sequence	Episomal Size
HPV-E1a	5'-ACGGGATGTAATGGATGGTTTTATG-3'	5'-AGGGGATGCTATATCAGATGACGAG-3'	5'-GATGCTGACGACTGATACCGG-3'	7.5 kb
HPV-E1b	5'-ATGTTACAGGTAGAAGGGCG-3'	5'-AGTCAGTATAGTGGTGGAAGTG-3'	5'-GATGCTGACGACTGATACCGG-3'	7.1 kb
HPV-E1c	5'-ACGCCAGAATGGATACAAAGACAAAC-3'	5'-ATGGTACAATGGGCCTACGATAATG-3'	5'-GATGCTGACGACTGATACCGG-3'	6.5 kb
HPV-E2a	5'-ACCCGCATGAACTTCCCATAC-3'	5'-TCAACTTGACCCTCTACCAC-3'	5'-GATGCTGACGACTGATACCGG-3'	2750 bp
HPV-E5a	5'-AGAGGCTGCTGTTATCCACAATAG-3'	5'-ATGTAGACACAGACAAAAGCAGC-3'	5'-GATGCTGACGACTGATACCGG-3'	3020 bp
HPV-L2a	5'-GTACGCCTAGAGGTTAATGCTGG-3'	5'-CCAAAAAGTCAGGATCTGGAGC-3'	5'-GATGCTGACGACTGATACCGG-3'	3500 bp
HPV-L1a	5'-ATCCACACCTGCATTTGCTGC-3'	5'-GCACTAGCATTTTCTGTGTCATCC-3'	5'-GATGCTGACGACTGATACCGG-3'	5.5 kb
HPV-E2b	5'-GTGGACATTACAAGACGTTAGCCTTG-3'	5'-CATGGATATACAGTGGAAGTGCAG-3'	5'-GATGCTGACGACTGATACCGG-3'	5.4 kb
HPV-E2c	5'-CGTCTACATGGCATTGGACAGG-3'	5'-GATAGTGAATGGCAACGTGACC-3'	5'-GATGCTGACGACTGATACCGG-3'	4.7 kb
HPV-L2b	5'-CCACTTTACATGCAGCCTCACC-3'	5'-CTGTACCCTCTACATCTTTATCAGG-3'	5'-GATGCTGACGACTGATACCGG-3''	3070 bp
HPV-E6a	5'-GTATTGCTGTTCTAATGTTGTTCC-3'	5'-GCAAAGTCATATACCTCACGTCG-3'	5'-GATGCTGACGACTGATACCGG-3'	7.7 kb

Table SIII.3. DIPS-PCR Primer Sequences and Predicted Episome-Only Amplicon Sizes for Integration Analysis.

HPV Primer	Sequence
HPV-E1a	5'-ACGGGATGTAATGGATGGTTTTATG-3'
HPV-E1b	5'-ATGTTACAGGTAGAAGGGCG-3'
HPV-E1c	5'-ACGCCAGAATGGATACAAAGACAAAC-3'
HPV-E2a	5'-ACCCGCATGAACTTCCCATAC-3'
HPV-E5a	5'-AGAGGCTGCTGTTATCCACAATAG-3'
HPV-L2a	5'-GTACGCCTAGAGGTTAATGCTGG-3'
HPV-L1a	5'-ATCCACACCTGCATTTGCTGC-3'
HPV-E2b	5'-GTGGACATTACAAGACGTTAGCCTTG-3'
HPV-E2c	5'-CGTCTACATGGCATTGGACAGG-3'
HPV-L2b	5'-CCACTTTACATGCAGCCTCACC-3'
HPV-E6a	5'-GTATTGCTGTTCTAATGTTGTTCC-3'

Chromosome 9 Primer	Sequence
Chrom9-A	5'-CCATCCTCTTGCCTCAGTTTTC-3'
Chrom9-B	5'-GAAAACTGAGGCAAGAGGATGG-3'
Chrom9-C	5'-TGCACTCAGCCCAGTGTGATAA-3'
Chrom9-D	5'-TTATCACACTGGGCTGAGTGCA-3'

Table SIII.4. HPV16 and Chromosome 9q31.1 PCR Primer Sequences for Interrogation of UPCI:SCC90 and UPCI:SCC152 Cell Lines.

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CHAPTER IV

High-Risk Human Papillomavirus Integration into Cellular Genes: Association with Recurrence and Progression in Oropharyngeal Squamous Cell Carcinoma

Abstract

High-risk HPV (hrHPV) is a known driver of carcinogenesis; multiple studies have shown that hrHPV is now the leading etiologic factor in oropharyngeal cancer. HPV-positive oropharynx tumors generally respond well to current therapies, with complete recovery in approximately 80% of patients. However, it is not yet known why a subset of HPV-positive oropharyngeal tumors fails to respond to treatment, with 20% of patients recurring within 5 years. We and others have hypothesized that viral integration into the host cellular genome may contribute to additional mechanisms of carcinogenesis. Furthermore, we have previously demonstrated evidence of hrHPV integration into cancer-related genes in head and neck squamous cell carcinoma (HNSCC) cell lines.

This study examines hrHPV oncogene expression for confirmation of viral activity and integration into the host cellular genome in oropharynx tumors with known outcome, to evaluate the predicative potential of viral integration sites in HPV-driven oropharynx tumors. Quantitative reverse-transcription PCR was used to assess viral oncogene alternate transcripts. Detection of integrated papillomavirus sequences (DIPS-PCR) and sequencing was used to establish viral integration and map specific sites of viral integration into the host cellular genome. Transcript analysis of viral integration into known cellular genes was used to assess the consequence of integration site on gene

expression. Ten oropharynx tumors were assessed, including 5 tumors that responded well to therapy and 5 tumors that recurred after failing to respond to therapy.

All of the tumors demonstrated active viral oncogenesis, indicated by expression of HPV E6 and E7 oncogenes and alternate E6 splicing. In the responsive tumors, HPV integration was found in extragenic chromosome regions, as well as one integration event into a known cancer-related gene, *TP63*. In the recurrent tumors, two HPV integration events were found in extragenic regions on chromosome 10, and each recurrent tumor exhibited HPV integration into known cellular genes, including cancer-associated genes *TNFRSF13B, SCN2A, SH2B1, UBE2V2, SMOC1, NFIA,* and *SEMA6D.* The difference in cellular sites of HPV integration seen between responsive and recurrent hrHPV-driven tumors may suggest that viral integration into intergenic regions is associated with more responsive tumors and viral integration into cancer-related genes is associated with those more likely to require additional or alternate therapies.

Introduction

High-risk human papillomaviruses (hrHPV) are known factors in the etiology of head and neck squamous cell carcinoma, particularly in association with the increasing incidence of oropharynx cancers. In oropharyngeal tumors, hrHPV is associated with better prognosis, suggesting that hrHPV-positive tumors may be responsive to alternate therapies that are more tolerable than those currently used[1-10]. However, a reduction in treatment intensity is precluded by our current inability to distinguish the responsive tumors from the minority of HPV-positive oropharynx tumors that fail to respond to current therapies.

Carcinogenesis in hrHPV-induced tumors is driven by sustained expression of viral E6 and E7 oncogenes. The HPV16 E6 gene contains two introns that can be spliced out, generating alternate E6*I-E7 and E6*II-E7 transcripts that have been linked to increased expression of E7, considered the more potent oncoprotein, at the expense of full length E6[11-16]. The E6*I and E6*II alternate transcripts result from a single donor site at nucleotide (nt) 226 of the viral genome and two acceptor sites at nt 407 (E6*I) and at nt 526 (E6*II) (Figure IV.1). It is not known whether viral integration contributes to progression or resistance to therapy by augmenting the viral oncogene expression or through additional mechanisms. Secondary carcinogenic mechanisms of viral integration could include disruption of tumor suppressor genes or upregulation of genes that promote cell-cycle progression. Integration of hrHPV into the host cellular genome has been reported to be associated with high E6 and E7 transcription and carcinogenic progression from cervical intraepithelial neoplasia (CIN) to invasive disease in many cervical cancer studies[17-20]. Cellular sites of viral integration in cervical cancer are primarily into extragenic regions or chromosome common fragile sites[21-23], but there are studies that report viral integration into known genes in cervical cancer[24-27].

We have previously demonstrated transcriptionally-active hrHPV integration into known cancer-related genes in all seven available HPV16-positive HNSCC cell lines, which are derived from tumors that failed to respond to therapy. We postulate that integration into important cellular genes may be a secondary driver of more highly malignant HPV-positive head and neck tumors, and may be a predictive tool to differentiate responsive and non-responsive HPV-positive oropharynx tumors.

Methods

Tumor specimens: We evaluated ten HPV-positive oropharyngeal tumors from patients who had provided written informed consent to investigate their tissue under a study approved by the Institutional Review Board for the University of Michigan medical school. Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor cores using the DNeasy Blood and Tissue Kit (Qiagen) or fresh-frozen tumor sections using a standard phenol extraction. Tumor tissue was microdissected for RNA from fresh-frozen tumor sections immediately following histological evaluation. Total RNA was isolated using the RNeasy Mini Kit with QIAzol (Qiagen), followed by on-column DNase treatment.

HPV genotyping and copy number analysis: hrHPV genotyping was performed on DNA from all tumors using the HPV PCR-MassArray assay[3,28-31]. Type-specific TaqMan quantitative PCR was used to determine HPV copies per cell, assessing both E6 and E7 amplicons, with a GAPDH assay as an endogenous two copy/cell endogenous reference control.

HPV E6 and E7 transcript analysis: HPV16 E6 and E7 transcripts were evaluated by reverse-transcription PCR (RT-PCR) with gel electrophoresis and TaqMan quantitative RT-PCR. To analyze the expression of HPV16 E6 and E7, transcript-specific assays were used that exclusively amplify each product: the intact, non-spliced, full-length E6-E7 transcript, the spliced E6*I-E7 transcript, and the spliced E6*II-E7 transcript, as illustrated in Figure IV.1 (Primer sets are listed in Table SIV.1). An assay for human endogenous GAPDH was included to verify the absence of contaminating genomic DNA. Quantitative RT-PCR was performed using similar transcript-specific TaqMan assays that individually interrogate each HPV E6 and E7 transcript: non-spliced full length E6, spliced E6*I, spliced

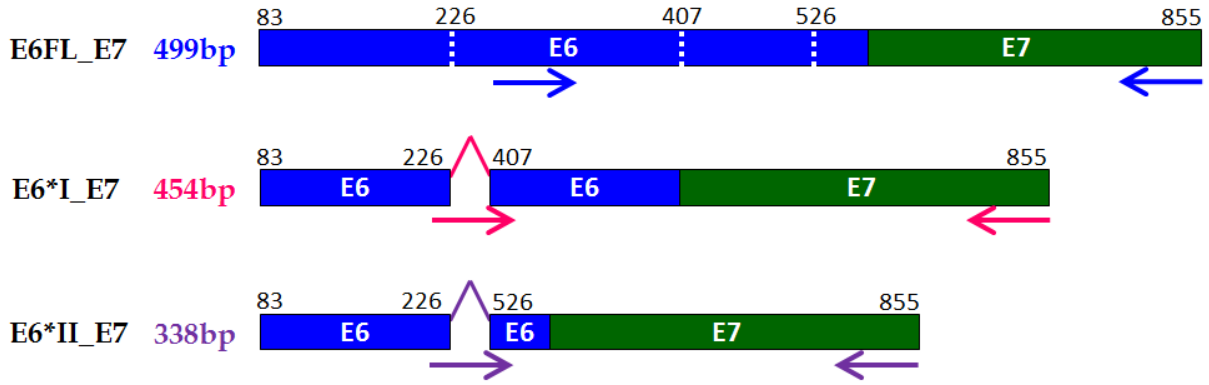

Figure IV.1. RT-PCR Strategy for Transcript-Specific E6-E7 Oncogene Evaluation in HPV16-Positive HNSCC Tumors. Primers within the splice region or across splice junctions allow for exclusive amplification of full length E6 or alternate E6 transcripts.

E6*II and E7 (Assays are portrayed in Figure IV.2 and primer sequences are listed in Table SIV.2). A TaqMan quantitative assay for GAPDH was included as an endogenous control to calculate relative viral gene expression.

Detection of Integrated Papillomavirus Sequences-Polymerase Chain Reaction (DIPS-PCR): Viral integration was evaluated using an adaptation (illustrated in Figure III.3) of the DIPS-PCR method previously published[23,25]. Genomic DNA from each tumor was subjected to Taq$^{\alpha}$1 restriction enzyme digestion, producing fragmented DNA. There are approximately 1.5 million Taq$^{\alpha}$1 restriction sites within the human cellular genome, but only one in the non-variant HPV16 genome, located in the E6 open reading frame (ORF) at nucleotide 505 (nt 505). Additional HPV16 Taq$^{\alpha}$1 restriction sites have been described in HPV16 variants at positions 311 and 2608. Following restriction digest, a ligation reaction attached a double-strand adapter oligo (5'-CGCAACGTGTAAGTCTG-NH2-3' annealed to 5'-GGGCCATCAGTCAGCAGTCGTAGCCGGAT CCAGACTTACACGTTG-3') to the overhanging ends of each fragment. Linear amplification of the ligated fragments was performed using 11 viral-specific primers, generating amplicons that originate in the viral genome, extend into adjacent cellular sequence, and terminate at the end of the adapter

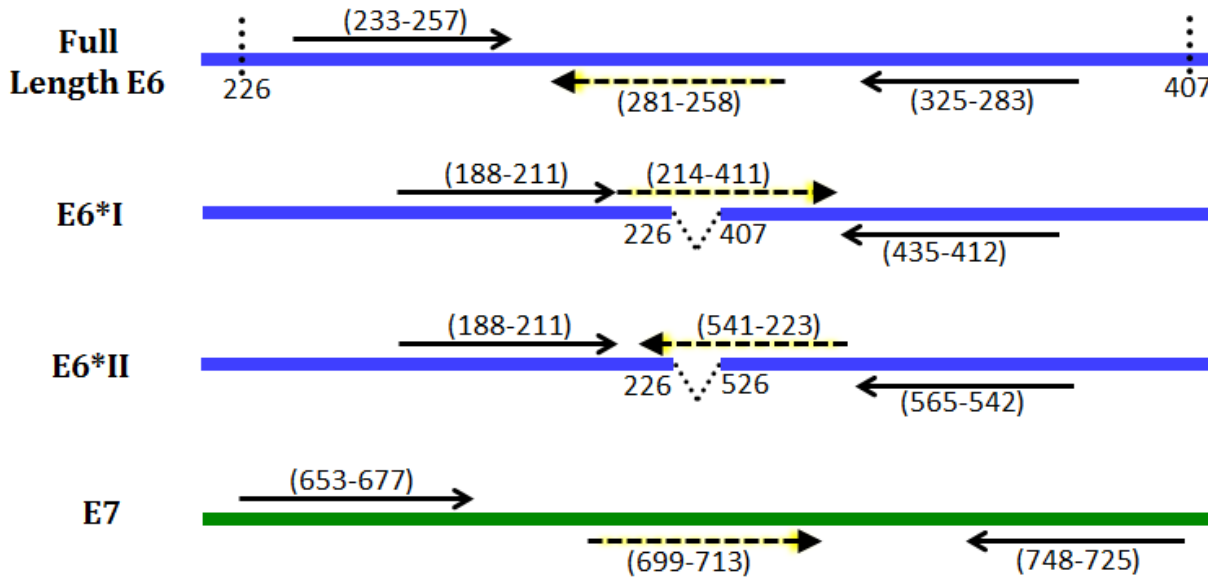

Figure IV.2. Illustration of TaqMan Primers and Probes for Quantitative RT-PCR in HPV16-Positive Tumors. Solid arrows represent primers and dashed arrows represent TaqMan probes.

oligo (Primers are listed in Table SIV.3). This was followed by a second, logarithmic, PCR using 11 nested viral primers with a reverse adapter-specific primer (Primers are listed in Table SIV.3). Thermocycling conditions used for linear and exponential PCR included 3 minute extension cycles, allowing limitation of amplicon size to 3kb or less, therefore excluding production of any of large (>3kb), episome-only fragments. PCR products were separated by gel electrophoresis.

Sequence analysis of HPV16 integration products: Viral-cellular fragments were distinguished from episomal virus fragments based on predicted viral-only amplicon sizes of 2750bp or larger (Table SIV.3). DIPS-PCR amplicons of approximately 2500bp or smaller were identified, the corresponding bands were excised, and the amplicons were purified and sequenced. Integration events into known cellular genes were confirmed by direct PCR and sequencing of the original tumor genomic DNA, using primers designed for each viral and cellular region.

Integration site transcript analysis: RT-PCR assays were designed to amplify viral-cellular fusion transcripts and cellular transcripts from tumor RNA in cases expected to be altered by confirmed viral integration into known cellular genes. Assays included virus-cellular fusion transcripts (although expected only in the single case where the integration into the cellular gene followed the same orientation as the virus) from HPV ORFs into cellular gene exons, cellular gene exon-exon transcripts spanning the integration site, and exon-exon or within-exon transcripts outside of the integration site region. All successfully amplified transcripts were sequenced for verification.

Results

HPV genotyping and copy number analysis: All 10 tumors were positive for HPV16 and negative for all other hrHPV types included in the PCR-MassArray assay. HPV16 copy number for the responsive tumors ranged from 16 to over 500 copies per cell; tumor 1733 had 22.5 HPV16 copies per cell, tumor 1769 had 475.6 copies per cell, tumor 1804 had 161.1 copies per cell, tumor 1971 had 538.9 copies per cell, and tumor 2148 had 16.1 copies per cell. The recurrent tumors had overall lower values, ranging from 6 to nearly 300 HPV16 copies per cell; tumor 0732 had 110.7 HPV16 copies per cell, tumor 0843 had 34.1 copies per cell, tumor 1040

Responsive Tumors	HPV16 copies/cell
1733	22.5
1769	475.6
1804	161.1
1971	538.9
2148	16.1
Recurrent Tumors	**HPV16 copies/cell**
0732	110.7
0843	34.1
1040	297.9
2049	6.3
2238	14.0

Table IV.1. Responsive and Recurrent Tumor HPV16 Copy Number as Determined by TaqMan Quantitative PCR.

had 297.9 copies per cell, tumor 2049 was the lowest with 6.3 copies per cell, and tumor 2238 had 14.0 copies per cell (Table IV.1). The average viral copy number was 242.8 for the responsive tumors, and 92.6 for the recurrent tumors.

HPV E6 and E7 transcript analysis: HPV16 E6 and E7 transcripts were expressed in all ten HPV16-positive tumors. In both responsive and recurrent tumor groups, the most abundant transcript in four of the five tumors was the alternate E6* transcript, which is known to be expressed in hrHPV-transformed tumor cells. In these tumors, the full length E6 transcript was much lower than the E6*I transcript; the exceptions were responsive tumor 1971 and recurrent tumor 0732, where the full length E6 transcript exhibited the highest level of expression (Figures IV.3 and IV.4).

Detection of Integrated Papillomavirus Sequences-Polymerase Chain Reaction (DIPS-PCR): All ten HPV16-positive tumor specimens demonstrated viral integration; representative DIPS-PCR gels for responsive and recurrent tumors are shown in Figures IV.5 and IV.6, respectively. A total of 207 hybrid viral-cellular amplicons were isolated and sequenced. Among the 99 amplicons generated from the responsive tumors, there were 20 for tumor 1733, 22 for tumor 1769, 26 for tumor 1804, 26 for tumor 1971, and 5 for tumor 2148. The 108 amplicons from the recurrent tumors included 30 for tumor 0732, 23 for tumor 0843, 13 for tumor 1040, 18 for tumor 2049, and 24 for tumor 2238. Viral-host DNA fusions were identified by sequence and BLAST analysis. The sequence reads mapped to viral-only sequence, viral-cellular hybrids as described below, or were unmapped due to poor sequence resolution. Diagrammatic representations of the viral integration events identified are depicted in Figure IV.7 for responsive tumors and Figure IV.8 for recurrent tumors; Tables IV.2 and IV.3 summarize the integration results for responsive

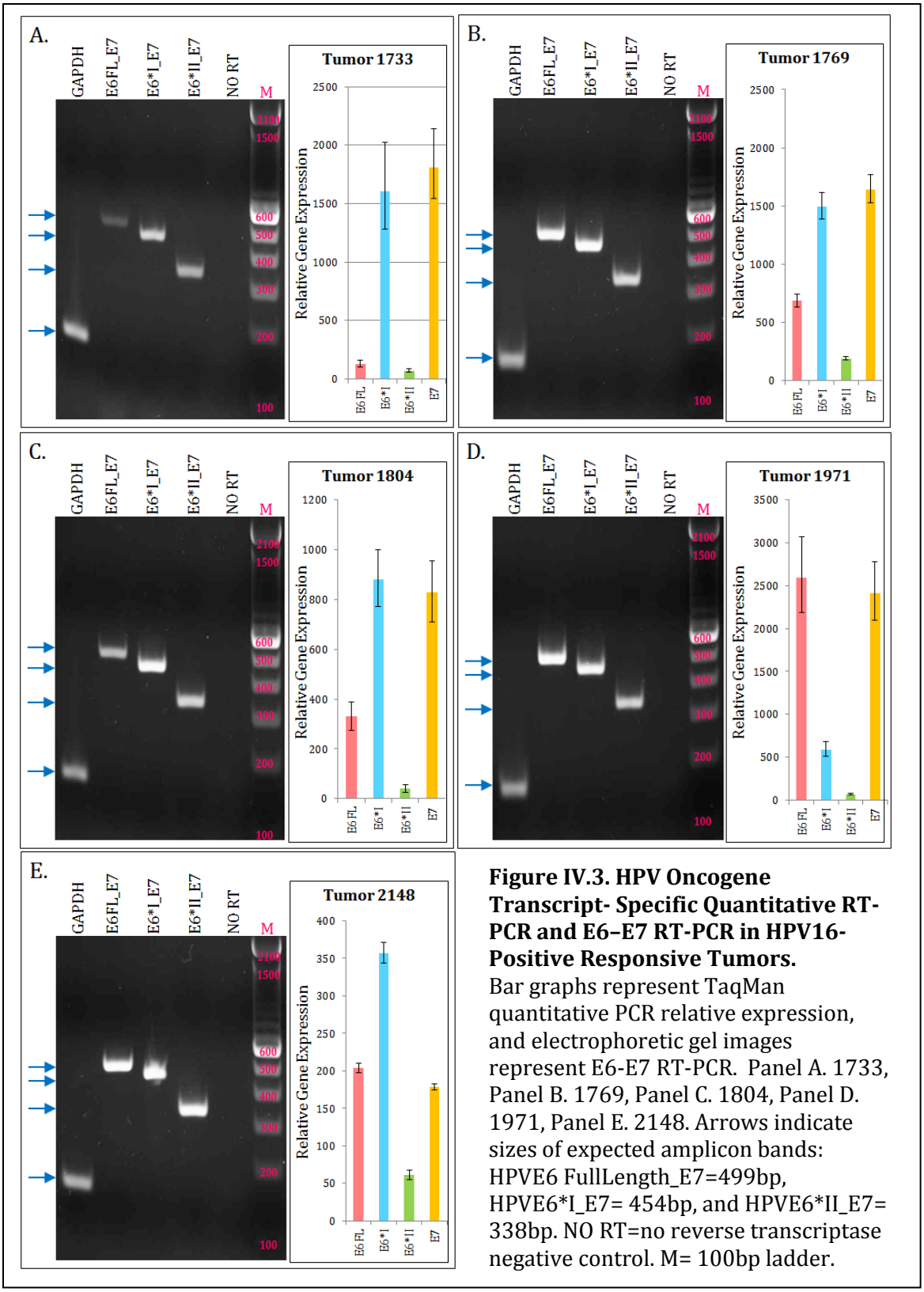

Figure IV.3. HPV Oncogene Transcript- Specific Quantitative RT-PCR and E6–E7 RT-PCR in HPV16-Positive Responsive Tumors.
Bar graphs represent TaqMan quantitative PCR relative expression, and electrophoretic gel images represent E6-E7 RT-PCR. Panel A. 1733, Panel B. 1769, Panel C. 1804, Panel D. 1971, Panel E. 2148. Arrows indicate sizes of expected amplicon bands: HPVE6 FullLength_E7=499bp, HPVE6*I_E7= 454bp, and HPVE6*II_E7= 338bp. NO RT=no reverse transcriptase negative control. M= 100bp ladder.

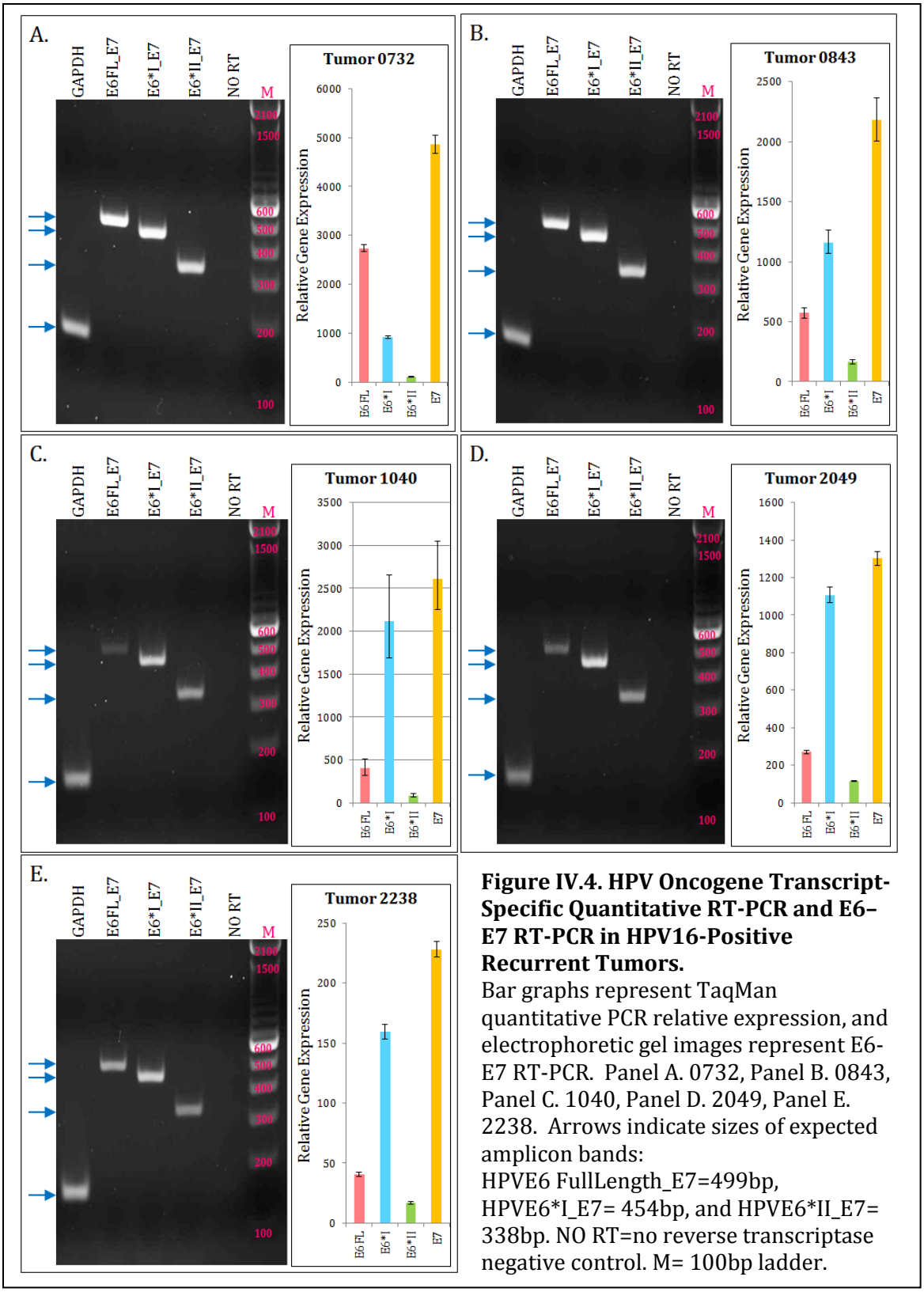

Figure IV.4. HPV Oncogene Transcript-Specific Quantitative RT-PCR and E6–E7 RT-PCR in HPV16-Positive Recurrent Tumors.
Bar graphs represent TaqMan quantitative PCR relative expression, and electrophoretic gel images represent E6-E7 RT-PCR. Panel A. 0732, Panel B. 0843, Panel C. 1040, Panel D. 2049, Panel E. 2238. Arrows indicate sizes of expected amplicon bands:
HPVE6 FullLength_E7=499bp, HPVE6*I_E7= 454bp, and HPVE6*II_E7= 338bp. NO RT=no reverse transcriptase negative control. M= 100bp ladder.

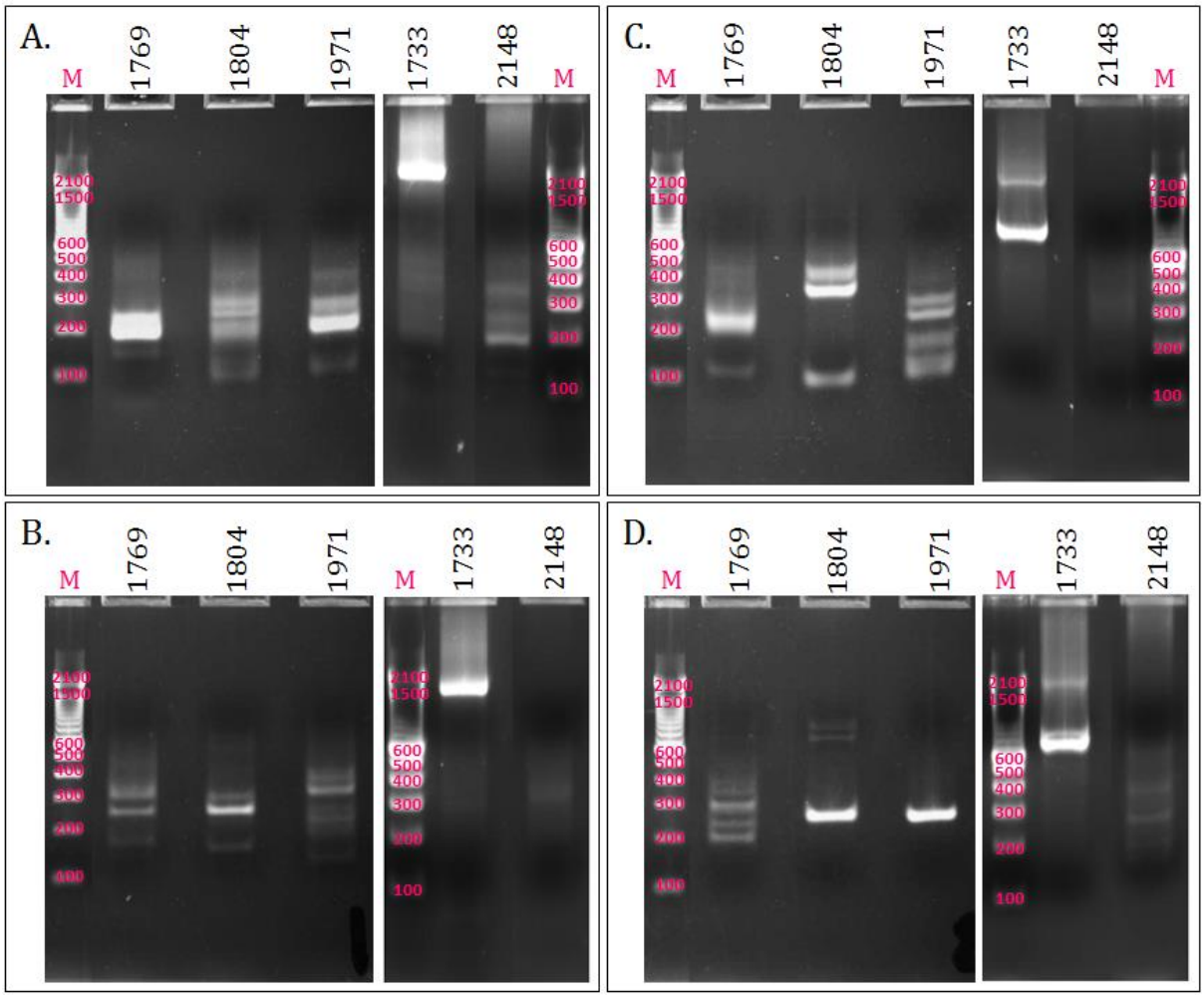

Figure IV.5. Representative Responsive Tumor DIPS PCR Gels. Panel A. HPV-E1a/Adapter primers, Panel B. HPV-E1b/Adapter primers, Panel C. HPV-E1c/Adapter primers, Panel D. HPV-E2a/Adapter primers, M=100 bp ladder.

and recurrent tumors, respectively, indicating the chromosome locus, known genes, and the regions of integration into the cellular gene.

Sequence analysis of integration events in responsive tumors: Eleven of the twelve HPV integration events identified in the responsive tumors involved intergenic chromosome regions. Tumor 1733 had an HPV E2 integration into 2p16, which is a known chromosome fragile site[32]; tumor 1979 had 3 integration events, HPV E2 into 9q21, HPV L1 into 16q11.2, and another L1 into 4q27; four integration events were identified in tumor 1804, HPV E1 into 6q16, HPV L2 into 10p11.1, HPV E5 into 16q11.2, and HPV E2 into

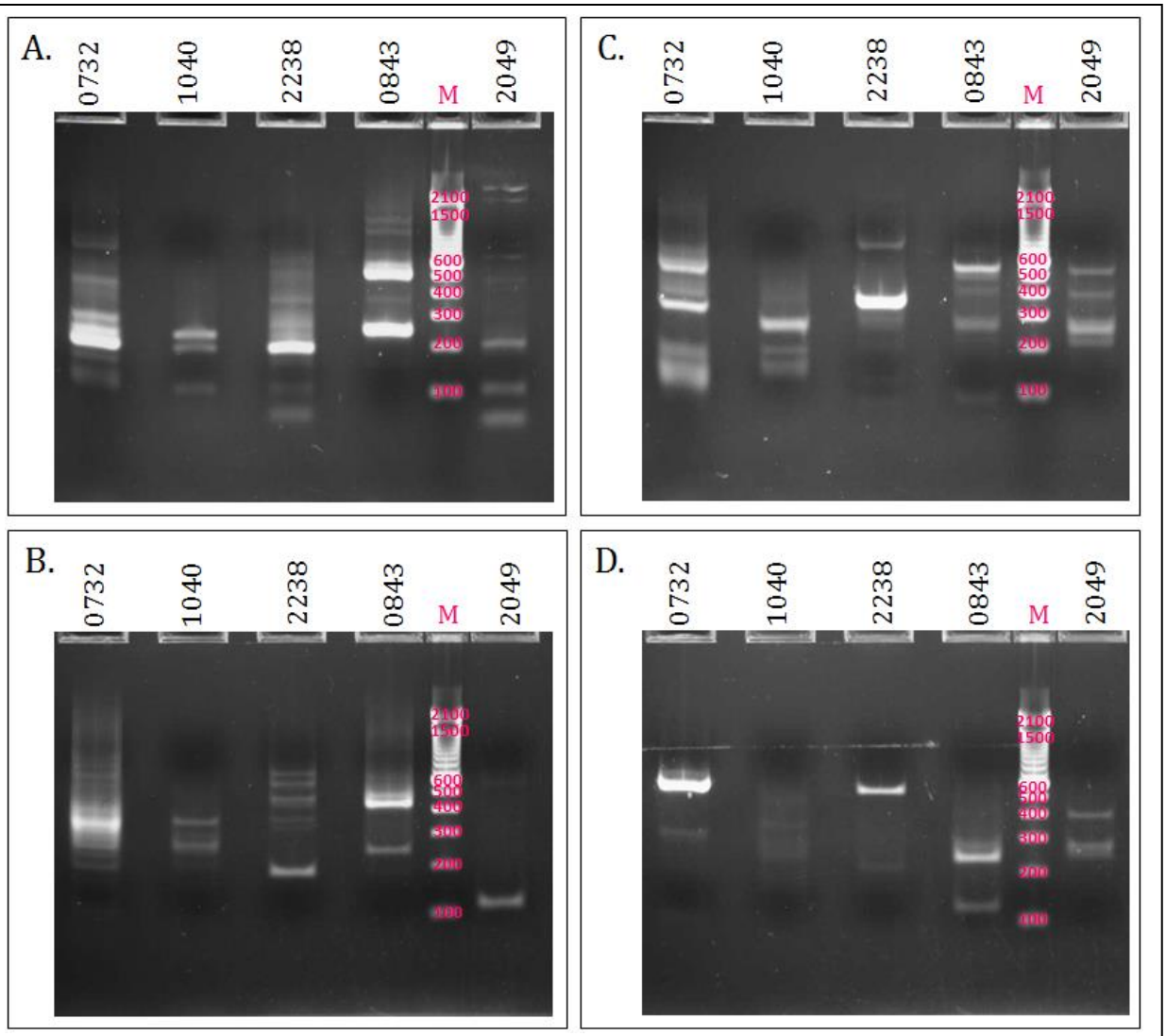

Figure IV.6. Representative Recurrent Tumor DIPS PCR Gels. Panel A. HPV-E1a/Adapter primers, Panel B. HPV-E1b/Adapter primers, Panel C. HPV-E1c/Adapter primers, Panel D. HPV-E2a/Adapter primers, M=100 bp ladder.

16q11.2; tumor 2148 had a single integration of HPV L2 into 7p22, which is a known chromosome fragile site[32]; and tumor 1971 had three integrations, HPV E1 into chromosome fragile site 7p22.3[32], L2 into 4p16.3, also a known chromosome fragile site[32], and HPV L1 into 3q28, where the virus inserted into intron 4 of *TP63*, the gene for tumor suppressor protein 63. This integration site is located within the region that codes for the DNA binding domain of the protein. Interestingly, we observed HPV integration into this same gene in the HNSCC cell line UM-SCC-47, where HPV E2 inserted into both intron 10

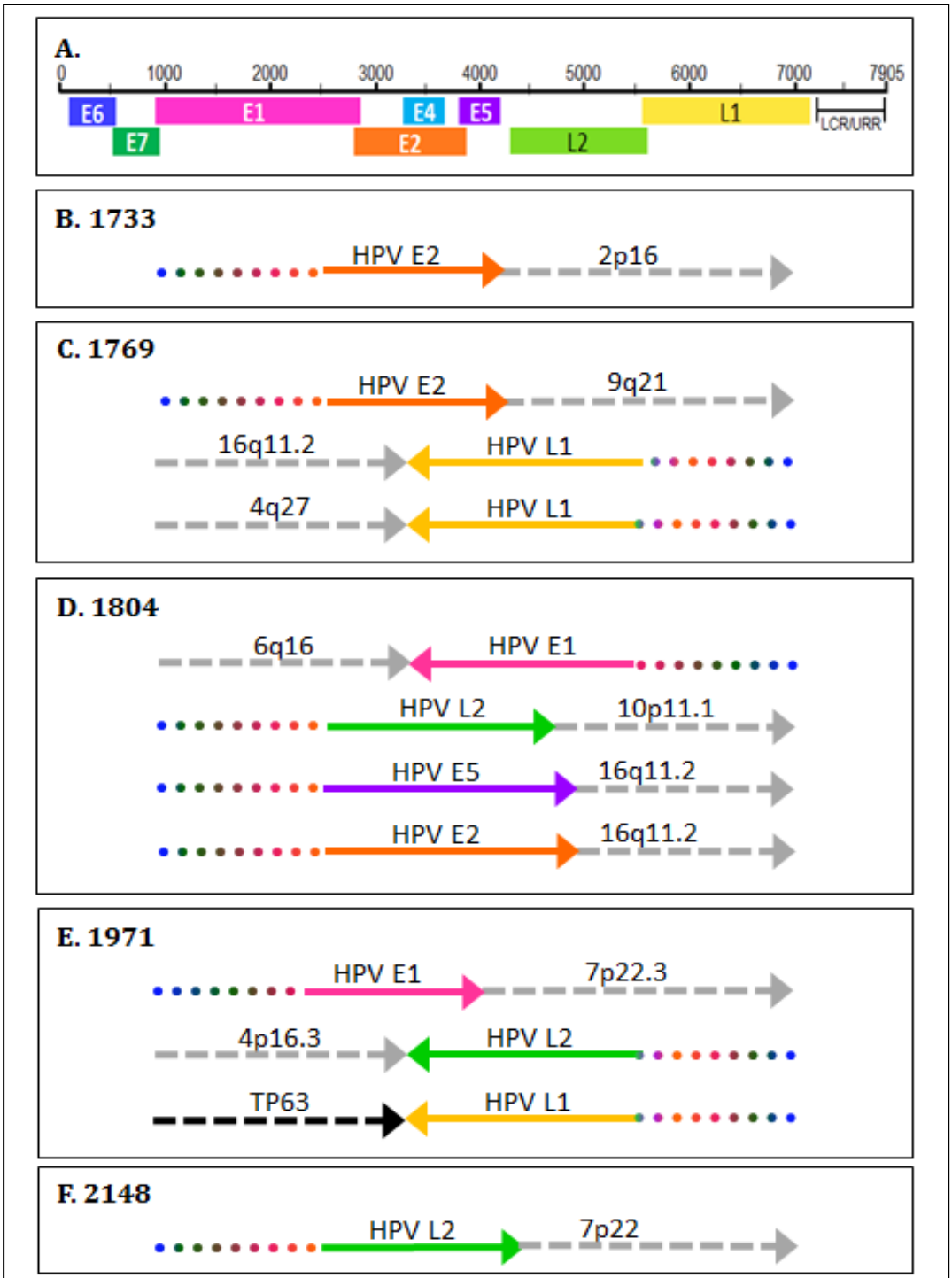

Figure IV.7. Schematic Representation of Integration Events in HPV16-Positive Responsive Tumors. Panel A. Linear organization of the HPV genome, Panel B. 1733, Panel C. 1769, Panel D. 1804, Panel E. 1971, Panel F. 2148. Arrow direction indicates orientation of genes. Solid colored arrows represent HPV, Dotted colored arrows indicate HPV sequence outside of mapped region, Dashed grey arrows are cellular intragenic regions, Dashed black arrows are cellular genes. The colors in the sequenced amplicons correspond to the color coded viral genome at the top of the figure.

and exon14 of *TP63*. Viral integration into *TP63* has also been reported in cervical cancers, and susceptibility for integration into this gene may be due to short segments of homologous sequence shared by HPV E1 and chromosome 3q28 within the *TP63* gene[24]. *TP63* belongs to the p53 family of tumor suppressor genes, and is a sequence-specific DNA binding transcriptional repressor and activator. The p63 protein participates in TGFβ and WNT signal transduction as well as differentiation and cell-cycle regulation[33], and as such, HPV integration into the *TP63* gene could cause disruption of these processes and may result in increased proliferation.

Tumor	HPV Site	Cellular				
		Locus	Gene	Gene Name	Region	Domain
1733	(F)E2	2p16	Intergenic			
1769	(F)E2	9q21	Intergenic			
	(R)L1	16q11.2	Intergenic			
	(R)L1	4q27	Intergenic			
1804	(R)E1	6q16	Intergenic			
	(F)L2	10p11.1	Intergenic			
	(F)E5	16q11.2	Intergenic			
	(F)E2	16q11.2	Intergenic			
1971	(F)E1	7p22.3	Intergenic			
	(R)L2	4p16.3	Intergenic			
	(R)L1	3q28	***TP63***	Tumor protein p63	Intron 4	DNA Binding domain
2148	(F)L2	7p22	Intergenic			

Table IV.2. Summary of Integration Events in HPV16-Positive Responsive Tumors. (F) and (R) = Forward or Reverse viral orientation in relation to the cellular gene.

Sequence analysis of integration events in recurrent tumors: The recurrent tumors exhibited viral integration into both intergenic and genic regions. Tumor 0732 had integration from HPV E2 into an intergenic region at 10p11.1, as well as HPV L2 into 17p11.2, inserting at intron 3 of *TNFRSF13B*, the gene coding for a member of the tumor necrosis factor receptor superfamily. This viral integration occurs within the region that produces the extracellular topological domain of the receptor protein, which participates in immunity by interacting with a TNF ligand. *TNFRSF13B* induces B-cell maturation and differentiation and activates multiple transcription factors, including NFAT, AP1, and NF-κB. It has been reported that hematological malignancies are induced by B-cell survival and aberrant proliferation caused by dysregulated signaling by TNFRSF family members[34], but how this pathway may be involved in HNSCC is not clear.

A single integration event was identified in tumor 0843, HPV L2 into 2q24.3, at intron 16 of *SCN2A*, which codes for the voltage-gated type II sodium channel α subunit. This integration takes place in the second helical transmembrane S6 region of the protein, which participates in a complex for action potential initiation and propagation in excitable cells, as well as proliferation, migration, and adhesion in non-excitable cells[35]. It has been reported that differential expression of voltage-gated sodium channels is associated with the metastatic activity of multiple malignancies such as leukemia and prostate, breast, and lung cancer, and these ion channels are currently being investigated as targets for cancer therapies[35-38]. Additionally, DIPS-PCR and sequencing revealed an HPV early gene rearrangement in tumor 0843, where the latter half of E6 was duplicated and joined within the E2 ORF (Figure IV.8C).

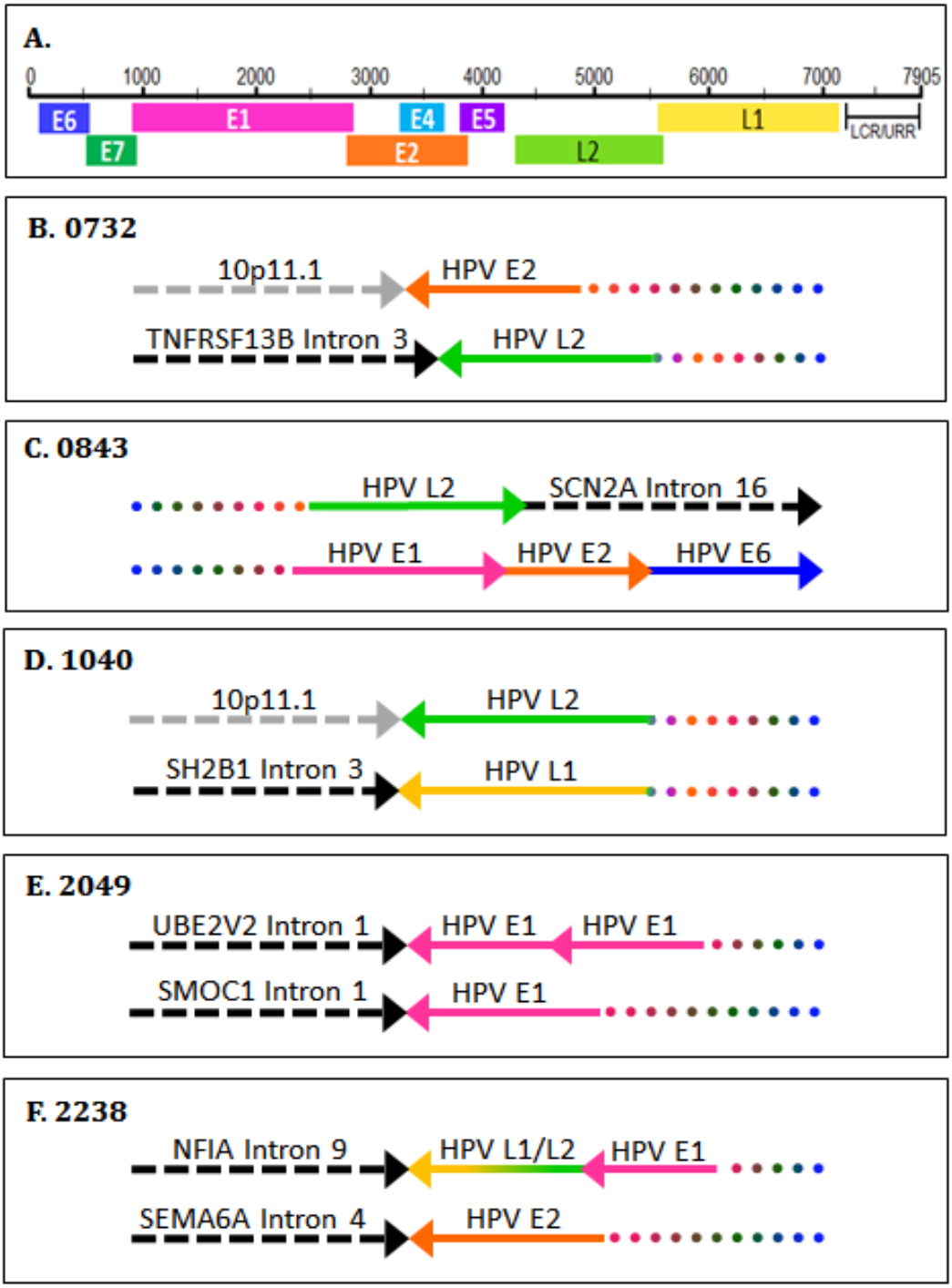

Figure IV.8. Schematic Representation of Rearrangements and Integration Events in HPV16-Positive Recurrent Tumors. Panel A. Linear organization of the HPV genome, Panel B. 0732, Panel C. 0843, Panel D. 1040, Panel E. 2049, Panel F. 2238. Arrow direction indicates orientation of genes. Solid colored arrows represent HPV, Dotted colored arrows indicate HPV sequence outside of mapped region, Dashed grey arrows are cellular intragenic regions, Dashed black arrows are cellular genes. The colors in the sequenced amplicons correspond to the color coded viral genome at the top of the figure.

Tumor	HPV Site	Cellular				
		Locus	Gene	Gene Name	Region	Domain
0732	(R)E2	10p11.1	Intergenic			
	(R)L2	17p11.2	***TNFRSF13B***	Tumor necrosis factor receptor superfamily, 13B	Intron 3	Extracellular Topological domain
0843	(F)L2	2q24.3	***SCN2A***	Sodium channel, voltage-gated, type II, α subunit	Intron 16	Helical Transmembrane S6 of repeat II region
1040	(R)L2	10p11.1	Intergenic			
	(R)L1	16p11.2	***SH2B1***	SH2B Adapter Protein 1	Intron 3	Plekstrin Homology domain
2049	(R)E1	8q11.21	***UBE2V2***	Ubiquitin-conjugating enzyme E2 variant 2	Intron 1	Ubiquitin-Conjugating domain
	(R)E1	14q24.1	***SMOC1***	SPARC related modular calcium binding 1	Intron 1	Kazal-like domain
2238	(R)L1	1p31.3-p31.2	***NFIA***	Nuclear factor I/A	Intron 9	DNA Binding domain
	(R)E2	15q21.1	***SEMA6D***	Semaphorin 6D	Intron 4	Extracellular Topological Sema domain

Table IV.3. Summary of Integration Events in HPV16-Positive Recurrent Tumors. (F) and (R) = Forward or Reverse viral orientation in relation to the cellular gene.

Tumor 1040 had integration of HPV L2 into an intergenic region of 10p11.1, as well as HPV L1 into 16p11.2, at intron 3 of *SH2B1*, the gene for SH2B adapter protein 1. This is a mediator protein for tyrosine kinase receptors, and is involved in Janus kinase (JAK) and receptor tyrosine kinase signaling pathways. Based on the common pathway, consequences of HPV integration into *SH2B* in this tumor could be similar to potential effects of HPV integration into *JAK1* as seen in the HNSCC cell line UD-SCC-2. JAK

mediates interferon receptors and STAT signaling, and viral integration may be associated with loss of interferon signaling within transformed cells[39].

Tumor 2049 had two integration events, the first involving a rearrangement of HPV E1 (where a duplicated region of E1 was inserted into E1 upstream of the integration) into 8q11.21, at intron 1 of *UBE2V2*, which codes for ubiquitin-conjugating enzyme E2 variant 2 (Figure IV.8E). The protein product of *UBE2V2* mediates transcriptional activation of target genes, regulates cell cycle progression and cellular differentiation, and is involved in DNA repair and cell survival after DNA damage. Deregulation of UBE2V2 expression has been reported to be associated with gastric cancer[40], and in ER-positive/HER2-negative breast cancer, UBE2V2 was linked to poor prognosis[41]. The second integration even identified in tumor 2049 was HPV E1 into 14q24.1, at intron 1 of *SMOC1*, the gene for SPARC-related modular calcium binding 1. *SMOC1* codes for a secreted protein localized to the basement membrane that is involved in cellular differentiation, and has been associated with brain cancer[42].

Two integration events were identified in tumor 2238, the first was comprised of a rearrangement within HPV, where the L2/L1 overlapping region was inserted into the E1 ORF and inserted into 1p31.3, at intron 9 of *NFIA*, which codes for nuclear factor I/A. The NFIA protein product is a sequence-specific transcription factor that regulates numerous viral and cellular genes, and is independently proficient in activating cellular transcription and replication. It was recently reported that an investigation of acute erythroid leukemia containing t(1;16)(p31;q24) uncovered a gene fusion between *NFIA/*C*BFA2T3*[43]. The second integration in tumor 2238 was HPV E2 into 15q21.1, at intron 4 of *SEMA6D*, the gene for semaphorin 6D. The product of *SEMA6D* is a transmembrane protein historically

characterized as an axon guidance molecule, but has more recently been shown to participate in differentiation, organogenesis, and angiogenesis, mediated by Plexin-A1 as the major Sema6D-binding receptor[44,45]. Furthermore, it has been reported that the Sema6D/Plexin-A1 complex binds VEGFR-2 to mediate survival and anchorage-independent growth of tumor cells[45,46].

HPV integration into the intergenic chromosome region 16q11.2 was identified 3 times among the responsive tumors examined; once in tumor 1769 and in two different events in tumor 1804. A second intergenic region was involved in 3 integration events among the tumors evaluated; responsive tumor 1804 and recurrent tumors 0732 and 1040 all exhibited viral integration into chromosome 10p11.1. These parallels suggest that there may be sequence or structural similarities that increase the probability of viral integration into these regions. Each integration into a cellular gene was confirmed by direct PCR and sequencing of the tumor genomic DNA, eliminating possible false-positive integration events induced by the DIPS-PCR method.

Integration site transcript analysis: Based on the integration results from the DIPS-PCR analysis, assays for integration site transcript analysis were designed as illustrated in Figure IV.9. Results of the transcript analysis (electrophoretic gel images of transcript amplicons) are shown in Figure IV.10, and Table IV.4 lists a summary of transcript RT-PCR and sequencing results. In responsive tumor 1971, a fusion transcript between HPV L1 and *TP63* exon 4 was not produced. The transcript across *TP63* exons 4 and 5, spanning the viral integration site in intron 4, was produced, and the sequence was in-frame. Additionally, the transcript across *TP63* exons 5 and 6 (outside of the integration region) was generated and was spliced in-frame.

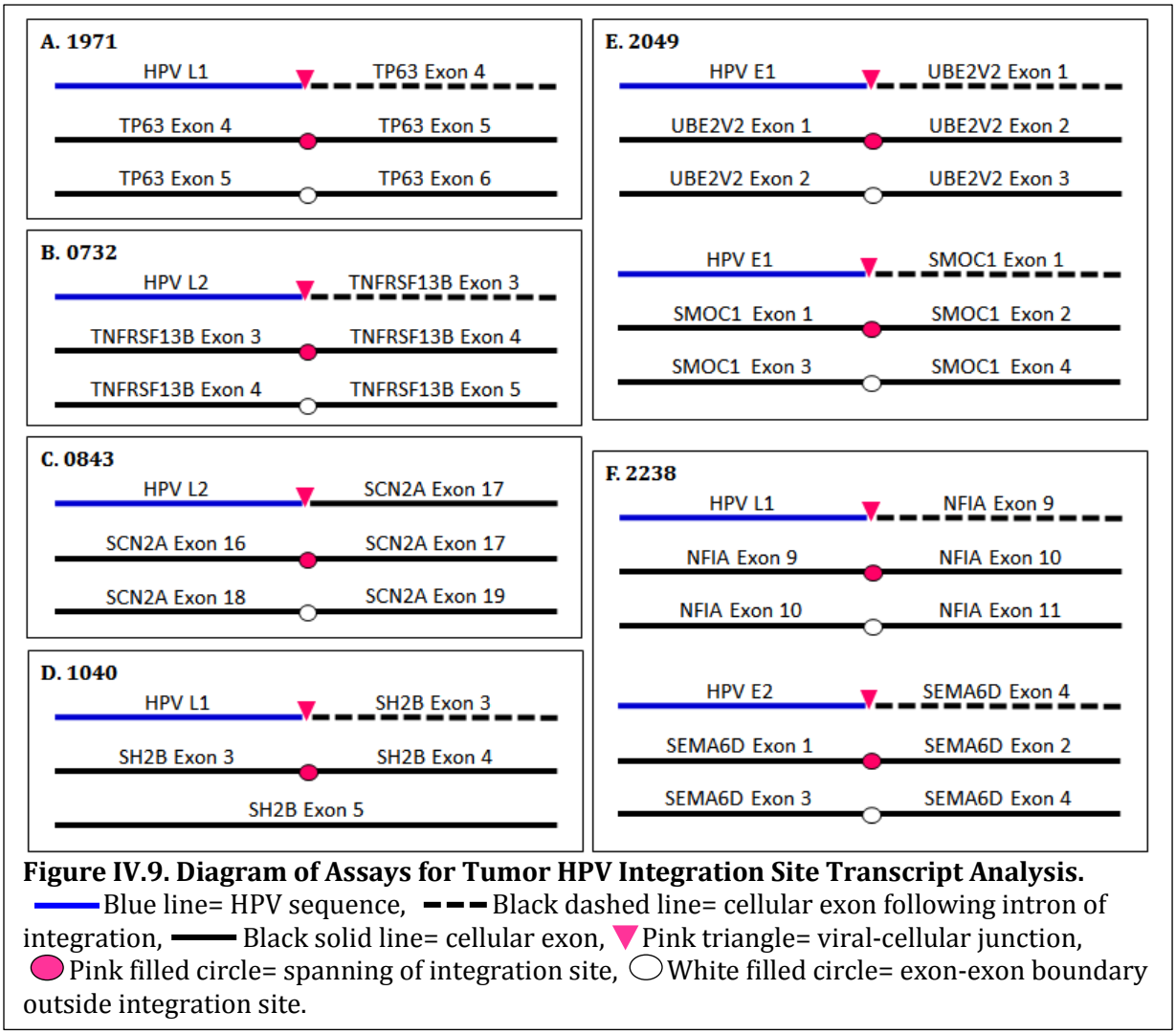

Figure IV.9. Diagram of Assays for Tumor HPV Integration Site Transcript Analysis. Blue line= HPV sequence, Black dashed line= cellular exon following intron of integration, Black solid line= cellular exon, Pink triangle= viral-cellular junction, Pink filled circle= spanning of integration site, White filled circle= exon-exon boundary outside integration site.

In the recurrent tumor 0732, no fusion transcript was generated between HPV L2 and *TNFRSF* exon 3. The transcript across *TNFRSF* exons 3 and 4, spanning the viral integration site in intron 3, as well as the *TNFRSF* transcript across exons 4 and 5, outside of the integration site, was generated and the both sequences were in-frame.

No fusion transcript was created in tumor 0843 between HPV L2 and cellular *SCN2A* exon 17. There was a transcript generated across the integration site in intron 16, but the transcript sequence did not map to any region of *SCN2A*. Sequence analysis of this

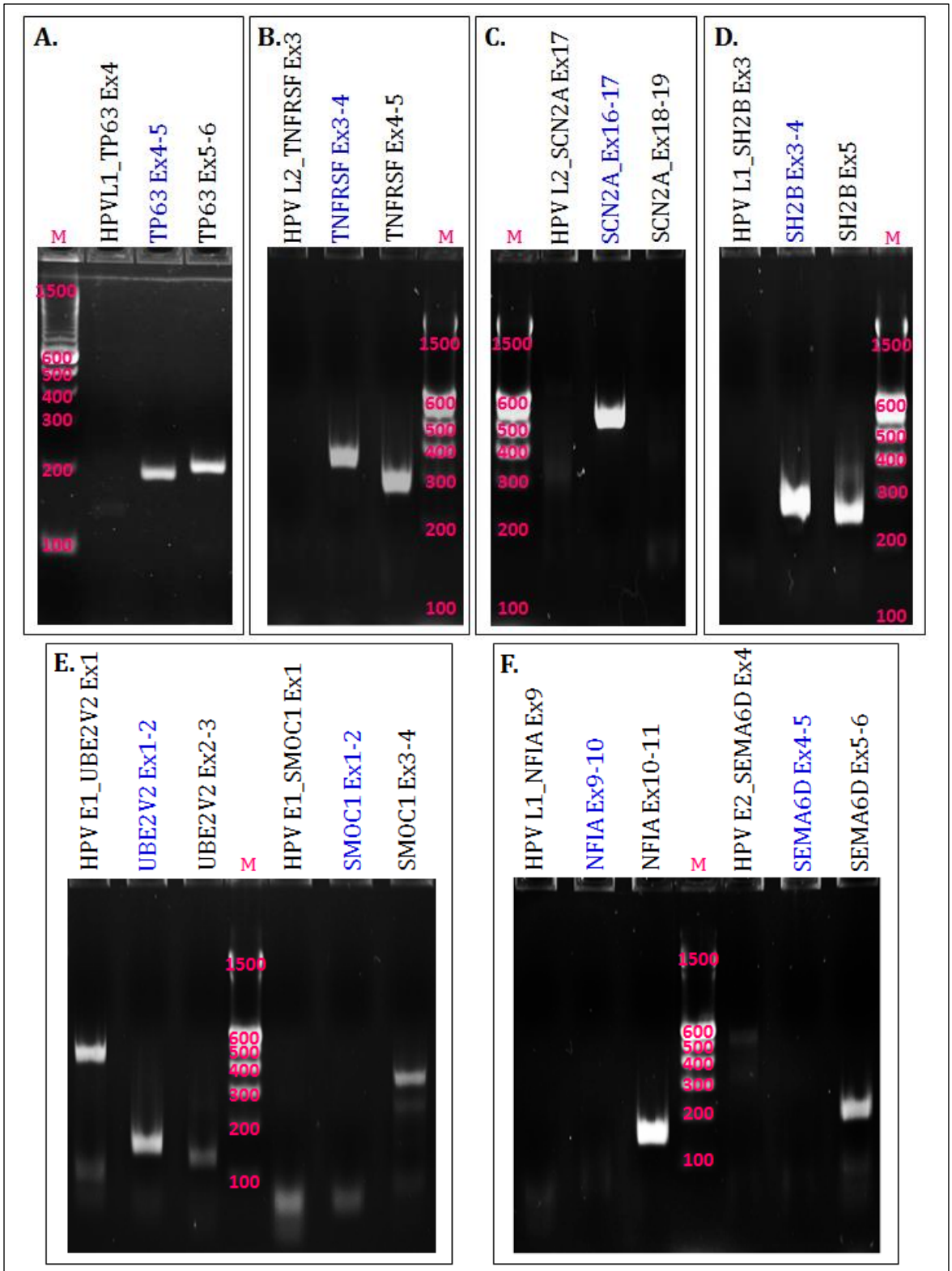

Figure IV.10. Gel Electrophoresis of Tumor Integration Transcript Analysis. Blue text indicates transcript spanning integration site. Panel A. Responsive tumor 1971, Panel B. Tumor 0732, Panel C. Tumor 0843, Panel D. Tumor 1040, Panel E. Tumor 2049, Panel F. Tumor 2238. M= 100bp ladder.

transcript amplicon identified a portion of HPV L1 flanked on one side by the cellular gene for the ATP-binding cassette, sub-family A, member 12 (*ABCA12*) located on chromosome 2q34, and on the other side by an intergenic region of chromosome 1q32. Furthermore, there was no transcript generated when *SCN2A* was queried downstream from the integration event, across exons 18 and 19.

In recurrent tumor 1040, there was no fusion transcript generated between HPV L1 and cellular *SH2B* exon 3. There were, however, transcripts generated across *SH2B* exons 3 and 4 (spanning the intron 3 integration site), and within exon 5 (outside of the integration region), and both sequences were in-frame.

Tumor	HPV Site	Gene	Region	Transcript Analysis		
1971	(R)L1	*TP63*	Intron 4	HPV L1_ p63 Ex4	p63 Ex4-5 ★	p63 Ex5-6 ★
0732	(R)L2	*TNFRSF13B*	Intron 3	HPV L2_ TNFRSF Ex3	TNFRSF Ex3-4 ★	TNFRSF Ex4-5 ★
0843	(F)L2	*SCN2A*	Intron 16	HPV L2_ SNC2A Ex17	SNC2A Ex16-17 +	SNC2A Ex18-19
1040	(R)L1	*SH2B*	Intron 3	HPV L1_ SH2B Ex3	SH2B Ex3-4 ★	SH2B Ex5 ★
2049	(R)E1	*UBE2V2*	Intron 1	HPV E1_ UBE2V2 Ex1 +	UBE2V2 Ex1-2 ★	UBE2V2 Ex2-3 ★
	(R)E1	*SMOC1*	Intron 1	HPV E1_ SMOC1 Ex1 +	SMOC1 Ex1-2 ✕	SMOC1 Ex3-4 ✕
2238	(R)L1	*NFIA*	Intron 9	HPV L1_ NFIA Ex9	NFIA Ex9-10	NFIA Ex10-11 ★
	(R)E2	*SEMA6D*	Intron 4	HPV E2_ SEMA6D Ex4	SEMA6D Ex4-5	SEMA6D Ex5-6 ✕

Table IV.4. Summary of Integration Transcription Analysis in HPV16-Positive Tumors. (F) and (R) = Forward or Reverse viral orientation in relation to the cellular gene. Green text= Viral/cellular fusion transcript, Blue text=Transcript spans integration site, Grey shade=No transcript produced, ✕= Nonsense sequence, no alignment, ★= Sequence was spliced in-frame, + = Sequence mapped to unexpected or rearranged sequences.

In both integration events in recurrent tumor 2049, fusion transcripts were generated between HPV E1 and *UBE2V2* and *SMOC1*. Sequence analysis revealed that a

portion of HPV L1 was fused with the entire *UBE2V2* exon 1, and the distal end of the transcript amplicon included the expected region of HPV E1 attached to chromosome 17q11.2, with nonsense sequence between. Both the *UBE2V2* transcript across exons 1 and 2, spanning the integration site in intron 1, as well as the transcript outside of the integration region across exons 2 and 3 were produced and the sequences were spliced in-frame. The second fusion transcript in tumor 2049 was sequenced and contained *SMOC1* exon 1 linked to chromosome 3p23, followed by nonsense sequence. There were transcripts generated across *SMOC1* exons 1 and 2 (spanning the intron 1 integration) and exons 3 and 4 (outside of the integration region), but the transcript sequences did not contain any homology to *SMOC1*, and were determined to be nonsense sequence.

In the recurrent tumor 2238, fusion transcripts were not generated between either HPV L1 and *NFIA* or HPV E2 and *SEMA6D*. There were also no transcripts generated across *NFIA* exons 9 and 10, spanning the intron 9 integration, or across *SEMA6D* exons 4 and 5, spanning the intron 4 integration site. The *NFIA* transcript across exons 10 and 11, outside of the integration site, was produced and the sequence was in-frame. The *SEMA6D* transcript across exons 5 and 6, outside of the integration site, was also generated, but was found to be nonsense upon sequence analysis.

Discussion

The incidence of HPV-positive oropharyngeal cancer is rising, and there remains a lack of understanding around factors that determine or influence tumor response to treatment[1-10,47-50]. There is significant interest in reducing treatment intensity for patients with HPV-positive tumors, but this would risk the possibility of increasing the proportion

of non-responsive tumors above the current 20-30% of patients who fail intensive concurrent therapy[2,51-53]. Based on our previous work and what is known from HPV in cervical cancer, we examined transcriptional activity and viral integration of hrHPV in responsive and recurrent tumors to determine whether these factors might be useful as clinically-relevant factors to predict response.

The ten tumors studied were positive for HPV16 and negative for all other high-risk HPV types assessed. HPV copy number was established for each tumor; the ranges of viral load values were similar for responsive and recurrent tumors (16-539 copies/cell for responsive tumors, 6-298 copies/cell for recurrent tumors). It is important to note that the values obtained for viral copy number may not be exact because the tumor DNA was extracted from tissue cores that may have contained normal cells. Nevertheless, the average viral copy for the responsive tumors (242.8 copies/cell) was more than twice that of the recurrent tumors (92.6). While the small number of tumors and wide ranges of copy number values limit our ability to draw conclusions from this result, it does agree with our hypothesis that earlier tumors are more likely to contain high numbers of episomal HPV, and more advanced cancers are more likely to have lost episomal copies and be driven by fewer copies of integrated virus.

All of the tumors demonstrated expression of the E6 and E7 oncogenes, suggesting that both the responsive and recurrent tumors are HPV-driven, and the virus is not an incidental passenger to an alternate carcinogenic mechanism. Four of the five tumors in each group (responsive and recurrent) exhibited the alternate E6*I as the most abundant E6 transcript; the full-length E6 transcript was highest in one tumor from each group. Each E6-E7 transcript is produced as a polycistronic mRNA derived from the first p97 promoter.

The E6 oncoprotein is translated from the full length E6-E7 transcript, and E7 is translated from the E6*I-E7 transcript[11-16]. This suggests that the tumors with more abundant full length E6 transcripts would have higher levels of the E6 oncoprotein, while the tumors with more abundant E6*I transcripts would produce higher levels of the E7 oncoprotein.

All of the tumors evaluated exhibited HPV16 integration into the cellular genome. In each of the responsive tumors, at least one viral integration event was identified in intragenic regions known to be chromosome fragile sites (2p16, 7p22, and 4p16 in tumors 1733, 1971, and 2148) or into intragenic regions that were found in multiple tumors (16q11.2 and 10p11.1 in tumors 1769 and 1804). This result suggests that these integrations may not be entirely random; viral integration is likely occurring into regions in the cellular genome that are already unstable, or into regions that share some amount of sequence homology with the virus. We postulate that the intergenic viral integrations seen in both the responsive and recurrent tumors contribute to the primary mechanism of HPV-driven carcinogenesis through disruption of the E6 and E7 transcriptional repressor E2. Viral integration into chromosome fragile sites occurs in cervical cancer[21-26], resulting in disruption of E2 and enhanced expression of E6 and E7[33,34,54,55].

The integration analysis of the recurrent tumors revealed viral integrations into celluar genes in each case. This supports our hypothesis that alterations in cellular genes as a consequence of viral integration may provide a second mechanism of oncogenesis in HNSCC. Cellular gene disruption caused by viral integration has been reported in rare cases of malignant transformation by low-risk HPV types that lack E6 and E7 oncogenic activity[56-59]. We postulate that hrHPV-induced cancers are driven by sustained activity of the E6 and E7 oncoproteins, which may be amplified upon viral integration through

disruption of the transcriptional repressor E2. We suspect that in most cases integration occurs into intragenic regions, and may be random, occur at cellular chromosome fragile sites, or occur in sites with sequence or structural characteristics that favor integration. We propose that viral integration events into genic regions alter cellular gene expression and mediate additional carcinogenic mechanisms, resulting in a more aggressive tumor phenotype. Not only was integration into a cellular gene identified in every recurrent tumor, each of the genes disrupted by viral integration (*TNFRSF13B*, *UBE2V2*, *SCN2A*, *SH2B1*, *SMOC1*, *NFIA*, and *SEMA6D*) is involved in a pathway or mechanism that is related to cancer, or is differentially expressed in some cancers[34-46].

Transcription analysis of these events indicates that viral integration does not necessarily eliminate cellular expression. In the five recurrent tumors, there were seven integration events into cellular genes. In three of the seven events (*TNFRSF13B*, *UBE2V2*, and *SH2B*), intact transcripts were detected both across the integration site and elsewhere in the gene. In all of these cases, the integration was intronic, and it is possible that the gene was spliced in-frame across the integration, eliminating the virus. A second possibility in these cases is generation of transcripts from additional copies of the gene that are unaffected by the virus. It is important to note that while intact UBE2V2 transcripts were identified both across the integration site and elsewhere in the gene, a fusion transcript between HPVE1 and *UEB2V2* was generated. Sequence analysis of this fusion transcript demonstrates the severity of chromosome disorder in the tumor cellular genome, as well as within the viral genome, with rearrangements resulting in production of a transcript containing exon 1 of *UBE2V2* (located on chromosome 8), HPV E1, nonsense sequence, HPV L1, and an intergenic region of chromosome 17q11.2.

In the remaining four recurrent tumor integrations (*SCN2A*, *SMOC1*, *NFIA*, and *SEMA6D*), some or all gene transcription was disrupted by viral integration. In two of these cases, genomic instability is again demonstrated by chromosome rearrangements. In tumor 0843, a transcript spanning the integration site in *SCN2A* was found to involve HPVL1, a portion of chromosome 2q34 (including part of the *ABCA12* gene), and an intergenic region of chromosome 1q32. In tumor 2049, a fusion transcript generated between HPV E1 and *SMOC1* included both exon 1 of *SMOC1* (located on chromosome 14) as well as a region of chromosome 3p23.

Viral integration into a gene does not inevitably cause loss of gene expression; we have shown that transcription of some or all of the gene can persist, possibly from additional, unaltered copies of the gene, or by splice removal of intron-integrated virus. Likewise, detection of gene transcripts does not definitively result in appropriate protein production. We cannot eliminate the possibility that the gene transcripts that were found were incomplete, inactive, or otherwise defective. An analysis of the full transcript would provide a better understanding of the effect of viral integration on the gene.

Upregulation of cellular genes is a possible consequence of viral integration as well, either through disruption of transcriptional repression, generation of fusion transcripts, or other mechanisms. Viral integration can both result from genomic instability and contribute to genomic instability. Oncogenic activities of E6 and E7 promote instability through unregulated cellular proliferation and alteration in cellular activities, thus providing access for integration, and viral integration results in increased viral oncoprotein expression (through disruption of E2) and can cause further chromosomal damage[60-65]. The process of HPV integration into the cellular genome may cause additional dsDNA

breaks, resulting in further rearrangement of the viral and cellular genomes. As tumor cells progressively acquire chromosome rearrangements from oncogenic processes, the genome becomes more disorganized and aberrant[63,64,66-68].

The limitations of the DIPS-PCR method restrict detection of cellular integration sites to those that have a Taq$^{\alpha}$1 restriction site in relatively close proximity, and the method assumes intact viral and cellular genomes. Viral rearrangement or convoluted integrations (multiple concatenated copies, alternate orientations) can reduce the sensitivity of the method and increase the complexity of analyzing the results. Identification of cellular genes affected by viral integration in all five recurrent tumors, together with detection of rearranged chromosomes, demonstrates the extent of cellular disorder present in the recurrent tumor cells.

Our evaluation of hrHPV transcriptional activity and integration in these tumors provides support to our hypothesis that viral integration analysis may be significant in distinguishing responsive tumors from those that require additional or alternate therapies. Locating cellular genes with viral integration, and assessing subsequent alterations in cellular expression may be a significant factor in predicting which tumors will respond to current or reduced-intensity treatments, and also possibly in discovering new treatment targets.

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CHAPTER V

Discussion and Summary

Introduction

The involvement of high-risk human papillomavirus in cervical carcinogenesis has been well-studied; screening, detection of early lesions, HPV testing, and colposcopy have been largely successful in reducing the incidence of cervical cancer[1,2]. An estimated 42% of women in the United States harbor cervical HPV; the worldwide annual incidence of cervical cancer is 530,000 and approximately half of these cases result in death[3]. High-risk HPV is the causative agent in nearly all cervical cancers, 71% of these are attributable to HPV16 or HPV18[4,5]. Additional HPV factors associated with increased risk in cervical cancer include infection with multiple hrHPV types, transcriptionally active viral oncogenes, and viral integration[6,7]. Non-HPV cofactors associated with cervical carcinogenesis and progression include smoking, co-infection with additional sexually transmitted diseases, and hormonal contraceptive use[6].

The rate of oral HPV infection is lower than that of cervical HPV infection, approximately 7% for adults in the United States. The worldwide incidence of head and neck squamous cell carcinoma (HNSCC) is 550,000, and 55% of these cases result in death[3]. Depending on tumor site, the proportion of HPV-positive HNSCC is between 10% and 80%, the highest occurring in OPSCC (83% in our study)[8]. As the incidence of cervical cancer is decreasing, head and neck cancers are increasing, due to the substantial rise in HPV-

induced oropharyngeal tumors. Tobacco and alcohol have historically been the strongest etiologic factors in HNSCC, but hrHPV is now recognized as the primary etiologic factor in oropharynx cancer[9-17]. This change in epidemiology is attributed to increased sexual activity involving multiple partners and HPV transmission between oral and anogenital regions. The characteristics shared by these locations make them appropriate environments for HPV infection, including thin mucosal epithelium or transition from squamous to columnar epithelium, an increased probability for inflammation from concurrent infection and trauma, or microabrasions facilitating viral access to basal cells for infection.

In contrast to cervical cancer, screening and early detection is lacking in HNSCC. The majority of patients with HPV-related oropharyngeal cancer present with advanced stage disease due to early dissemination to regional lymph node, and primary tumors are small and often located in inaccessible regions[15,18-25]. Nevertheless, response to therapy and survival is dramatically better in patients with HPV-positive tumors than in those with HPV-negative oropharynx cancer[10,13,15,16,26-32], prompting wide interest in reduction of treatment intensity for these patients[33,34]. However, there is a minority of HPV-positive oropharyngeal tumors that fail to respond to treatment, and patients with these tumors have local recurrences or distant metastasis[35]. Identification and characterization of factors that could be used to classify HPV-positive tumors into appropriate treatment groups would allow patients with responsive tumors to receive less intense treatments than those currently used. This type of reduction in treatment intensity could prevent treatment related morbidities such as post-radiation loss of salivary flow, swallowing difficulty, osteoradionecrosis, and chemotherapy-induced neuropathies[33]. Likewise, if

potentially non-responsive tumors could be recognized at the time of diagnosis, further evaluation could be performed to identify potential targets for effective personalized therapy that could increase likelihood of successful treatment.

Summary of Chapters

In Chapter II, hrHPV detection by three methods was assessed in oropharyngeal, oral cavity and nasopharyngeal tumors. $p16^{INK4a}$ immunohistochemistry, HPV in situ hybridization, and HPV PCR-MassArray were compared, using L1 consensus PCR and sequencing as the definitive assay for resolution of discordant test results. Among the oropharyngeal tumors evaluated, 83% were HPV-positive, and of these 94% were HPV-16. In the nasopharyngeal tumors, 44% were positive for HPV with a more equivalent distribution among the 4 types detected: HPV16 (37.5%), HPV18 (25%), HPV39 (12.5%) and HPV59 (25%). Only 10% of the oral cavity tumors were HPV-positive, 40% of these were HPV16, and the remaining 60% consisted of other high-risk types or dual hrHPV infections.

HPV-positive oropharynx cancers have a better prognosis than HPV-positive oral cavity or nasopharynx tumors, in which HPV has been associated with worse outcome[36,37] (unpublished data, Stemark et al., 2013). Oropharyngeal tumors arising in lymphoid tissue of the tonsils may be inherently easier to treat based on their proximity to lymphocytes and likely generation of an immune response to viral antigens. However, it is possible that the non-responsive HPV-positive tumors that constitute the minority subset share viral characteristics with HPV-positive tumors at the other HNSCC sites. We have not performed viral copy number, HPV transcription, or integration analysis on tumors other than OPSCC

or in HPV types other than HPV16. It would be interesting to investigate these factors to determine whether they are similar or significantly different from HPV16 in oropharynx tumors.

When HPV detection methods were compared, PCR-MassArray had 99.5% sensitivity and 100% specificity, p16 immunohistochemistry had 94.2% sensitivity and 85.5% specificity, and HPV in situ hybridization had 82.9% sensitivity and 81% specificity. The correlation between p16-positive and HPV-positive results suggests that the virus is transcriptionally active and contributing to carcinogenesis in these tumors. The discordant cases negative for p16 expression and positive for HPV by PCR-MassArray may represent tumors with inactive HPV, or disruption of p16 expression due to mutation, deletion, or methylation affecting the CDKN2A locus that encodes p16. The discordant cases positive for p16 expression and negative for HPV by PCR-MassArray may include tumors that are negative for HPV but have another mechanism for upregulation of p16, such as tumors induced by a type of hrHPV not represented in the genotyping assay, or tumors with viral rearrangement or nucleotide polymorphism in the region of E6 that is assessed in the assay. Consensus PCR and sequencing is a time- and resource-heavy method, but is a valuable tool for resolving discordant cases. L1 consensus primers should detect any known HPV type present in a sample, including those with E6 rearrangements that are false-negative by PCR-MassArray and rare HPV types or variants, which can subsequently be identified by sequencing the L1 consensus PCR product. While this study demonstrates that PCR-MassArray is the most accurate and informative test, it is important to include p16 expression as an indicator of viral activity. Viral DNA may be detected in latent or

inactive infections, and it is essential to identify tumors in which HPV is active and driving carcinogenesis in order to use HPV status as a predictive factor in HNSCC.

In chapter III, we examined viral copy number, early transcripts, and viral integration in all seven known HPV-positive cell lines from HNSCC, representing tumors that failed to respond to therapy. All seven of the cell lines were positive for HPV16 and no other hrHPV types were detected in the cell lines when tested in our PCR-MassArray assay for 15 high-risk types. Each cell line demonstrated HPV E6-E7 viral oncogene expression, with dominant expression of the E6*I-E7 alternate transcript, indicating active viral oncogene expression. HPV16 was integrated in each of the cell lines evaluated, and integration occurred into cellular genes *TP63*, *DCC*, *JAK1*, *TERT*, *ATR*, *ETV6*, *PGR*, *PTPRN2*, and *TMEM237*. Remarkably, each of these genes is associated with cancer pathways or differential expression in one or more cancers. The integration events were confirmed by direct PCR and sequencing of the viral-cellular fusions, eliminating false-positive results that could have been artifacts induced by the DIPS-PCR method. Transcript analysis of the integration events evaluated HPV-cellular fusion transcripts, cellular exon-exon transcripts spanning the integration site, and exon-exon or within-exon transcripts outside the region involved in the integration. Our results indicate that *DCC* is disrupted in UM-SCC-104 and *PGR* is disrupted in UPCI:SCC154, since no transcripts for either of these genes were produced. However, our analysis does not prove that this loss is definitively caused by HPV integration into these genes. Our analysis revealed one HPV-cellular fusion transcript, which occurred in UM-SCC-47 between HPV E2 and *TP63*, and was out of frame. One or more transcripts were produced for the remaining integration events in the cell lines, but were not consistent within each gene for each event. In UPCI:SCC152, the *ATR* transcript

upstream of the integration event was generated, but the transcript spanning the integration site was not in-frame, and the transcript downstream from the integration site was not produced at all. Similarly, in UPCI:SCC90 the transcript spanning the integration site was not produced, but the transcript downstream from the integration site was generated. Disruption of a cellular gene due to viral integration may or may not determine knockout of the gene, depending on whether the second copy (or multiple copies, in the case of aneuploid tumor cells) is affected. The affected cellular gene may be upregulated, disrupted, or unaffected, contingent on strand orientation, as well as the precise viral-cellular junction relative to sequence elements such as promoters and splice sites.

Our assessment of cellular transcripts affected by viral integration provides important but limited information on the consequence of HPV integration on cellular gene expression. A more comprehensive analysis of cellular gene transcripts and resulting protein expression would provide improved understanding of the effects of viral integration into cellular genes, and how resulting changes in cellular activity contribute to tumor response.

We postulate that while integration into gene poor or chromosome fragile sites occurs in the majority of HPV-driven cancers, secondary integration events into cellular genes, specifically tumor suppressor genes or those involved in cancer pathways may separate responsive tumors from those with more aggressive malignant behavior. In our model for differentiation of responsive and non-responsive HPV-positive head and neck tumors, early responsive tumors are driven by sustained E6 and E7 oncoprotein activity (associated with intergenic or fragile site viral integration, loss of E2 transcriptional repression, and E6*I alternate transcripts) alone, while more advanced tumors that will fail

to respond to treatment have viral integration into cellular genes, which alters cellular expression or activity and provides additional carcinogenic drivers. We will continue to investigate the hypothesis to determine if supporting or refuting evidence can be generated from the study of more tumors from patients whose outcome is known.

In chapter IV, responsive and recurrent tumors from patients whose treatment is known, were evaluated for viral copy number, early transcripts, and viral integration. The average HPV16 copies/cell for the responsive tumors was 242.8, more than twice the average HPV16 copy number for both the recurrent tumors (average value of 92.6) and the HPV16-positive cell lines evaluated in Chapter III (average value of 100.6). This observation, while not significant due to small sample size, is concordant with our hypothesis that earlier, responsive tumors often contain many episomal HPV copies, but that more advanced cancers lose episomal copies with fewer copies of HPV which are mostly integrated in the cellular genome.

All of the tumors we evaluated demonstrated active viral transcription. The majority of tumors in each group (4/5 in the responsive and 5/5 in the recurrent tumors) expressed E6*I as the most abundant transcript, suggesting that E7 is the primary translated oncoprotein in these tumors. One recurrent and one responsive tumor expressed full length E6 as the most abundant transcript, suggesting that E6 is the dominant oncoprotein translated in these tumors[38-43]. Relative levels of viral transcripts and oncoprotein translation may be associated with different tumor characteristics, but our limited sample size and comparative equivalence between responsive and recurrent tumors prevents this analysis in our study.

Viral integration into intergenic chromosome sites was found in each of the responsive tumors evaluated, whereas HPV integration into a cancer-related cellular gene was found in only one responsive tumor. The integration events were confirmed by direct PCR and sequencing of the viral-cellular fusions, eliminating false-positive results that could have been artifacts induced by the DIPS-PCR method. The intergenic sites of viral integration included three known chromosome fragile sites and two chromosome sites that were involved in multiple integration events, suggesting that these integrations may not be entirely random. We suspect that HPV integrates into regions in the cellular genome that are unstable or into regions that share some amount of sequence homology with the virus[44]. This supports our hypothesis that intergenic viral integration seen in both responsive and recurrent tumors contributes to the primary mechanism of HPV-driven carcinogenesis through disruption of E2, the transcriptional repressor of E6 and E7. Viral integration into chromosome fragile sites is similarly seen in most cervical cancers[44-49], typically resulting in disruption of E2 and enhanced expression of E6 and E7[33,34,50,51].

We identified HPV16 integration into cellular genes (*TNFRSF13B*, *UBE2V2*, *SCN2A*, *SH2B1*, *SMOC1*, *NFIA*, and *SEMA6D*) in each of the recurrent tumors. Each of the cellular genes identified is involved in a pathway or mechanism related to cancer, or is differentially expressed in one or more cancers[52-64]. However, the precise activity of each gene haboring an integrated viral segment and how viral integration might alter that activity is unknown. Our analysis indicates that integration into a gene does not inevitably result in complete loss of gene expression of the host gene harboring the integrated virus. We found that at least partial transcription of some of the cellular gene involved can persist, possibly from additional, unaltered copies of the gene, or by splice removal of

intron-integrated virus. In contrast, detection of a transcript does not guarantee quality; it conceiveable that gene transcripts are generated but are incomplete, inactive, or otherwise ineffective for accurate protein production. Upregulation of cellular genes is an additional possible consequence of viral integration, either through disruption of transcriptional repression, generation of fusion transcripts, or other mechanisms.

Our transcript analysis of viral-cellular fusion transcripts, exon-exon cellular transcripts spanning the integration sites, and exon-exon or within-exon cellular transcripts outside of the integration region demonstrates that effects of viral integration on cellular genes is not straightforward. In four cases (*TP63* in responsive tumor 1971, *TNFRSF13B* in recurrent tumor 0732, *SH2B* in recurrent tumor 1040, and *UBE2V2* in recurrent tumor 2049) the cellular transcript across the integration site and downstream of the integration was generated and spliced in frame. In other cases, at least one of the cellular transcripts was produced, but failed sequence alignment. In three cases, viral and cellular rearrangements were discovered in viral-cellular fusion transcripts or cellular transcripts spanning the integration site. In recurrent tumor 0843, a transcript across the integration site in *SCN2A* included HPVL1, a portion of chromosome 2q34 (including part of the *ABCA12* gene), and an intergenic region of chromosome 1q32. In tumor 2049, a fusion transcript generated between HPV E1 and *SMOC1* included exon 1 of *SMOC1* (located on chromosome 14) as well as a region of chromosome 3p23. The fusion transcript between HPVE1 and *UEB2V2* in recurrent tumor 2049 contained exon 1 of *UBE2V2* (located on chromosome 8), HPV E1, nonsense sequence, HPV L1, and an intergenic region of chromosome 17q11.2. These results demonstrate the severe disorder and instability present in the cellular genome as well as the limitations of the DIPS-PCR assay.

Oncogenic activities of E6 and E7 promote instability through unregulated cellular proliferation and alteration in cellular activities, and viral integration results in increased viral oncoprotein expression through disruption of E2. Increased viral oncogene expression inhibits p53 and Rb and can lead to further accumulation of chromosomal damage[65-70]. The process of HPV integration into the cellular genome may cause additional dsDNA breaks, resulting in further rearrangement of the viral and cellular genomes. In cervical cancer, HPV integration has been shown to cause structural alterations in the cellular genome at the site of integration, including genomic rearrangements, local amplifications, and genomic deletions[71,72]. As tumor cells progressively acquire chromosome rearrangements from oncogenic processes, the genome becomes more disorganized and aberrant[68,69,73-75].

Identification of viral integration into *TNFRSF13B*, *UBE2V2*, *SCN2A*, *SH2B1*, *SMOC1*, *NFIA*, and *SEMA6D* into the recurrent tumors supports our hypothesis that integration into cellular genes is a secondary event that could contribute to a more aggressive tumor behavior, recurrence, and metastasis by deregulation of cellular genes. Identification of tumors with viral integration into and disruption of cellular genes may be one factor for identifying a subset of tumors unlikely to respond to concurrent chemo-RT. Furthermore, evaluation of integration-induced alterations in cellular expression may provide evidence for new treatment targets.

Future Directions

The mechanism for differences in outcome for HPV-positive head and neck cancers at different tumor sites is not fully understood. Investigation of viral copy number, HPV

early gene transcription, and integration analysis on HPV-positive oral cavity and nasopharynx tumors would allow elucidation of viral activity, physical status, and possible alterations in cellular gene expression. It is possible that HPV-positive tumors at non-oropharynx sites may share characteristics with the subset of non-responsive HPV-positive oropharyngeal tumors that allow these tumors to be resistant to current therapies.

In our transcription and integration studies, we evaluated cell lines and tumors that were HPV16-induced, as these were most abundant and readily available. It would be interesting to examine tumors and cell lines with non-HPV16 high-risk types to determine whether E6 splicing and integration patterns mimic those seen in HPV16. This may be of particular interest in nasopharynx and oral cavity tumors, where we have observed higher relative incidence of non-HPV16 high-risk types.

HPV early gene transcript analysis would perhaps be more meaningful if we knew the p53 status and expression in the tumors examined. The E6*I alternate transcript lacks several key amino acids in the homodimerization region, required for interaction with E6AP and p53; perhaps tumors with more abundant E6*I compared to full length E6 have differences in p53 protein stability and activity.

To better understand the consequences of viral integration into cellular genes, and to determine whether this is a useful strategy for tumor categorization, a more comprehensive analysis of expression cellular genes affected by integration should be performed. This would include evaluation of full transcripts and protein expression. A more thorough exploration of HPV-cellular fusion transcripts may reveal alternate splicing from upstream viral open reading frames to cellular exons. Additionally, examination of

the HPV genome distal to the integration site would discern whether the virus has integrated into multiple different sites in possibly rearranged chromosomes.

To determine whether there is an association between alterations in cellular gene expression due to HPV integration and increased malignancy, gene knock-in and post-transcriptional silencing experiments could be performed for the cancer-related genes that contained viral integrations. The HOK-16A and HOK-16B cell lines are human oral keratinocytes transformed by transfection with recombinant HPV16, and contain approximately 40 and approximately 25 copies of integrated HPV16 DNA per cell, respectively[76]. While HOK-16A and HOK-16B cell lines are immortal, they are not tumorigenic, making them ideal models in which to study mechanisms of multistep oral carcinogenesis with knock-in and silencing experiments to simulate altered gene expression caused by viral integration.

Resolution of the allelic origin of transcripts from cellular genes involved in viral integrations could be approached with allele frequency matching studies; intact transcripts may have been generated from the alternate gene copy, and viral integration may not change gene expression. Minor allele frequency studies may also reveal aneuploidy within tumor cells, as seen in the UM-SCC-47 cell line where pseudotetraploidy and other chromosome rearrangements were verified by SKY analysis.

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