Integration of high-risk human papillomavirus into cellular cancer-related genes in head and neck cancer cell lines

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ABSTRACT: *Background.* Human papillomavirus (HPV)-positive oropharyngeal cancer is generally associated with excellent response to therapy, but some HPV-positive tumors progress despite aggressive therapy. The purpose of this study was to evaluate viral oncogene expression and viral integration sites in HPV16- and HPV18-positive squamous cell carcinoma lines.

Methods. E6/E7 alternate transcripts were assessed by reverse transcriptase-polymerase chain reaction (RT-PCR). Detection of integrated papillomavirus sequences (DIPS-PCR) and sequencing identified viral insertion sites and affected host genes. Cellular gene expression was assessed across viral integration sites.

Results. All HPV-positive cell lines expressed alternate HPVE6/E7 splicing indicative of active viral oncogenesis. HPV integration occurred within cancer-related genes *TP63*, *DCC*, *JAK1*, *TERT*, *ATR*, *ETV6*, *PGR*, *PTPRN2*, and *TMEM237* in 8 head and neck squamous cell carcinoma (HNSCC) lines but UM-SCC-105 and UM-GCC-1 had only intergenic integration.

Conclusion. HPV integration into cancer-related genes occurred in 7 of 9 HPV-positive cell lines and of these 6 were from tumors that progressed. HPV integration into cancer-related genes may be a secondary carcinogenic driver in HPV-driven tumors. © 2017 Wiley Periodicals, Inc. *Head Neck* 39: 840–852, 2017

KEY WORDS: human papillomavirus (HPV), integration, head and neck squamous cell carcinoma (HNSCC), cancer, oropharynx

INTRODUCTION

High-risk human papillomaviruses (HR-HPVs) are frequently identified as etiologic factors in the increasing incidence of head and neck cancer, particularly HR-HPV–positive oropharyngeal cancers.[1,2] 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 nasopharyngeal cancers, 14% of laryngeal cancers, and 10% of oral cavity cancers are positive for HR-HPV.[3] In the oropharynx, HR-HPV is generally considered to be associated with better prognosis.[4,5]

Clinical trial data from the University of Michigan shows that selected patients with stages 3 and 4 oropharyngeal cancer who could be treated with radiation fields that spare a parotid gland and the swallowing musculature had 88% 3-year progression-free survival after treatment with concurrent platinum-taxol–based chemotherapy and intensity-modulated radiotherapy (chemo-RT).[6] Similarly, a retrospective analysis of a Radiation Therapy Oncology Group randomized trial of tumor HPV status and survival in patients with stages III and IV oropharyngeal cancer found patients to have 3-year overall survival of 82.4%.[7] These high rates of response and outcome data have

stimulated a national dialogue on deescalating treatment intensity to reduce treatment-related morbidity in patients with HPV-positive oropharyngeal squamous cell carcinomas (SCCs).[8,9] However, there are no indicators that distinguish those HPV-positive tumors most likely to respond from those that progress even after intensive therapeutic regimens.

There are relatively few cell lines established from HPV-positive head and neck tumors. To better understand the characteristics of HPV-positive tumors, we collected and studied HPV oncogene transcription, physical status, integration sites, and identification of the cellular genes affected by integration in 7 HPV16-positive cell lines, 1 HPV18-positive head and neck squamous cell carcinoma (HNSCC) cell line, and 1 HPV16-positive cervical carcinoma cell line (UD-SCC-2, UM-SCC-47, UM-SCC-104, UM-SCC-105, UPCI:SCC090, UPCI:SCC152, UPCI:SCC154, VU-SCC-147, and UM-GCC-1). It is well established that the viral oncogenes, E6 and E7, are potent drivers of malignant behavior,[10,11] chromosomal instability, and that viral integration into the host genome is associated with malignant transformation, progression to high-grade cervical intraepithelial neoplasia (CIN), and invasion in cervical lesions,[12,13] as well as high level expression of viral oncogene transcripts.[14] Surveys of anogenital HPV-related tumor specimens commonly exhibit integration into either intragenic regions of the cellular genome or integration into fragile sites.[15–17] Viral integration is reported to occur in 65% to 75% of HPV16-positive head and neck tumors,[18,19] but until recently identification of cellular sites of integration and effects on cellular gene expression has been lacking. Ragin et al[15] examined the HPV16-positive oropharyngeal cell line UPCI:SCC090 and observed multiple viral integration sites in chromosomes 3, 6, 9q, 13q, and a t(1;8)(q;?), and suggested that these occurred at common fragile sites. Wald et al[20] examined the HPV-positive oropharyngeal cancer lines UD-SCC-2, UM-SCC-47, UPCI:SCC090, and 93-VU-147T (also known as VU-SCC-147) and found that characteristic microarray profiles were observed in the HPV-positive lines that distinguished them from HPV-negative lines from the oropharynx. Akagi et al[21] analyzed genomewide analysis of HPV integration in several cervical and oropharyngeal cell lines and found significant clastogenic effects of HPV integration, including extensive host genomic amplifications, rearrangements, deletions, inversions, and chromosomal translocations, which they linked to a looping HPV integrant-mediated DNA replication and recombination that were associated with gene disruption and amplification of viral oncogenes. We previously investigated UD-SCC-2, UM-SCC-47, UM-SCC-104, UPCI:SCC090, UPCI:SCC152, and UPCI:SCC154 for HPV copy number and virus–host fusion-transcripts using primarily Amplification of Papillomavirus Oncogene Transcripts.[22] In that study, fusion transcripts were found in 4 of the cell lines,[22] but viral loads were not associated with integration status. UD-SCC-2 expressed a fusion transcript into exon 20 of *DIAPH2*, UM-SCC-47 expressed a fusion transcript into exon 7 of *TP63*, and UPCI:SCC090 and UPCI:SCC152 both expressed a fusion transcript into *NAP1*. In the present study, we assessed HPV viral oncogene alternate transcript expression, the cellular genomic site of viral integration in 8 head and neck and 1 cervical cancer HPV-induced human tumor cell lines, and determined the effect of viral integration on effected host genes.

MATERIALS AND METHODS

Cell lines

Seven HPV16-positive and 1 HPV18-positive HNSCC tumor cell lines and 1 cervical carcinoma cell lines were studied. Four were developed in our laboratory: UM-SCC-47,[23,24] UM-SCC-104,[23] and UM-SCC-105, all were HNSCC and UM-GCC-1, which is a cervical glassy cell carcinoma variant of SCC. UD-SCC-2[25] was obtained from H. Bier and T. Hoffmann from the University of Düsseldorf,[25] VU-SCC-147 (previously called 93-VU-147T)[26] was obtained from R. Brakenhoff from the Vrije Universiteit, Amsterdam, and UPCI:SCC090, UPCI:SCC152, and UPCI:SCC154 were obtained from S. Gollin from the University of Pittsburgh Cancer Institute.[27–29] UM-SCC-47 was established in 1985 from a surgical resection of a previously untreated T3N1M0 carcinoma of the lateral tongue in a 53-year-old male smoker. The patient was referred for RT closer to his home but he died within a year of diagnosis. UM-SCC-104 was from a 56-year-old male heavy smoker with recurrent T4N2bM0 SCC of the floor of the mouth after prior treatment at an outside hospital. He was treated at the University of Michigan with surgical resection in 2009 and had postoperative RT, but the tumor persisted and he died in 2010. UM-SCC-105 was derived from a biopsy of a T4N0M0 glottic mass in a 51-year-old male patient who had never smoked and who had been treated symptomatically for hoarseness for 18 months before referral to an otolaryngologist who discovered the large laryngeal tumor. The patient was treated with RT and remains free of disease 5 years later. UM-GCC-1, an HPV16-positive cell line, was derived from a glassy cell cervical carcinoma from a 27-year-old female patient who was treated with surgical resection and remains healthy and free of disease 30 years later. UD-SCC-2 was derived from surgical resection from a 58-year-old male smoker with T1N3 carcinoma of the pyriform sinus who died of pulmonary metastases 1 year after diagnosis.[30] UPCI:SCC090 and UPCI:SCC152 were established from a 46-year-old male smoker with an oropharyngeal T2N1M0 HNSCC arising in the base of the tongue. The histology was moderately to poorly differentiated invasive SCC with basaloid features. UPCI:SCC090 was from the surgical resection of the primary; UPCI:SCC152 was established from a recurrent tumor in the same patient 1 year later. The patient died of his disease 4 years after diagnosis. VU-SCC-147 was derived from a 57-year-old male smoker with a T4N2 carcinoma of the floor of the mouth/lower alveolar ridge. He was treated with surgery and postoperative RT, but developed an untreatable second primary tumor after 6.5 years and was lost to follow-up after 7 years. UPCI:SCC154 was derived from a 52-year-old male smoker with T4N2 previously untreated SCC of the tongue. The patient was alive at his most recent follow-up, which was 10 years and 2 months after his surgery.

All cell lines established at the University of Michigan were from donors who gave written informed consent to use the resected tissue from their tumors for laboratory study, including cell line development. The IRBMED institutional review board approved the studies. The cell lines from other institutions were obtained directly from the originators in 2010. Primary tumor tissue from which the cell lines were derived was unavailable, with the exception of UM-SCC-104, UM-SCC-105, and UM-GCC-1. Like the cell lines, these patients' tumor tissues were positive for HPV16, HPV18, and HPV16, respectively. All lines were genotyped in the University of Michigan Genomics Core using ProfilerPlus, which interrogates 10 tetranucleotide short tandem repeats, and were confirmed to have unique genotypes. UPCI:SCC090 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 originators and repeat confirmatory tests were performed immediately before the integration experiments carried out between 2012 and 2013. Because there was only one HPV-positive HNSCC cell line that was obtained from a known survivor, UM-GCC-1, an HPV16-positive cervical carcinoma cell line, and UM-SCC-105, an HPV18-positive laryngeal carcinoma, were also tested for viral integration site. 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.

Human papillomavirus detection

All cell lines were grown on glass slides and examined for expression of p16^{INK4A} (inhibitor of cyclin-dependent kinase 4) using the CINtec (Roche/Ventana, Tucson, AZ) assay per supplier protocol. HPV in situ hybridization (ISH) was performed using the INFORM HPVIII assay (Ventana; which detects 12 HR-HPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66) per supplier protocol. All cell lines were tested for the presence and type of HPV using the HPV PCR-MassArray assay.[3,23,31–33] As part of an earlier study to characterize UM-SCC-47, 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 and treated with 0.075M potassium chloride hypotonic solution. Spectral Karyotyping of UM-SCC-47 was performed at the Van Andel Institute (Grand Rapids, MI).

Human papillomavirus E6 and E7 transcript analysis

Two complementary 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 226 of the viral genome and 2 acceptor sites at nucleotide 407 (E6*I) and at nucleotide 526 (E6*II). To examine the expression of HPV E6 and E7 transcripts, primer sets were designed that specifically and discretely amplify the intact, nonspliced, full-length E6 and E7 transcript, the spliced E6*I-E7 transcript, and the spliced E6*II and E7

transcript. The full-length E6/E7 transcript was generated using a forward primer located within the region that is eliminated by splicing, whereas the transcripts for the alternate splice forms were generated using unique forward primers that span the respective splice junctions (primer sets are listed in Supplementary Table S1, online only). Primers for *GAPDH* were used as a negative control in the no reverse transcriptase lane to confirm the absence of contaminating genomic DNA.
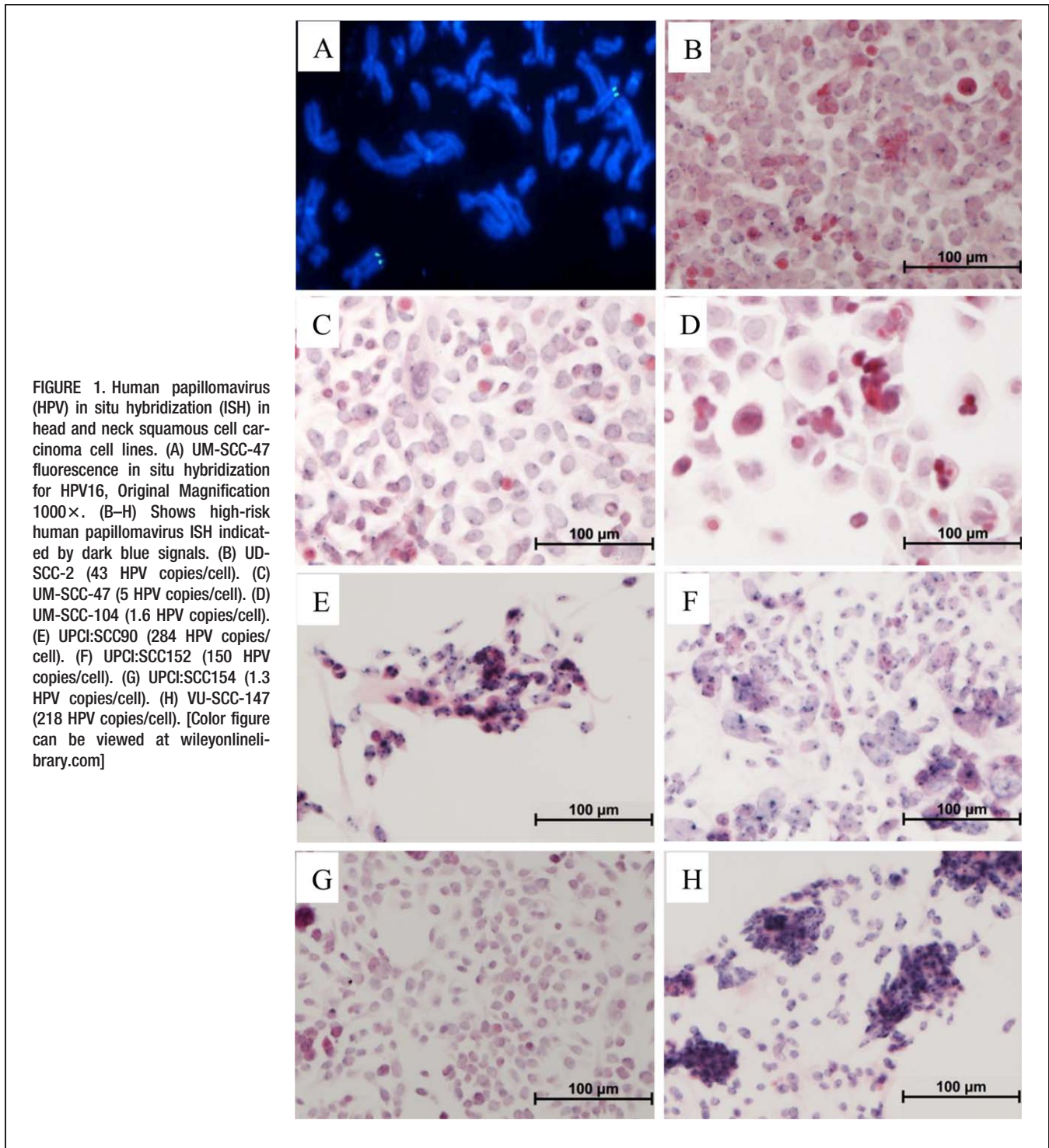
Quantitative real-time polymerase chain reaction (qRT-PCR) was similarly performed using TaqMan assays designed to exclusively amplify each HPV early gene transcript: E1, E2, E5, nonspliced full length E6, spliced E6*I, spliced E6*II, and E7 (primers are listed in Supplementary Table S2, online only). A prepared *GAPDH* endogenous control primer/probe assay was used to quantify relative viral gene expression.

Detection of integrated papillomavirus sequences–polymerase chain reaction

Integration analysis was performed using a modified method (detection of integrated papillomavirus sequences [DIPS]-polymerase chain reaction [PCR]) based on previously published work.[34,35] 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 adapter oligo (5′-CGCAACGTG-TAAGTCTG-NH2-3′ annealed to 5′-GGGCCATCAGT-CAGCAGTCGTAGCCGGATCCAGACTTACACGTTG-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 (Supplementary Table S3, online only). Thermocycling conditions used for both rounds of PCR included 3-minute extension cycles that limited amplification of large (>3 kb) episome-only fragments. PCR products were separated by agarose gel electrophoresis. To search for a previously reported HPV insertion into 9q31[15] that was not detected by DIPS-PCR in UPCI:SCC090, we used primers from multiple regions of HPV16 and within 9q31 for direct PCR using DNA from UPCI:SCC090 and the second cell line from the same patient, UPCI:SCC152 (primers are listed in Supplementary Table S4, online only). 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 nonintegrated 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

FIGURE 1. Human papillomavirus (HPV) in situ hybridization (ISH) in head and neck squamous cell carcinoma cell lines. (A) UM-SCC-47 fluorescence in situ hybridization for HPV16, Original Magnification 1000×. (B–H) Shows high-risk human papillomavirus ISH indicated by dark blue signals. (B) UD-SCC-2 (43 HPV copies/cell). (C) UM-SCC-47 (5 HPV copies/cell). (D) UM-SCC-104 (1.6 HPV copies/cell). (E) UPCI:SCC90 (284 HPV copies/cell). (F) UPCI:SCC152 (150 HPV copies/cell). (G) UPCI:SCC154 (1.3 HPV copies/cell). (H) VU-SCC-147 (218 HPV copies/cell). [Color figure can be viewed at wileyonlinelibrary.com]

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 open reading frames 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.

Protein isolation and Western blot analysis

The cells were lysed with 1% Nonidet-P40 lysis buffer containing protease and phosphatase inhibitors (Pierce, Rockford, IL). The supernatant was collected and the protein content was measured using the BCA Protein Assay
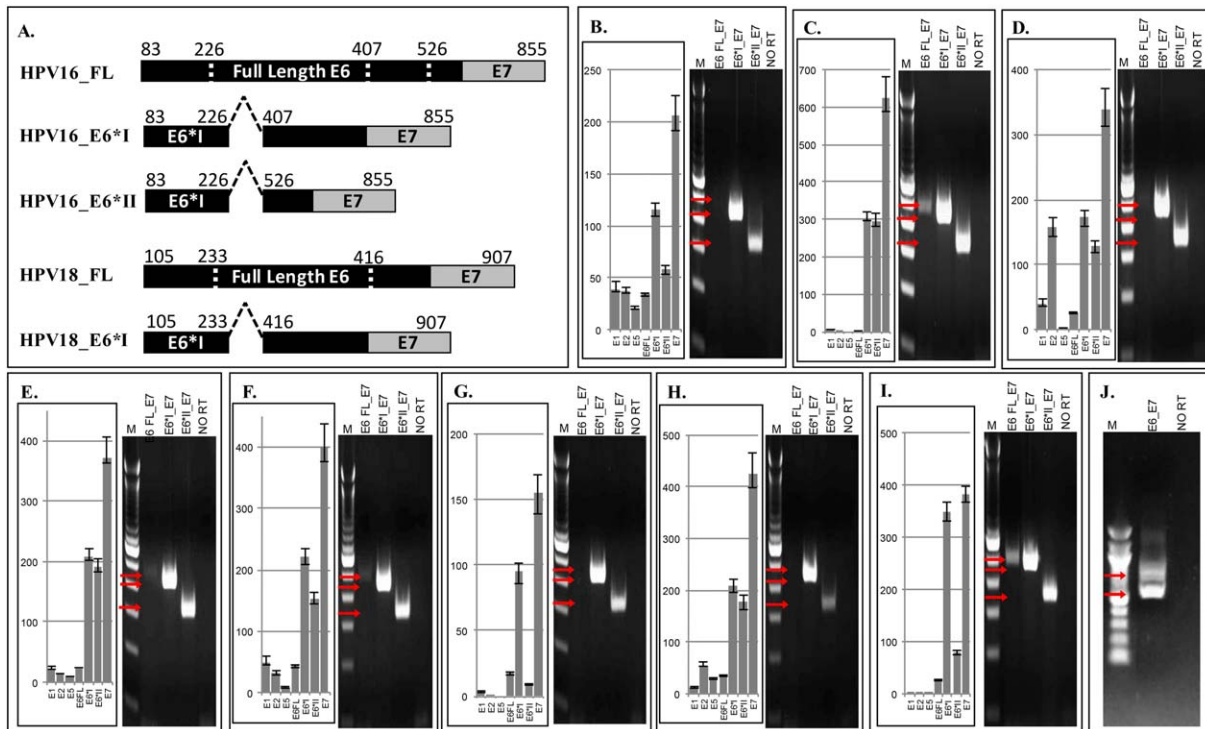
FIGURE 2. Human papillomavirus (HPV) oncogene transcript–specific quantitative real-time polymerase chain reaction (RT-PCR) and E6/E7 RT-PCR in HPV-positive head and neck squamous cell carcinoma cell lines. Bar graphs represent TaqMan quantitative PCR relative expression, and electrophoretic gel images represent E6/E7 RT-PCR. (A) Depiction of HPV16 and HPV18 splicing. Primers within the splice region or across splice junctions allow for exclusive amplification of full length E6 or alternate E6 transcripts. (B) UD-SCC-2. (C) UM-SCC-47. (D) UM-SCC-104. (E) UPCI:SCC90. (F) UPCI:SCC152. (G) UPCI:SCC154. (H) VU-SCC-147. (I) UM-GCC-1. (J) UM-SCC-105. Arrows indicate sizes of expected amplicon bands: HPV16 E6 Full-Length_E7 = 499bp, HPV16 E6*I_E7 = 454bp, and HPV16 E6*II_E7 = 338bp, HPV18 E6_E7 FullLength = 723bp, HPV18 E6*I_E7 = 540bp, NO RT = no reverse transcriptase negative control. [Color figure can be viewed at wileyonlinelibrary.com]

Kit (Pierce). Equal amounts of protein were electrophoresed on NuPAGE Bis-Tris gels and transferred to Immoblin-P (Millipore Corporation, Billerica, MA). Membranes were blocked in 5% milk in Tris Buffered saline with 0.1% Tween. The membranes were incubated overnight with primary TP63 C-Terminus (Boster Biological Technology, Pleasanton, CA) and p63 delta N (Biolegend, San Diego, CA) rabbit antibodies at 4°C for 2 hours at room temperature, followed by incubation with secondary anti-antibody horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA) and detected by chemiluminescence (Amersham, Little Chalfont, UK).

RESULTS

All 9 cell lines were verified to contain HPV16 or HPV18 by PCR-MassArray and all cell lines strongly expressed viral oncogene transcripts.

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 1A). Spectral karyotype analysis of UM-SCC-47 shows rearranged chromosome 3 (Supplementary Figure S1, online only). All of the cell lines were examined for nuclear viral DNA by ISH (Figure 1B–1H) with deep blue hybridization signals

indicating the presence of HPV DNA. UM-SCC-104 (Figure 1D) and UPCI:SCC154 (Figure 1G) have very faint hybridization signals, consistent with the low viral copy number in these cell lines reported previously.[22] The Ventana ISH assay was no longer available for testing UM-SCC-105 or UM-GCC-1.

Viral oncogene expression

All HPV16-positive HNSCC cell lines strongly express viral oncogene transcripts, particularly the alternate E6/E7 transcripts expressed in HR-HPV transformed tumor cells (see Figure 2). The HPV16 E6 gene contains 2 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.[36] The E6*I and E6*II alternate transcripts result from a single donor site at nucleotide 226 of the viral genome and 2 acceptor sites at nucleotide 407 (E6*I) and at nucleotide 526 (E6*II). The HPV18 E6 alternate transcript results from a donor site at nucleotide 233 and acceptor site at nucleotide 416 (E6*I) of the viral genome. As shown in Figure 2B to 2I, all of the HPV16-positive 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, at the expense of full length E6 (quantitative real-time polymerase chain reaction [qRT-PCR]) or full length E6/E7 (RT-PCR). The HPV18-
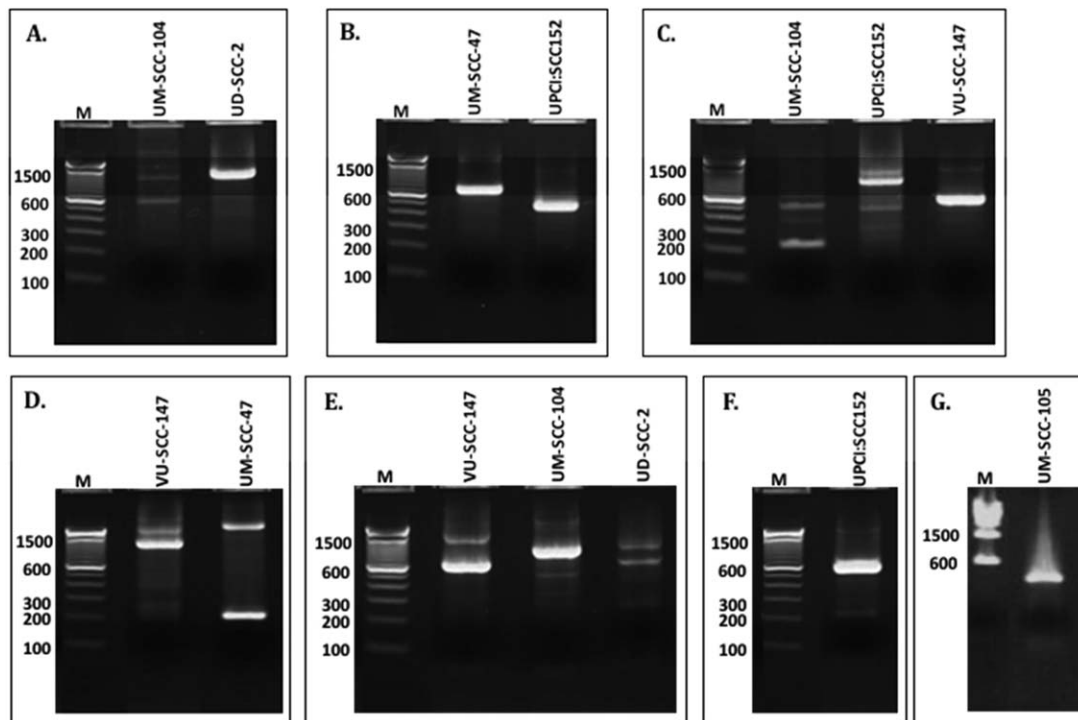
FIGURE 3. Representative cell line detection of integrated papillomavirus sequences polymerase chain reaction gels. (A) Human papillomavirus (HPV)16-E1a/adapter primers. (B) HPV16-E1c/adapter primers. (C) HPV16-E5a/adapter primers. (D) HPV16-L2a/adapter primers. (E) HPV16-E2b/ adapter primers. (F) HPV16-E6a/adapter primers. (G) HPV18-L2f/adapter primers. M, 100 bp marker ladder.

positive cell line UM-SCC-105 also shows the alternate E6*I transcript as a smaller band when E6 and E7 is amplified end-to-end (Figure 2J). These findings are consistent with the viral oncogenes particularly the alternate E6* transcripts and E7 as drivers of tumor development. In all of the cell lines, the expression of E1 and E2 is severely reduced compared to the E6/E7 transcripts, as measured by TaqMan qRT-PCR relative to *GAPDH*. Only UM-SCC-104 showed moderate levels of E2, but still expressed very low E1, E5, and full length E6 expression. These results support an important role for alternate E6 transcripts in viral oncogenesis together with disruption of the viral E1/E2 region and loss of full length E6 expression.

Identification of viral integration sites

Separated amplicon DIPS-PCR bands are shown in the representative gels in Figure 3. A total of 104 hybrid viral-cellular amplicons were isolated and sequenced, ranging from 5 to 16 amplicons for each cell line. Viral-host DNA fusions were identified by sequence and basic local alignment search tool analysis. All sequence reads were studied. Reads mapped to viral-only sequence, viral-cellular hybrids, as described below, or were unmapped because of poor sequence resolution. Diagrammatic representations of the viral insertion sites determined by this method are shown in Figure 4, and Table 1 summarizes the integration results for all 9 cell lines, indicating the HPV integration site, chromosome locus, cellular gene, and the cellular gene region of integration.

A single integration event was identified in UM-GCC-1. The L1 region of HPV16 was joined to an intergenic region of chromosome 20p13 (Figure 4I). Two integration events were found in UM-SCC-105; both involved the HPV18 L1 region. One involved a complex rearrangement with the viral read going into 8q12.3/4p15.33. Both host regions were in noncoding regions of the cellular genome. The other integration event, HPV18 L1, read into 17q12, also a noncoding region of chromosome 17 (Table 1 and Figure 4J). Two fusion events were detected in UD-SCC-2. The first was integration of HPV from E2 into an intergenic region of chromosome 17q12, and a second fusing HPV E1 to intron 14 of *JAK1*. *JAK1* is a large membrane protein tyrosine kinase involved in the proper function of the interferon receptor complexes and signaling through the signal transducers and activators of transcription (STAT 1–4) pathway. How integration of HPV into *JAK1* might be advantageous to an HPV-positive tumor is uncertain, but could relate to loss of interferon signaling within a virally infected/transformed cell. This hypothesis is supported by the association of STAT-1 with impaired induction of isolated nodal failure β.[37] UM-SCC-47 exhibited 2 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 finding is consistent with the FISH result (Figure 1A) 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) (Supplementary Figure S1, online only). Integration into *TP63*
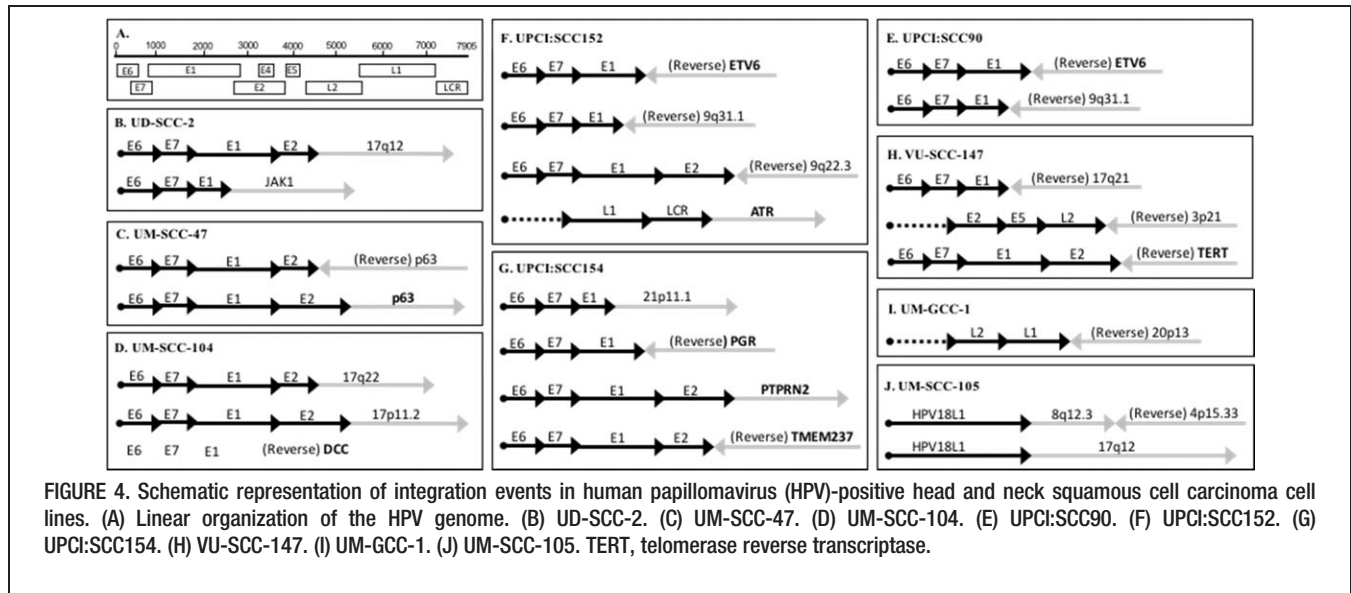
FIGURE 4. Schematic representation of integration events in human papillomavirus (HPV)-positive head and neck squamous cell carcinoma cell lines. (A) Linear organization of the HPV genome. (B) UD-SCC-2. (C) UM-SCC-47. (D) UM-SCC-104. (E) UPCI:SCC90. (F) UPCI:SCC152. (G) UPCI:SCC154. (H) VU-SCC-147. (I) UM-GCC-1. (J) UM-SCC-105. TERT, telomerase reverse transcriptase.

has been observed in cervical cancers and Schmitz et al[38] reported a region of homology between the HPV16 E1 region and a segment of chromosome 3q28 within *TP63* that may facilitate this integration. We detected a fusion transcript between HPV16 E2 and *TP63* (Figure 5B). To investigate this further, we investigated the expression of TP63 protein using Western blotting. A subset of HPV-

positive cell lines and UM-SCC-38, an HPV-negative oro-pharyngeal cancer cell line, was tested (Supplementary Figure S2, online only). UD-SCC-2, UM-SCC-104, UPCI:SCC-154, VU-SCC-147, and UM-SCC-38 express TP63-alpha, as detected by the c-terminus antibody, but UM-SCC-47 expresses only a truncated version of this TP63α isoform. Further, UD-SCC-2, UM-SCC-104, and

TABLE 1. Summary of integration events in human papillomavirus–positive cell lines.

Cell line	Patient information	HPV site	Cellular integration site		
			Locus	Gene	Region
UD-SCC-2	58-year-old male smoker with T1N3 SCC of the pyriform sinus, died of pulmonary metastases 1 year after diagnosis	E2	17q12	Intergenic	
		E1	1p32.3	*JAK1*	Intron 14
UM-SCC-47	53-year-old male smoker with T3N1M0 lateral tongue SCC, died of disease within 1 year of diagnosis	E2	3q28	*TP63*	Intron 10
		E2	3q28	*TP63*	Exon 14
UM-SCC-104	56-year-old male smoker with recurrent floor of mouth T4N2bM0 SCC, died of disease within 1 year after treatment	E2	17q22	Intergenic	
		E2	17p11.2	Intergenic	
		E1	18q21.3	*DCC*	Intron 1
UPCI:SCC90	46-year-old male smoker with recurrent base of tongue T2N1M0 SCC, died of disease 4 years after diagnosis*	E1	9q31.1	Intergenic	
		E1	12p13	*ETV6*	Intron 1
UPCI:SCC152	47-year-old male smoker (donor of UPCI:SCC90) with recurrent hypopharyngeal SCC, died of disease 4 years after diagnosis*	E2	9q22.33	Intergenic	
		E1	9q31.1	Intergenic	
		LCR	3q23	*ATR*	Intron 36
		E1	12p13	*ETV6*	Intron 1
UPCI:SCC154	52-year-old male smoker with base of tongue T4N2 SCC, alive 10 years after surgery	E1	21p11.1	Intergenic	
		E2	2q33.2	*TMEM237*	Exon 14
		E2	7q36	*PTPRN2*	Intron 3
		E1	11q22–23	*PGR*	Intron 3
VU-SCC-147	57-year-old male smoker with floor of mouth T4N2 SCC, developed untreatable second primary after 6.5 years	E1	17q21	Intergenic	
		L2	3p21	Intergenic	
		E2	5p15.33	*TERT*	Promoter
UM-GCC-1	26-year old female, stage IB cervical carcinoma, alive without disease 30 years after diagnosis	L1	20p13	Intergenic	
UM-SCC-105	51-year old male never-smoker with T4N0M0 laryngeal carcinoma, alive without disease 5 years after treatment	L1	8q12.3/4p15.33	Intergenic	
		L1	17q12	Intergenic	

Abbreviations: HPV, human papillomavirus; SCC, squamous cell carcinoma; TERT, telomerase reverse transcriptase; LCR, long control region of HPV16 genome.
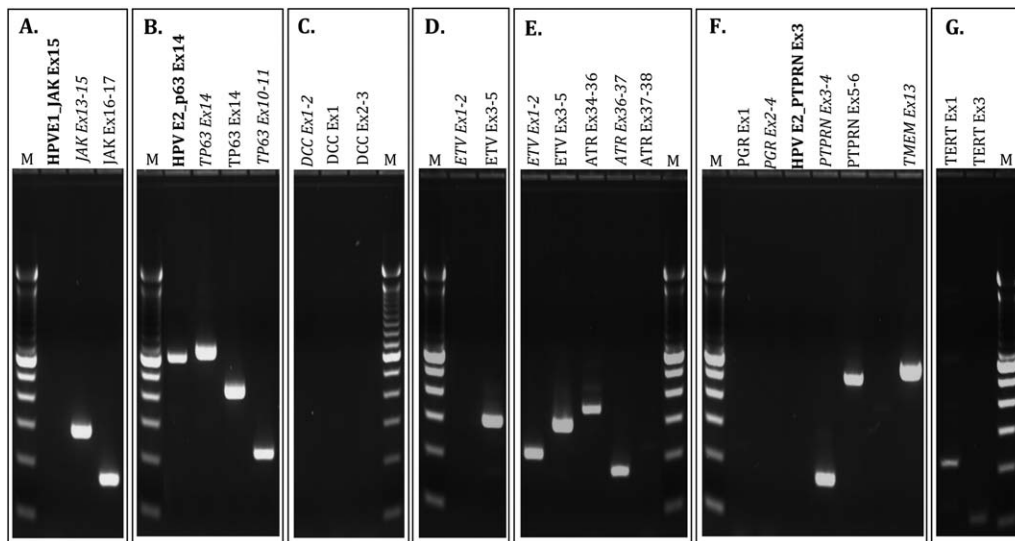* UPCI:SCC90 and UPCI:SCC152 are from the same patient.

FIGURE 5. Gel electrophoresis of cell line integration transcript analysis. Bold text = viral/cellular fusion transcript. Italicized text = transcript spans integration site. (A) UD-SCC-2. (B) UM-SCC-47. (C) UM-SCC-104. (D) UPCI:SCC090. (E) UPCI:SCC152. (F) UPCI:SCC154. (G) VU-SCC-147. M = 100 bp ladder. HPV, human papillomavirus; PTPRN2, protein tyrosine phosphatase, receptor type, N2; TERT, telomerase reverse transcriptase.

UM-SCC-38 strongly express the alpha isoform of ΔNp63, but UM-SCC-47, as well as VU-SCC-147, and UPCI:SCC-154 fail to express ΔNp63. This suggests that the HPV integration in UM-SCC-47 affects the size of the tumor suppressor form of TP63. It is accompanied by loss of the oncogenic ΔNp63 isoform, which is also observed in 2 other HPV-positive tumor cell lines that did not exhibit viral integration into TP63. Clearly, this is an area for further investigation. *TP63* is a homolog to *TP53* and *TP73*, and is a tumor suppressor gene, functioning as both a sequence-specific DNA binding transcriptional repressor and activator. The p63 protein product of *TP63* is involved in differentiation and cell-cycle regulation, as well as transforming growth factor-beta and WNT signaling.[39] In contrast, ΔNp63 lacks the transactivation domain and acts as a dominant negative inhibitor of the transactivating p63 isoforms.[40] Thus, HPV integration may cause reduced p63 signaling and increased WNT function leading to increased proliferative signaling. UM-SCC-104 exhibited multiple integration events, including 2 HPV E2 integration events into intergenic regions of 17q22 and 17p11.2 (Figure 4D). 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 SCCs.[41,42] In UM-SCC-104, there was no DCC transcript detectable (data not shown), suggesting that 1 copy may have been disrupted by HPV integration and the other lost or silenced by methylation.[42] UPCI:SCC090 and UPCI:SCC152 (tumors from the same patient) are interesting in that both share the identical integration from HPV E1 into intron 1 of *ETV6*, which is consistent with this being an early event 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, including *ETV6-PDGFRB*, *ETV6-NTRK3*, *ETV6-ABL1*, *ETV6-ABL2*, *ETV6-JAK2*, and *ETV6-EVI1* fusions.[43] There is also evidence suggesting mutational inactivation of *ETV6* in prostate carcinoma.[44] Interestingly, an *ETV6* fusion oncogene was recently identified in a subset of salivary gland tumors.[45] These tumors, designated as mammary analog secretory carcinomas, have been shown to contain a clinically significant *ETV6-NTRK3* gene fusion that is also present in secretory carcinoma of the breast.[45,46] It is possible that in UPCI:SCC090 and UPCI:SCC152 cell lines, viral integration into *ETV6* causes alteration of gene expression. Previous studies of UPCI:SCC090 had reported a complex rearrangement of HPV into a rearranged chromosome 9 with fusions between HPV16 and 9q31.1 and 9p24.[15,21,22] Because we did not find this by integration by DIPS-PCR using Taq^α1, we confirmed its presence by targeted PCR. Sequence analysis revealed HPV E1 integrated into the same sequence, as reported by Ragin et al,[15] which was confirmed by National Center for Biotechnology Information (NCBI) basic local alignment search tool analysis to map to 9q31.1. DIPS-PCR in UPCI:SCC152, in addition to the HPV E1- *ETV6* integration, identified a viral rearrangement resulting in fusion of HPV E2 into an intergenic region of reverse chromosome 9q22.33, which is similar to the integration reported by Olthof et al[22] and Akagi et al.[21] In addition, DIPS-PCR revealed integration of HPV long control region (LCR) into *ATR* intron 36 on chromosome 3q23. *ATR* codes for a cell-cycle checkpoint protein kinase required arrest and repair in response to DNA damage. Disruption of this gene by integration of HPV at the viral noncoding LCR region could result in uncontrolled cell-cycle progression and uninhibited tumor cell replication and growth. UPCI:SCC152 was also evaluated for the 9q31.1 integration that was previously reported, and was detected by direct PCR exactly as in

UPCI:SCC090. The multiple viral integrations into chromosome 9 in UPCI:SCC090 and UPCI:SCC152 seem to be complex, involving both the 9p and 9q arms. Thus far, our indications are that these chromosome 9 integrations involve exclusively intergenic regions of the chromosome. UPCI:SCC154 exhibited 4 integration events detected by DIPS-PCR, including 1 from HPV E1 into an intergenic region of chromosome 21p11.1, HPV E1 into reverse *PGR*, and 2 involving HPV E2; 1 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 nuclear factor-kappa B, activating protein-1, or STAT. Overexpression of *PGR* has been associated with disease-related mortality and recurrence in breast and gastric cancers.[47,48] Protein tyrosine phosphatase, receptor type, N2 (*PTPRN2*) 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 SCC of the lungs.[49] This suggests that a similar inactivating event, such as gene disruption by integrated HPV, alone or in combination with methylation, may also result in functional loss of *PTPRN2* tumor suppressor activity and contribute to tumor cell malignancy and resistance to therapy. *TMEM237* is a tetraspanin membrane protein that is thought to participate in the WNT signaling pathway. Although the specific interactions of this protein are not yet entirely understood, it is feasible that disruption of this gene by HPV integration would affect the WNT signaling pathway, possibly resulting in dysregulated differentiation and proliferation of the tumor cells. Three integration sites were identified in VU-SCC-147, 1 from HPV E1 into reverse chromosome 17q21, a second from HPV L2 into reverse chromosome 3p21, and a third from HPV E2 into reverse telomerase reverse transcriptase (*TERT*) in the promoter region. Disruption in the promoter region of *TERT* leading to increased TERT expression could provide a growth advantage to tumor cells. In a study that evaluated the frequency of *TERT* promoter mutations in 60 tumor types, HNSCCs were among the highest, with 17% of tumors having mutations in the promoter region of the gene.[50] These results show that, in every cell line, viral integration into one or more cancer-related genes was identified. Table 1 summarizes the integration results for all 7 cell lines, indicating the chromosome locus, known genes, and the regions of integration into the cellular gene.

Integration site transcript analysis: based on the DIPS-PCR integration results, RT-PCR assays were designed to assess virus-cellular fusion transcripts from HPV open reading frames (ORFs) into cellular gene exons, cellular gene exon-exon transcripts across the integration site, and distant cellular gene transcripts. HPV fusion transcript and cellular gene transcript RT-PCR amplicon products are shown in Figure 5.

Transcript analysis in the UD-SCC-2 cell line revealed that there was no HPV-E1/*JAK* exon 15 fusion transcript generated, but there were transcripts generated across the splice insertion site, including *JAK* exons13 to 15, as well as downstream from the integration site for *JAK* exons 16 and 17. In the UM-SCC-47 cell line, transcripts across the integration sites in *TP63* exon 14 and intron 10 were generated, as well as a transcript for *TP63* exon 14 downstream of the integration site. There was also an HPV-E2/*TP63* exon 14 fusion transcript generated for which the sequence was correct but the splicing was out of frame. 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 1 copy may have been disrupted by HPV integration and the other lost or silenced by methylation.[42]

In both UPCI:SCC090 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:SCC090. The transcripts that were generated in UPCI:SCC090 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 34–36). 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.

DISCUSSION

The incidence of HPV-positive oropharyngeal cancer is increasing.[2,33,51] Unlike cervical cancers that are detected early by Papanicolaou test 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 because of high-risk HPV, the incidence of oral, oropharyngeal, and laryngeal cancers is increasing and the incidence of oropharyngeal cancers exceeded that of cervical cancer in 2013.[52]

HPV-related oropharyngeal cancers are significantly more responsive to current therapeutic regimens than are HPV-negative cancers arising at the same anatomic sites,[5,53–56] prompting interest in reducing the intensity of

treatment for this disease.[9] However, even with rigorous therapeutic approaches combining concurrent chemotherapy with RT, 20% to 30% of HPV-positive cancers progress and become unresponsive to further treatment efforts.[33,54,57] 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. Most of the HPV-positive HNSCC cell lines studied thus far are derived from tumors that failed to respond to therapy, and may represent a more aggressive subset of tumors with features consistent with tumor progression. All of the HPV16-positive head and neck cancer cell lines express p16[INK4a], and exhibit HPV E6/E7 viral oncogene expression, with dominant expression of the E6/E7 alternate transcripts. UM-SCC-105, which contains HPV18, also strongly expresses p16[ink4a] and expresses HPV18 E6 and E7 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. In UM-SCC-105, we found HPV18 integration of L1 into a complex t(8;4) translocation. Similarly, HPV16 integration into *TP63* on chromosome 3 was also located near the site of a t(3;7) translocation. Similar clastogenic events were also reported for complex rearrangements at sites of HPV integration in head and neck tumors, as reported by Seiwert et al.[58] A somewhat surprising finding in our study was that in 8 of the cell lines, 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,[59] which lack the transforming ability of the high-risk E6 and E7 genes.[60] It should be noted that of the HNSCC cell lines, all but UM-SCC-105 were obtained from patients with a history of heavy smoking; we found no evidence of smoking by the donor of UM-GCC-1, but it is unknown if a smoking history was elicited at the time of diagnosis. Contribution of additional genomic alterations resulting from the added carcinogenic effects of tobacco use is not unlikely.[33]

High-risk HPV integration has been widely examined in uterine cervix samples, and is strongly associated with high-grade CIN and cancer development.[61] HPV E2, a transcriptional repressor of E6 and E7, is frequently reported to be disrupted upon integration, resulting in prolific 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 (approximately 90% of the genome is intragenic), and in chromosome-fragile sites,[15,16] although integration into cellular genes has also been reported in a minority of cases.[35,38,62]

In this study, we detected integration sites that differed from other investigators studying the same cell lines.[15,21,22] 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[α]1, which has a single restriction site within the HPV genome, or Sau3AI, with 10 restriction sites in the HPV genome. Both enzymes cut at numerous sites in the host cellular genome, but because the sites occur at different locations in the genome, the enzyme used will determine the cellular regions amplified in the assay. In fact, Olthof et al[22] used Sau3AI, whereas in the current study we used Taq[α]1 and found different integration sites in the same cell lines. 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 the 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 2 kb to minimize analysis of virus-only amplicons. The DIPS-PCR approach used in this study found integration sites previously unreported using Taq[α]1, but did not find the chromosome 9 intergenic insertion in UPCI:SCC090 that was found by a similar approach using Sau3AI,[22] a focal sequencing approach,[15] and whole genome sequencing (WGS).[21] With directed PCR, we confirmed the presence of this insertion in the UPCI:SCC090 cells we studied. Another common method used to detect HPV integration, Amplification of Papillomavirus Oncogene Transcripts,[63] which detects fusion transcripts from integrated HPV, has similar challenges in that this method will detect some but not all events because of the limitations of viral primer location, possible gene rearrangement, absence of fusion transcripts, or insufficient assay sensitivity. Even a whole genome sequencing approach failed to find some of the cell line integration sites detected by DIPS-PCR. Clearly, viral integration can be complex and affect multiple cellular sites.[64] Evaluation is further complicated by the possibility of multiple viral copies existing in episomal or integrated forms as complete or partial genomes.

Disruption of a cellular gene because of 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 unchanged, contingent on strand orientation, as well as the precise viral-cellular junction relative to sequence elements, such as promoters and splice sites. Genomic amplification at HPV integration sites is not uncommon.[21,64]

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, such as 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. DCC 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, but 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 and 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 cellular transcripts across viral-cellular integration junctions suggests the existence of at least one intact copy of the genes evaluated (UD-SCC-2 *JAK1*; UM-SCC-47 *p63*; UPCI:SCC090 *ETV6*; UPCI:SCC152 *ETV6*; and *ATR*; and UPCI:SCC154 *PTPRN2*). In the majority of these cases, the viral integration occurs in an intron, 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. It is of interest that of the studies examining HPV16 integration in *TP63* in the UM-SCC-47 cell line, several different loci within the gene were affected. Olthof et al[22] reported integration of HPV E1 into exon 7 of *TP63*, Akagi et al[21] reported HPV E2 integration into *TP63* intron 13 reading into a rearranged repeated segment of exon 9a and fusion transcripts of E2-*TP63*, as well as E2 fusion transcripts reading through exon 9, 10, 11, and 12, whereas, in the current study, we found integration of HPV E2 into *TP63* exon 14 and intron 10. Akagi et al[21] presented a rolling loop model of integration to explain their observations. We speculate that the viral integrations are unstable or that there may be multiple clones with varying integration sites in *TP63*. We assessed expression of the different p63 isoforms in the UM-SCC-47 cell line, using some of the other cell lines as a reference. Although the oncogenic ΔNp63 is the most prominent isoform expressed in HNSCC,[65,66] other studies have demonstrated that the TAp63 tumor suppressor isoform is expressed at a higher level than the ΔNp63 oncogenic isoform in HNSCC cell lines.[25] ΔNp63 has also been implicated in blockade of keratinocyte differentiation as well as acting as a positive and negative transcriptional regulator.[67] Our analysis of protein expression indicates that the ΔNp63 isoform is absent from the UM-SCC-47 cell line and that the TAp63 isoform is present as a truncated protein. Further research is underway to determine the nature of this truncated form and its possible oncogenic function. Interestingly, previous work in this cell line has shown HPV integration-mediated gene amplification resulting in aberrant expression of a novel truncated p63 protein that functions as a dominant-negative regulator of the TAp63 tumor suppressor isoform.[21]

The integration of HR-HPV into cancer-related genes in 7 of the HNSCC cell lines, but only intergenic integration into UM-SCC-105 and UM-GCC-1, suggests a basis for further investigation of this as one possible factor in 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.

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. Studies of the tumors submitted to The Cancer Genome Atlas had a bias for large primary cancers, although many HPV-induced primary tumors are small and may be detected by the appearance of a nodule in the neck from early lymphatic spread. Responses of T1 to T3 HPV-positive tumors, even with positive N classification, are generally better than those with high T classification.[9] Thus, those larger primary tumors may be enriched for complex HPV integration.[64] Design of a model to distinguish responsive from nonresponsive HPV-positive head and neck tumors assumes viral integration as a primary carcinogenic event, associated with disruption of the E1/E2 region, and alternate E6*I, E6*II transcription, which leads to increased E7 viral oncogene expression.[68–70] 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.

Comprehensive investigation to understand the specific cellular alterations caused by HPV integration may provide insight for development of alternate therapies for nonresponsive tumors. Implementation of viral integration analysis to differentiate responsive from nonresponsive HPV-positive head and neck tumors may provide further insight into the factors that distinguish responsive and nonresponsive oropharyngeal cancers. Improved knowledge of genomic factors may be valuable in patient selection to avoid undertreatment 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.

Acknowledgments

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