Human Papillomavirus Infection as a Prognostic Factor in Oropharyngeal Squamous Cell Carcinomas Treated in a Prospective Phase II Clinical Trial

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Abstract. *The aim of this study was to determine the presence of high-risk HPV-16 in patients with HNSCC, assess the impact of HPV status on treatment response and survival in this select cohort treated with combined modality therapy and to identify the differences in HIF-1α and VEGF expression in HPV-positive and -negative tumors. Patients and Methods: Patients had resectable, untreated stage III, IV HNSCC of the oral cavity, oropharynx, hyopharynx or larynx, and stage II cancer of the base of tongue, hypopharynx and larynx. HPV status was determined by conventional PCR in fresh frozen biopsy samples and by Taqman PCR assay on formalin-fixed, paraffin-embedded specimens. HIF-1α and VEGF expression were assessed by quantitative real-time PCR (RT-PCR). Multivariate Cox proportional hazards regression analysis was used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) based on HPV status. Results: HPV-16 was detected in 14 of 24 evaluable cases. There were no significant differences in response rates after neoadjuvant chemotherapy (86% vs. 90%) in HPV-positive and HPV-negative patients, respectively. There was a trend toward better progression-free (HR=0.15, 95% CI=0.002-12.54; p=0.06) and overall survival (HR=0.14, 95% CI=0.001-14.12; p=0.10) for HPV-positive patients. In a subset of 13 fresh frozen samples, RT-PCR revealed a significant increase in VEGF mRNA levels in HPV-positive tumors (p<0.01). No difference was seen for HIF-1α expression. Conclusion: HPV presence portended a better prognosis in patients with oropharyngeal SCC treated with a multimodality treatment in a prospective clinical trial. The level of VEGF mRNA was up-regulated in HPV-16-positive tumors possibly through an HIF-1 independent manner.*

Human papillomavirus (HPV) is a DNA tumor virus with oncogenic potential and is the primary causal agent of cervical cancer (1). Of the more than 130 subtypes identified to date, HPV16 and 18 are high-risk strains and are the most closely linked to cervical carcinoma (1). HPV viral proteins, E6 and E7, play vital roles in cervical carcinogenesis. The proteins bind to and disrupt the function of two tumor suppressor genes, p53 and retinoblastoma gene product (pRB), respectively, resulting in cell immortalization and transformation (2, 3). Since the 1980's, there is growing evidence of an etiological link between HPV infection and a subset of head and neck squamous cell carcinomas (HNSCC), especially those involving the oropharynx (4-7). The implications of such an association could impact future diagnostic, prognostic, therapeutic and prevention strategies for this subset. However, the oncogenic pathway from infection to transformation for head and neck cancer has not been fully defined.

In recent years, much attention has been given to the prognostic role of HPV status in HNSCC. Various studies have reported conflicting results. Some investigators have demonstrated no association between HPV and survival (7, 8) while others have confirmed a more favorable prognosis for patients with HPV-positive tumors (9-13). The variable outcomes are likely due to the heterogeneity of the patient populations, treatments received and HPV detection methods used. Furthermore, studies were limited by their retrospective nature. Recently, Fakhry *et al*. reported on the prognostic value of HPV status in a prospective phase II clinical trial of HNSCC conducted by ECOG in which patients received

Abbreviations: HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia-inducible factor-1α, EGFR, epidermal growth factor receptor.

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homogeneous treatment (14). Patients with HPV-positive tumors had better survival, with increased response rates to induction chemotherapy. To the Authors' knowledge, this is the second effort to further evaluate the outcomes associated with HPV infection in HNSCC in a prospective phase II study.

The mutagenic and transforming events accounting for the observed differences in clinical behavior of the HPV-positive subset are not well defined. Tumor-mediated angiogenesis is a fundamental prerequisite for sustained cancer growth and metastases. Vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1 (HIF-1) are two of the most important proangiogenic factors for most tumor types, including head and neck cancer (15, 16). In cervical carcinoma models, preclinical data have shown that HPV-16 oncoproteins E5, E6 and E7 are able to induce VEGF expression directly, *via* a p53-independent pathway (17) or indirectly by way of epidermal growth factor receptor (EGFR) (18) or HIF (19). However, the HPV-associated angiogenic pathways for HNSCC are not as well defined. It is hypothesized that similar interactions between HPV, HIF-1 and VEGF could be observed in HNSCC tumors.

The objectives of this study were to retrospectively determine the presence of high-risk HPV-16 in patients with HNSCC enrolled in a prospective phase II clinical trial of multimodality treatment, to assess the impact of HPV status on treatment response and survival in this select cohort, and to identify the differences in HIF-1α and VEGF expression in HPV-positive and -negative tumors to better understand the biology of HPV tumorigenesis.

Patients and Methods

Patients and therapy. Between June 2000 and November 2003, 31 patients with biopsy-proven newly diagnosed HNSCC were enrolled in a prospective phase II trial of multimodality management at the City of Hope (Duarte, CA, USA). Eligible patients were those with previously untreated, resectable, non-metastatic clinical stage III or IV SCC of the oral cavity, oropharynx, hypopharynx or larynx. Stage II cancer of the larynx, hypopharynx and base of tongue were also eligible. All patients were treated with neoadjuvant chemotherapy consisting of docetaxel (T) 60 mg/m^2, then a 96-hour continuous intravenous infusion of cisplatin (P) 25 mg/m^2/d, 5-fluoruracil (F) 700 mg/m^2/d and leucovorin (L) 500 mg/m^2/d (TPFL) administered every 28 days. In selected cases, those with at least a partial response received a third cycle followed by surgery and then radiation. Patients with progressive or stable disease following cycle 2 proceeded directly to surgery. Adjuvant radiation (standard fractionation external beam radiation therapy, total dose 70 Gy in 35 to 39 fractions in 7 to 8 weeks) was delivered to the primary tumor and upper neck in all patients subsequent to surgery. Concurrent chemotherapy with weekly gemcitabine 25 mg/m^2 with or without cisplatin 25 mg/m^2 was given to 4 patients due to persistent clinical disease following induction chemotherapy.

Specimen collection. Of the 31 patients enrolled, pretreatment biopsy specimens were available for 24 patients either in the form of fresh frozen tissue (13 samples) or formalin-fixed paraffin-embedded (FFPE) blocks (11 samples). All frozen specimens were obtained from the Medical Oncology Department Tissue Bank where samples were snap-frozen in liquid nitrogen within 30 minutes of biopsy and stored at –80˚C until use. FFPE tumors were obtained from outside facilities where initial biopsies were performed (6 samples) if not available at City of Hope. Histopathological diagnoses were reviewed by the institution's pathologists. All patients gave informed written consent for collection, storage and analysis of tissue for this study per the protocol approved by the City of Hope Institutional Review Board.

HPV detection and DNA sequencing in fresh frozen tissue. Thirteen fresh frozen samples were available for HPV typing. DNA and RNA extraction were accomplished by using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA). HPV detection was performed as previously described (20). In brief, β-globin primers were used to confirm the presence of amplifiable DNA in the extracted specimens. The DNA was amplified with consensus primers to the L1 region MY09/MY11 (21). All fresh frozen tumor DNAs were also tested for HPV-16 and HPV-18 by amplification of the viral E7 (HPV-16) and E6 (HPV-18) region by use of type-specific primers as previously described (7). The presence of an amplicon of the expected length was considered a positive test. HPV subtype was confirmed in all DNA from tumors shown to be HPV-positive by sequencing using BigDye Terminator Sequencing (Applied Biosystems, FosterCity, CA, USA) at the City of Hope Sequencing core facility. Of the 13 samples, 7 were HPV-16-positive. None of the specimens harbored HPV-18. No other HPV types were identified by consensus primers.

Genomic DNA extraction and HPV detection in FFPE tissue. FFPE cancer biopsies from 11 patients were analyzed for HPV status. For each sample, 10 cuts of 10 μm-thick sections containing tumor were used for genomic DNA extraction. DNA purification was performed according to a published protocol with a slight modification (22).

Because of DNA degradation inherent to formalin-fixation, TaqMan real-time PCR was employed to detect HPV-16 and HPV-18 in FFPE cancer biopsies. The primers and probes used for real-time PCR are listed in Table I. Genotype-specific primers and probe for HPV-16 targeted to the E7 gene amplifying a 98 bp product were designed. A HPV-18-specific PCR for E6 gene (78 bp product) was also tested. As a positive control for amplification, human β-globin PCR was performed with gene-specific primers amplifying a 141 bp product. Probes were labeled with 6-FAM at the 5' end and TAMRA at the 3' end. All real-time PCR experiments were run on an ABI 7900HT SDS system (Applied Biosystems). Duplicate reactions for each gene were performed in 20 μL volume using the TaqMan® Universal PCR Master Mix (Applied Biosystems) containing 250 nm primers, 250 nM probes and 50 ng DNA template. The PCR procedures were as follows: initial denaturation and hot-start activation at 95˚C for 10 min followed by 40 cycles of 95˚C for 15 s, 60˚C for 30 s. Each PCR was performed with negative (genomic DNA of HPV-negative FFPE tissues and ddH_2O samples) and positive controls (genomic DNA of SiHa cells for HPV-16, genomic DNA of Hela cells for HPV-18). Normal liver tissue for beta-globin was also included in each gene amplification. PCR amplification of positive controls displayed a typical amplification curve with a Ct value of ≤28; none of the negative controls displayed a measurable amplification for any of the PCR assays. Because of the high

Table I. *List of primers and probes used for real-time quantitative PCR analysis.*

Assay	Name	Sequence (5' → 3')
HPV16	HPV16 E7-F	GAGGAGGAGGATGAAATAGATGGT
E7	HPV16 E7-R	AGCGTAGAGTCACACTTGCAACA
	HPV16 E7-P	CTCTGTCCGGTTCTGCTTGTCCAGCT
HPV18	HPV18 E6-F	CTGGGCACTATAGAGGCCAGT
E6	HPV18 E6-R	GTGTTTCTCTGCGTCGTTGG
	HPV18 E6-P	TGCAACCGAGCACGACAGGAACGA
β-globin	β-globin-F	AAGTGCTCGGTGCCTTTAGTG
	β-globin-R	AACATCAAGCGTCCCATAGACTC
	β-globin-P	TGGCCTGGCTCACCTGGACAACCT
β-Actin	β-Actin-F	CGAGCGCGGCTACAGCTT
	β-Actin-R	CCTTAATGTCACGCACGATT
	β-Actin-P	ACCACCACGGCCGAGCGG
VEGF	VEGF-F	TCTACCTCCACCATGCCAAGT
	VEGF-R	TGCGCTGATAGACATCCATGA
	VEGF-P	CCAGGCTGCACCCATGGCAGA
HIF-1α	HIF-1α-F	CCAAATCCAGAGTCACTGGAACTT
	HIF-1α-R	AGGTGAACTTTGTCTAGTGCTTCCAT
	HIF-1α-P	TACCATGCCCCAGATTCAGGATCAGACAC

F: Forward primer, R: reverse primer, P: probe.

sensitivity of the Taqman assay, no sequencing was performed to confirm HPV subtype. Of the 11 samples, 7 were HPV-16 positive and no sample was HPV-18 positive.

Quantitative RT-PCR for HIF and VEGF from fresh frozen tissue. cDNA was prepared from 3-40 μg RNA in a 100 μL volume using MMLV reverse transcriptase enzyme and random hexamers as primers (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed for 45 min at 42˚C, followed by 5 minutes denaturation at 75˚C. The reaction was boosted by addition of 1,000 units MMLV reverse transcriptase enzyme and repeating the 42˚C step for another 60 minutes. The enzyme was inactivated for 5 minutes at 95˚C and the cDNA was stored at –20˚C until used. The quantification of the level of gene expression was carried out from the cDNA samples with a real-time PCR method. The primers and probes were designed according to the Applied Biosystems guidelines (Primer Express software; Applied Biosystems) to fit the real-time PCR requirements and are listed in Table I. Genomic DNA amplification was excluded by designing the primers around the exon-intron splicing sites.

PCR reaction was set up in a 20 μL final volume adding 1 μL cDNA from each sample, using TaqMan Universal PCR mix (Applied Biosystems). For each target gene, the probe concentration was 0.3 μmol/L and the primer concentrations for the detection of VEGF and HIF-1α genes were 0.4 μmol/L. The PCR amplification was performed on 384 well plate using the default cycling conditions. During the PCR reaction, the Taq DNA polymerase cleaved the probe and released the 5' end reporter fluorescence dye (6-FAM) whose fluorescence was detected by the ABI 7900HT Sequence Detection System (Applied Biosystems). For the absolute calibration curve of the target genes and the housekeeping gene (β-actin), serial dilutions of the plasmids (10^7 to 1 copy range) containing the gene insert were used. Relative gene expression was determined as the ratio of the gene of interest to the internal reference gene expression based on the standard curves.

Table II. *Clinicopathological characteristics of HPV-positive and -negative patients.*

	HPV-16 status number (%)		
	Positive (N=14)	Negative (N=10)	*P*-value*
Average age (years)	62	58	
Gender			
Male	12 (86)	6 (60)	0.19
Female	2 (14)	4 (40)	
Tobacco status			
≥10 Pack-years	9 (64)	8 (80)	0.65
<10 Pack-years	5 (36)	2 (20)	
Alcohol status†			
≥Several drinks/wk	6 (46)	6 (60)	0.68
<Several drinks/wk	7 (54)	4 (40)	
Performance status†			
≥80	12 (86)	9 (90)	0.50
<80	2 (14)	0	
Primary tumor			
Oropharynx	13 (93)	1 (10)	<0.001
All others	1 (7)	9 (90)	
T Stage			
T1/T2	10 (71)	3 (30)	0.10
T3/T4	4 (29)	7 (70)	
N Stage			
N0/N1	6 (43)	8 (80)	0.10
N2/N3	8 (57)	2 (20)	
Tumor grade†			
Poor	7 (50)	2 (20)	0.23
Well/Moderate	7 (50)	7 (70)	

†Data for 1 individual is missing. *Fisher's exact test.

Statistics. Statistical analyses were performed using the SAS statistical package, release 9.1 (SAS Institute, Cary, NC, USA). Tests for association of HPV detection with gender, age, performance status, smoking and alcohol usage, TNM status, treatments received and response to therapy were performed using a two-sided Fisher's exact test. Statistical significance in median levels of VEGF and HIF-1α expression by HPV status was examined using the Wilcoxon rank-sum test. Progression-free survival and overall survival were estimated using the Kaplan–Meier method. Multivariate Cox proportional hazards regression analysis of time to disease progression or death was used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for HPV-positive status with adjustments for patient age, gender, performance status, TNM status, smoking and alcohol usage and VEGF expression. All tests were considered to be of statistical significance at $p<0.05$.

Results

Detection of high-risk HPV-16 and risk profile of patients with HPV positive tumors. The clinicopathologic characteristics of the 24 evaluable patients are summarized in Table II. Most tumor specimens were of squamous cell origin with the

Table III. *HPV-16 status and treatment modality**.

Treatment modality	HPV-16 status number (%)	
	Positive (N=14)	Negative (N=10)
Induction chemotherapy completed	13 (93)	8 (80)
Dose reduction required	10 (71)	4 (40)
Reason for not completing chemotherapy		
Stable disease	1	1
Toxicity	0	1
Surgery	13 (93)	7 (70)
Adjuvant XRT ± concurrent chemotherapy completed	11 (79)	9 (90)

*$p>0.05$, Fisher's exact test, two-sided.

Table IV. *Association of HPV status and response to induction chemotherapy**.

	HPV-positive		HPV-negative	
Outcome	No.	%	No.	%
Partial or complete response	12	85.7	9	90.0
Pathological complete response†	5	38.5	3	42.9

• $p>0.05$, Fisher's exact test, two-sided. †Assessable in surgical patients only, including 13 HPV-positive and 7 HPV-negative specimens.

exception of one diagnosed as an undifferentiated carcinoma, lymphoepithelioma type. HPV-16 was detected in 14 out of 24 tumor specimens (58%). HPV-18 was not detected in any tumor. Thirteen out of 14 HPV-16-positive tumors (93%) were found in the oropharynx (Table II; $p<0.001$). Seven HPV-positive tumors were in the tongue base, six in the tonsillar fossa and one in the lateral tongue. HPV-positivity was also associated with lower T classification ($p=0.10$) as well as more advanced N stage ($p=0.10$) at diagnosis although this was not statistically significant. There was no correlation between tobacco, alcohol use, gender, age, tumor grade and HPV-positivity.

HPV status and response to treatment. Thirteen out of 14 (93%) HPV-positive patients completed the planned three courses of induction chemotherapy compared to 8 of 10 (80%) for the HPV-negative counterparts (Table III). The majority of patients in each group required dose reductions due to toxicities, 10 (79%) and 4 (40%) in the HPV-positive and -negative group, respectively. One patient in each group did not complete chemotherapy due to lack of response while another HPV-negative patient discontinued chemotherapy because of toxicities. No patient progressed during induction therapy.

Following induction chemotherapy, surgery most commonly consisted of unilateral or bilateral supraomohyoid neck dissection with biopsy and/or resection of the primary tumor as deemed appropriate by the surgeon. No patient required either laryngectomy or base of tongue resection. In the HPV-positive cohort, 13 out of 14 (93%) underwent surgery compared to 7 out of 10 (70%) in the HPV-negative group (Table III). One HPV-positive patient was lost to follow-up and did not undergo surgery. Another HPV-negative patient with clinical stage T4, N0 SCC of the oral cavity refused surgery following a clinical complete response to chemotherapy. Surgery was not recommended for the two HPV-negative patients with early-stage disease following clinical complete responses to induction therapy.

Adjuvant radiation was completed in nearly all patients, 11 (79%) and 9 (90%) in the HPV-positive and -negative groups, respectively (Table III). Concurrent chemotherapy was given to 4 patients (all HPV-positive) with advanced disease or poor response to induction therapy. Radiation was discontinued in 3 patients (2 HPV-positive) due to toxicities. One HPV-positive patient was lost to follow-up and did not receive radiation. There were no treatment-related deaths.

Following induction chemotherapy, all patients underwent clinical, radiographic and if appropriate, surgical evaluation for response. Overall, there was no association between HPV status and response to induction chemotherapy (Table IV). In addition, no difference in the rate of pathological complete responses were noted in the surgical subset of patients, 5 (38.5%) for tumors harboring HPV-16 and 3 (42.7%) for HPV-negative tumors.

The Kaplan-Meier survival curves revealed a trend for better progression-free (HR=0.15, 95% CI=0.002-12.54; $p=0.06$; Figure 1A) and overall survival (HR=0.14, 95% CI=0.001-14.12; $p=0.10$; Figure 1B) for patients with HPV-positive tumors. One out of 14 (7%) HPV-positive patients died of their disease compared with 4 out of 10 (40%) in the HPV-negative group ($p=0.17$). Recurrence was observed in local or regional sites in 5 patients, all of whom died. No distant sites of relapse were noted.

VEGF and HIF-1α expression. *VEGF* mRNA transcript levels were measured by RT-PCR in a smaller subset of patients (13 in total) with available fresh-frozen tumor specimens. Comparing HPV-16-positive and -negative tumors, *VEGF* mRNA was significantly higher in specimens harboring HPV ($p<0.01$; Figure 2A and C). In contrast, no

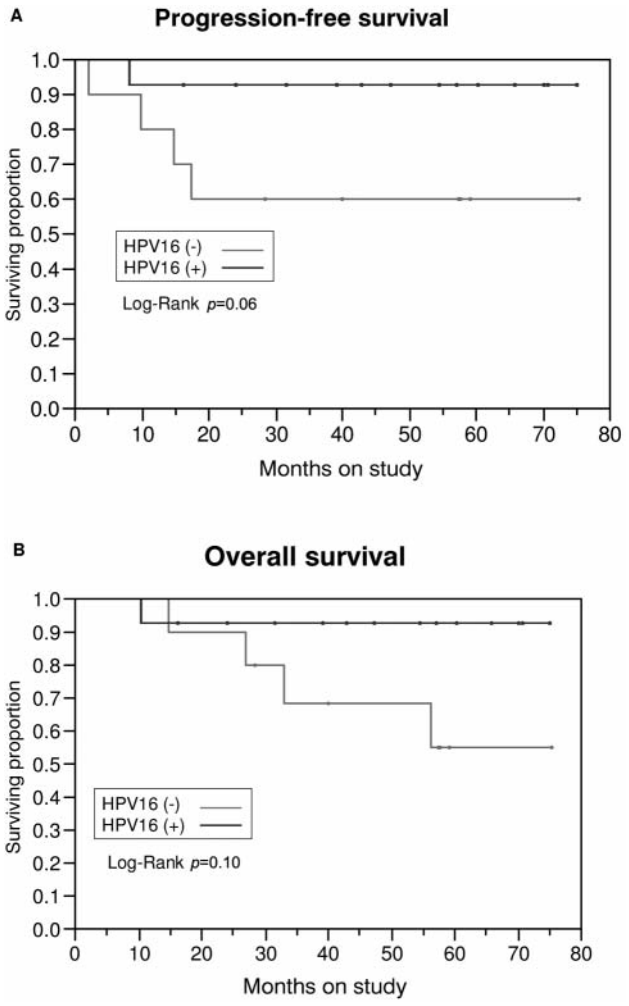
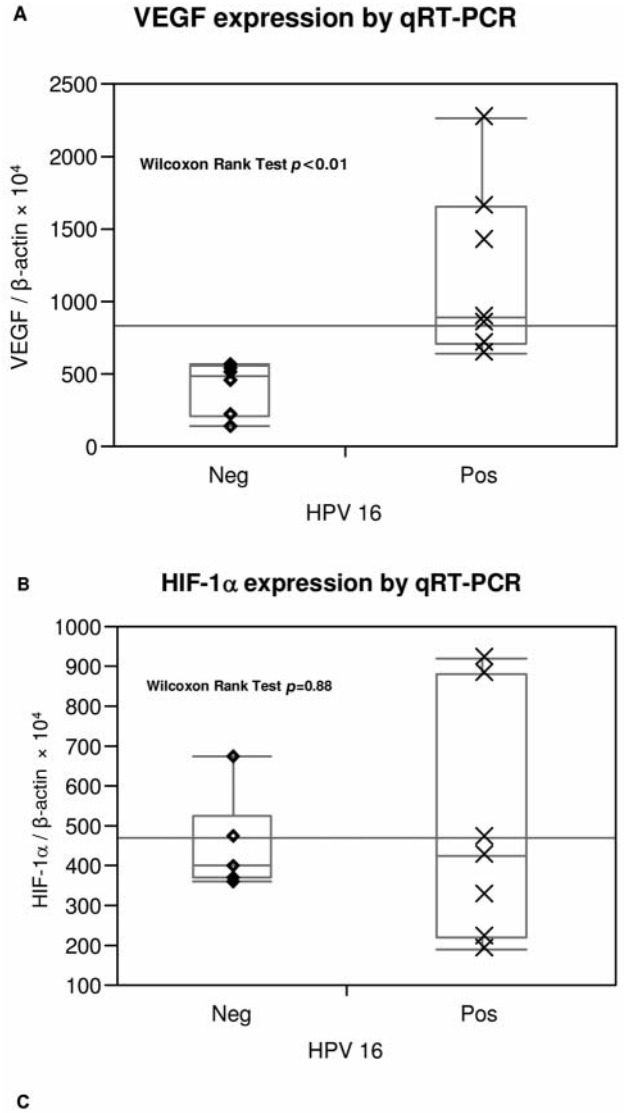


Figure 1. *Kaplan-Meier survival curves for progression-free and overall survival with respect to HPV status.*



C

	Median (percentile 25%-75%)		*P*-value*
	HPV-16-Negative	HPV-16-Positive	
VEGF	483.0 (203.3-552.5)	895.0 (712.0-1658.0)	<0.01
HIF-1α	402.0 (368.8-524.3)	425.0 (218.0-879.0)	0.89

* Wilcoxon rank test

Figure 2. *Scatter plots for the ratio of VEGF (A) and HIF-1α (B) mRNA expression levels to β-actin (housekeeping gene) by quantitative RT-PCR; (C) comparison table of the levels of VEGF and HIF-1α mRNA transcript by HPV status.*

difference was seen in the level of HIF-1α mRNA by HPV status (p=0.88; Figure 2B and C). Furthermore, no difference in the rate of VEGF or HIF-1α overexpression was noted by immunohistochemistry in HPV-positive and -negative tumors (data not shown).

Discussion

There is increasing epidemiological evidence for a causal association between HPV and a subgroup of head and neck cancers, especially of the oropharynx (6, 23, 24). In addition, much of the data suggests HPV-positivity portends a more favorable prognosis (5, 13) albeit the reported outcomes were based on case series of patients who received heterogeneous treatments for their disease. This is one of the first reports on the prognostic significance of HPV in a cohort uniformly treated in a prospective phase II trial.

In a recent systematic review, Kreimer *et al*. reported that overall, HPV prevalence was 25.9% in 5,046 HNSCC cancer specimens from 60 studies conducted worldwide (25). A much higher prevalence was found in this study, with 58% of the HNSCC tumors testing positive for HPV-16. No HPV-

18 was detected in any sample. The finding of only HPV-16 in these individuals is likely due to the small sample size, but is consistent with the 90% detection rate of HPV-16 among HNSCC tumors (5, 23). The prevalence of HPV-positivity has also been observed to be higher in oropharyngeal tumors compared to other HNSCC primaries (5, 7, 13). Kreimer *et al*. reported an overall HPV prevalence of 47.0%, a much higher rate than for any other head and neck site, with HPV-16 accounting for the large majority (86.7%) in oropharyngeal SCC from North America (24). In this study, HPV-16 DNA was found almost exclusively in oropharyngeal tumors: 93% of HPV-positive tumors were located in the oropharynx. The seemingly high rate could have been a selection bias associated with enrollment of patients on an organ preservation protocol at a tertiary referral center. In addition, geographic variation of HPV-positivity was likely a factor. HPV prevalence in oropharyngeal SCC varied from as low as 28.2% in Europe to the highest rate of 47.0% in North America (24). But these findings are consistent with a strong association between HPV and oropharyngeal cancer (5).

Most of the data investigating the prognostic value of HPV-positivity report better outcomes for HPV-associated tumors, especially of the oropharynx (5, 9, 10, 12, 13). Until recently, all such prognostic data were derived from various case series which were limited by the retrospective nature and the heterogeneous treatments patients received in these studies. In 2007, Fakhry *et al*. were the first to report on the prognostic significance of HPV tumor status for patients with HNSCC in a prospective, multi-center phase II clinical trial conducted by ECOG (14). In this study, eligible patients included those with newly diagnosed, resectable, stage III or IV HNSCC of the oropharynx (65%) or larynx (35%). All patients were uniformly treated with two cycles of induction chemotherapy (consisting of paclitaxel 175 mg/m^2 and carboplatin AUC 6) followed by chemoradiation (weekly paclitaxel 30 mg/m^2 administered concurrently with standard fractionation external beam radiation therapy, total dose of 70 Gy in 35 fractions over 7 weeks). Of 96 pateints included in the analysis, HPV-16, -33 or -35 was detected in 40%. After a median follow-up time of 39 months, patients with HPV-positive tumors had a risk of progression that was 72% lower (HR=0.28, 95% CI= 0.07-1.0) and a risk of death that was 79% lower (HR=0.21, 95% CI= 0.06-0.74) than patients with HPV-negative tumors, after adjustment for age, gender, race, smoking, tumor site and TNM staging. In line with this report, after adjustment for similar confounders including VEGF expression, the presented study also showed a trend towards better progression-free (HR=0.15) and overall survival (HR=0.14) for patients with HPV-positive tumors treated in a phase II study with neoadjuvant chemotherapy followed by adjuvant radiation with or without chemotherapy. The association was slightly stronger for progression-free survival, although the overall survival benefit was likely blunted by the limited patient number typical for a phase II study.

The reason for these better outcomes in HPV-positive HNSCC remains elusive. In this study, HPV-positive patients demonstrated better survival despite the finding of a trend towards more advanced nodal stage at diagnosis. Similar associations between the presence of HPV and larger tumor size and nodal involvement have been reported by others (7, 9). Increased radiosensitivity has been proposed as a possible mechanism. In a retrospective review of HNSCC patients who had undergone curative irradiation, Lindel *et al*. reported a higher local control rate and a subsequent increased overall survival in HPV-positive patients (10). Fakhry *et al*. also reported significantly higher response rates to induction chemotherapy in patients with HPV-positive tumors (82% *vs*. 55%, p=0.01; for HPV-positive and negative patients, respectively) (14). Although treatment compliance was not reported, interestingly, concurrent chemoradiation did not add significantly to the percentage of responders following induction therapy, likely as a result of the already high response rate to chemotherapy in the HPV-positive cohort (response rates following concurrent chemoradiation: 84% *vs*. 57%, for HPV-positive and -negative patients, respectively). In contrast, although a non-statistically significant higher percentage of HPV-positive patients required a dose reduction during treatment in this study (76.9% *vs*. 50%, for HPV-positive and -negative patients, respectively), there was no difference in the response rates seen following induction chemotherapy when stratified by HPV status (85.7% *vs*. 90%, for HPV-positive and –negative patients, respectively). This finding is likely explained by the different chemotherapy regimens used as part of induction therapy in each of the phase II protocols. The presented study implemented a much more aggressive regimen (TPFL) with reported response rates >90% in various trials regardless of HPV status (26, 27). Taken together, the early data suggest that improved survival in HPV-positive HNSCC may be explained, in part, by higher sensitivity to both chemotherapy and radiation.

To further explore the role of HPV in the carcinogenesis and clinical behavior of HNSCC, the expression of HIF-1 and VEGF, two factors that play an important role in tumor-mediated angiogenesis and may possibly have prognostic implications were analyzed. The interplay between HPV-16, VEGF and HIF-1 is one that is actively being studied, further characterized and proving to be quite complex in invasive cervical squamous cell carcinoma models. It is well known that hypoxia is an inducer of VEGF expression under the control of HIF-1 (28). Preclinical data also show that the HPV-16 oncoproteins E5, E6 and E7 are able to induce VEGF expression directly, *via* a p53-independent pathway (17) or indirectly by way of epidermal growth factor receptor (EGFR) (18) or HIF-1 (19). Furthermore, VEGF

has also been shown to induce expression of E6 in what may be a positive feedback mechanism (28). HPV-16-related angiogenic pathways are not as well defined in HNSCC. However, the findings of the present study are in line with the reported data for cervical cancer models. Despite the fact that there was no difference in the level of VEGF protein expression by IHC, in a smaller subset analysis of frozen biopsy specimens, *VEGF* mRNA transcript levels were up-regulated in HPV-positive tumors compared to HPV-negative specimens. Unfortunately, it was not possible to assess mRNA transcript levels in all specimens due to the poor quality of RNA in paraffin-embedded tissue blocks. In contrast, there was no difference in HIF-1 expression at either the protein or mRNA transcript level suggesting that in HPV-16-positive oropharyngeal cancers, the induction of VEGF expression occurs in a HIF-independent manner. However, these results must be interpreted with caution given the very limited number of patients.

Further studies will be required for a clearer interpretation of these complex angiogenic pathways in HPV-associated HNSCC. An explanation was not obtained on why the up-regulation of *VEGF* mRNA levels did not translate into higher VEGF protein expression, but several studies have demonstrated that the regulation of VEGF occurs at various tiers from the transcriptional to post-translational level (18,29). In contrast to the present study, another group has shown that in HPV-16-positive tonsillar cancer specimens, integration of HPV DNA into the host genome was strongly associated with HIF-1α overexpression (30). However, it is becoming evident that even among the subset of head and neck cancers associated with HPV, there is a clear variability in the cytogenetic characteristics, resulting tumor biology and clinical behavior in these complex tumors (31-33).

In conclusion, this study demonstrated HPV-16 infection in a significant proportion of oropharyngeal carcinomas. Furthermore, HPV-positivity portended a better prognosis in patients with oropharyngeal SCC treated with induction chemotherapy and adjuvant radiation. The level of *VEGF* mRNA was up-regulated in HPV-16-positive tumors possibly by a HIF-1-independent manner. Larger, prospective studies will be required to further validate these findings. Continued characterization of these complex HPV-associated angiogenic pathways will provide future targets for therapy.

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