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High-Risk HPV, Biomarkers, and Outcome in Matched Cohorts of Head and Neck Cancer Patients Positive and Negative for HIV

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Abstract

In this study, high-risk HPV (hrHPV) incidence, prognostic biomarkers, and outcome were assessed in HIV-positive (case) and HIV-negative (control) patients with head and neck squamous cell cancer (HNSCC). HIV-positive cases were matched to controls by tumor site, sex, and age at cancer diagnosis. A tissue microarray (TMA) was constructed and DNA isolated from tumor tissue. MultiPlex-PCR MassArray, L1-PCR and In Situ Hybridization were used to assess hrHPV. TMA sections were stained for p16ink4a, TP53, RB, CCND1, EGFR, and scored for intensity and proportion of positive tumor cells. The HNSCC cohort included 41 HIV-positive cases and 41 HIV-negative controls. Tumors from 11/40 (28%) cases, and 10/41 (24%) controls contained hrHPV. p16 expression, indicative of E7 oncogene activity, was present in 10/11 HPV-positive cases and 7/10 HPV-positive controls. Low p16 and high TP53 expression in some HPV-positive tumors suggested HPV-independent tumorigenesis. Survival did not differ in cases and controls.

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RB expression was significantly associated with poor survival ($p=0.01$). High TP53 expression exhibited a trend for poorer survival ($p=0.12$), but among cases, association with poor survival reached statistical significance ($p=0.04$). The proportion of HPV-positive tumors was similar, but the heterogeneity of HPV types was higher in the HIV-positive cases than in HIV-negative controls. High RB expression predicted poor survival, and high TP53 expression was associated with poorer survival in the HIV-positive cases but not HIV-negative controls.

Implications—HIV infection did not increase risk of death from HNSCC, and HPV-positive tumors continued to be associated with a significantly improved survival, independent of HIV status.

Keywords

HPV; HIV; HNSCC; prognostic biomarkers

Introduction

Cumulative evidence suggests that a proportion of patients with HIV-1 infection develop head and neck squamous cell carcinoma (HNSCC)(1–4). Although it is suspected that immunodeficiency in these patients contributes to lower immune surveillance against a malignant process, little is known about mechanisms of immune suppression that lead to the development and progression of HNSCC in some HIV-positive subjects. Likewise, it is not known whether cancer progression or its aggressiveness is greater in HIV-positive subjects who develop HNSCC relative to HIV-negative subjects with HNSCC. This gap in knowledge limits our ability to successfully treat this cohort of patients with HNSCC. It is postulated that due to compromised immunity, these cancers are aggressive and rapidly progressing. In fact, clinical observations suggest that HNSCC in this cohort of patients are aggressive and difficult to treat(5, 6). Therefore, an increased understanding of mechanisms that operate *in vivo* and contribute to the development of HNSCC in HIV-positive subjects is crucial for the selection of the most effective therapies. Studies are necessary to define molecular mechanisms that especially endanger HIV-positive subjects with HNSCC to predict outcome and more effectively treat such patients. In this inter-SPORE collaboration, five Head and Neck SPOREs sought to address these gaps in knowledge. Herein we assess the presence and relative frequency of high-risk HPV in HIV-positive cases and HIV-negative controls with HNSCC matched for sex, age, and tumor site and examine prognostic tumor biomarkers.

Materials and Methods

Study Population

This study is a case control series of HIV-infected and HIV-non-infected head and neck cancer patients seen at five tertiary care referral centers: Emory, Johns Hopkins, MD Anderson, Michigan, and Pittsburgh. IRB approval or exemption to share de-identified data with the study data center was obtained at each study site. This study was funded by a National Cancer Institute Translational Research Program (AARA) supplement to the Head and Neck Cancer Specialized Programs of Research Excellence (HNC-SPORE)

collaborative project. HIV-infected patients also diagnosed with HNSCC were retrospectively identified through medical record review at each center and HNSCC tissue located in the pathology archive. To identify controls, pathology records were searched for patients with HNSCC matched to the cases by tumor site, sex, and age at diagnosis (within 18 years to get sufficient matches) and for the presence of archived material. Cases were contributed from five HNC-SPORE sites including MD Anderson Cancer Center (n=4), Emory University (n= 22), University of Pittsburgh (n= 10), University of Michigan (n=2), and Johns Hopkins University (n=3). HIV-negative site, sex, and age matched head and neck cancer controls were contributed by Emory University (n= 2), University of Michigan (n= 35), and Johns Hopkins University (n= 4). Clinical-pathologic data and outcome were obtained from the medical record and tumor tissue was used for biomarker testing. Of the 82 cases and controls (Table 1), follow-up information was provided by each center for 72 patients (36 case/control pairs) with median follow-up of 65 months. Patients with follow-up information represented a similar distribution of calendar time as the dataset overall, with 54% diagnosed between 2006 and 2011, 31% diagnosed between 2001 and 2005, and 15% diagnosed between 1990 and 2000.

Tumor Biomarker and HPV Testing

Formalin-fixed, paraffin-embedded (FFPE) tumor tissues from all cases and controls with adequate specimens were collected, barcoded, and tested in a single centralized laboratory (University of Michigan). Slides from each FFPE block were evaluated by a board certified head and neck pathologist (JBM) in the University of Michigan Pathology Laboratory and tumor-rich areas were marked on the blocks for construction of a TMA and for DNA extraction. Specimens with too little tissue to be included on the TMA were tested for biomarker expression using individual slides. The TMA was examined using immunohistochemistry for expression of p16 (CINtec, mtm Laboratories), TP53 (Ab-6 clone DO-1), RB, (Ab-1 clone 1F8), CyclinD1 (clone SP4) (all from Thermo Scientific), and EGFR (Clone 31G7, Life Technologies). IHC staining was quantified using the product of proportion and intensity scores. Scores for proportion of stained tumor cells and for staining intensity were each on a four-point scale. Intensity of tumor cell staining: 1: no staining, 2: low, 3: moderate, and 4: high; proportion of tumor cells staining: 1: <5%, 2: 5–20%, 3: 21–50%, 4: 51–100%. IHC scores from each core or tissue section were averaged for each patient and IHC scores of 1–4 were considered negative or low expression; scores of 5–11 were considered moderate expression; and scores of 12–16 were considered positive or high expression. The IHC assays were scored at 400X magnification by the pathologist (JBM) who was blinded to the origin of the individual samples.

The TMA was also tested for high-risk HPV by *in situ* hybridization (ISH) (Ventana INFORM HPV III, Medical Systems, Tucson, AZ) according to the manufacturer's protocol. The INFORM HPV III assay (Ventana, AZ) is designed to detect the presence of 12 high-risk (oncogenic) HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66), but does not distinguish the specific HPV type(s) present. ISH was scored for the presence of blue signals indicating the presence of HPV DNA in tumor cell nuclei as single punctate (integrated) or diffuse (episomal) signals.

DNA isolated from tumor cores was tested for oncogenic HPV DNA using the HPV MultiPlex PCR-MassArray assay designed to detect and identify 15 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 73), using multiplex, type-specific, competitive PCR and single base extension followed by MALDI-TOF mass spectrometry analysis, as previously described (7). For any sample with adequate DNA that was positive by p16^{ink4a} or ISH but negative by HPV PCR-MA, the DNA was re-examined by consensus PCR targeting the L1 region of the viral genome using PGMY primers(8) and sequencing of the PCR product to identify the unknown type. All specimens that were found to contain an identifiable high-risk HPV type were scored as HPV-positive.

Statistical Analysis

Correlations among biomarkers were explored using Spearman correlation coefficients and differences in patient characteristics between HIV positive/negative or HPV positive/negative groups were analyzed using logistic regression. Kaplan-Meier estimates for overall survival and Cox models taking into account the matched nature of the data were employed to analyze outcome. Overall survival was defined as time (months) from cancer diagnosis to date of death or last follow-up. Patients lost to follow-up were censored at date they were last known to be alive. All analysis was performed using SAS v9.3.

Results

Patient Characteristics

Forty-four HIV-positive cases with head and neck cancer were diagnosed between June 1990 and July 2011 at five institutions. HIV-negative patients with comparable tumors were selected from the general HNSCC patient population. Control patients were matched based on gender, disease anatomic subsite, and age at cancer diagnosis. One reported HIV-positive case with esophageal cancer was later found to be HIV-negative and was excluded with its control from further consideration. There was insufficient tissue for any testing from two cases, leaving 82 HNSCC patients, 41 evaluable HIV-positive cases and 41 HIV-negative controls (Table 1). For the entire group, the median age at cancer diagnosis was 52 years (range 29–76 years). Ninety-six percent of patients were male. After the analysis was completed it was discovered that one pair had incorrectly matched a female HIV case, EU1852, to a male non-infected control, UM2499. The data from these subjects was retained in the study and data analysis. Median follow-up for survival was 65 months. At the time of survival analysis, 38 patients (19 cases and 19 controls) were deceased. There was no evidence of a significant difference in survival between HIV-positive and HIV-negative cases ($p=0.58$). The majority of tumors were from the oral cavity (22 HIV-positive cases/22 HIV-negative controls), roughly a quarter were oropharynx tumors (11 cases/11 controls), 15% were larynx tumors (6 cases/6 controls), and a minority of tumors were hypopharynx (1 case/1 control) or classified as other (1 case/1 control) (Table 1 summary).

HPV Detection and Biomarker Expression in HPV positive tumors

Of the 82 cases and controls, sufficient valid DNA for HPV testing by PCR-MA was available from 74 samples, including 34 HIV-positive cases and 40 HIV-negative controls. In the PCR-MA assay, 16 of 74 tumors were positive for high-risk HPV. Of the 58 remaining

samples with adequate DNA that were HPV negative by PCR-MA, 13 were strongly positive for p16 expression and were retested by L1 consensus HPV PCR. Of these, 5 were found to contain hrHPV (Table 1), for a total of 21/74 hrHPV-positive tumors (Table 2). Three of the five detected by the L1 consensus primer assay were HPV types not included in the PCR-MA assay: HPV26, HPV69, and HPV82. These HPV types all belong to alpha papillomaviruses group 5, and have been implicated in cancer or rare aggressive dysplastic lesions(9–11). Two of the five contained HPV16 but were not detected by PCR-MA, suggesting a possible rearrangement in the E6 region where the PCR-MA primers bind. Seventy-three tumors were tested by *in situ* hybridization; of these 7 (2 cases and 5 controls) were HPV-positive by ISH (Table 1 and Figure 1). As with the PCR-MA assay, HPV types 26, 69 and 82 are not represented in the INFORM HPV III ISH assay (Figure 2). All 7 HPV ISH-positive tumors contained HPV16 or HPV18 and all were strongly positive for p16 expression (Table 1). Two examples of HPV-positive case tumors with positive *in situ* HPV detection are illustrated in Figure 1. Examples of HPV33 and HPV69 positive tumors from HIV-positive cases not positive for HPV *in situ* hybridization are illustrated in Figure 2 (Note that both tumors strongly express p16). HPV DNA was detected in tumor tissue of 11/34 (32%) evaluable HIV-positive cases and 10/40 (25%) site-matched HIV-negative controls (Tables 1 and 2). Overall, HPV16 was present in 16/21 (76%) of the HPV-positive tumors.

There was greater diversity of hrHPV types in the tumors of the HIV-positive cases compared to the HIV-negative controls. While the majority (7/11) of HPV-positive cases contained HPV16, 4 of 11 cases had less common hrHPV types: HPV26, HPV33, HPV69, and HPV82. In the HPV-positive controls, 9/10 tumors contained HPV16 and one contained HPV18 (Table 2). Among the HPV-positive cases, 10/11 tumors expressed high p16 expression, indicating HPV E7 oncogene activity, whereas of the HPV-positive controls, 3/10 tumors failed to express strong p16 (Table 2). Of HPV-positive tumors, 10/21 (47.6%) were oropharynx and 11/21 (52.4%) were from other sites. Of the 21 HPV-positive tumors, 5/11 in the HIV-positive case group and 5/10 in HIV-negative control group were from tonsil, base of tongue, or vallecula; other sites of HPV-positive tumors in the cases were: tongue, pyriform sinus, mandible, hard palate, alveolar ridge, and medial canthus of the eye. In the HPV-positive controls, tumor sites included tongue (1), floor of mouth (2), conjunctiva (1), and larynx (1) (Table 2).

p16^{ink4a}, TP53, and RB expression differed in HPV-positive and HPV-negative tumors

HPV-positive tumors had higher p16 ($p<0.001$) and lower RB ($p<0.0001$) expression (Figures 1 & 2). Among HPV-positive tumors, RB was moderately expressed in only 3/21 (14%) tumors (Table 2). In contrast, among the HPV-negative tumors (Table 1, Figures 3 & 4), RB was strongly expressed (IHC score 12) in 15/59 (25%) samples. In 14/15 (93%) RB-positive, HPV-negative tumors, p16 expression was moderate or absent. Expression of TP53 was generally low in the HPV-positive tumors and only 4/21 (19%) of these exhibited IHC scores 12, and three of these had low to moderate (1, 8, 8.5) p16 scores (Table 2). Of the HPV-negative tumors, 22/60 (37%) exhibited strong TP53 staining (Table 1, Figures 3 & 4). Cyclin D1 expression was absent in all of the HPV-positive cases and was strongly expressed in only 3 of the HPV-negative tumors. Two Cyclin D1 positive tumors and one

Cyclin D1 moderate tumor are shown in Figure 4. EGFR overexpression was common in the entire set with 32/82 tumors exhibiting IHC scores ≥ 12 ; among the HPV-positive tumors, 9/21 had EGFR scores ≥ 12 (Tables 1 & 2, Figure 1). HIV-positive patients had higher p16 (7.6 vs 5.1, $p=0.05$) and lower TP53 (5.6 vs 8.3, $p=0.03$) scores than HIV-negative controls. Strong TP53 staining was significantly more common ($p=0.03$) in the HIV-negative group (18/41, 44%) than in the HIV-positive group (8/40, 20%) (Table 1, Figure 4). Among HPV-positive tumors, the biomarker expression levels were not significantly different ($p=0.34$ – 0.78) between HIV-positive patients and HIV-negative controls (Table 2 summary).

Biomarkers and Outcome

HPV-positive patients had significantly better survival than HPV-negative patients ($p=0.03$, Cox; HR (95% CI) = 0.49 (0.25, 0.94)). There was no significant difference in overall survival in HIV-positive patients compared to HIV-negative patients ($p=0.58$, Cox; HR (95% CI) = 0.85 (0.46, 1.54)). Among all patients, high TP53 expression was associated with a trend for poorer survival ($p=.20$). In subset analysis, high TP53 expression was significantly associated with decreased survival ($p=0.04$) in the HIV-positive cases but not the HIV-negative controls ($p=0.92$, Figure 5). A test for interaction in the combined model was not significant ($p=0.34$) failing to provide evidence that the effect of TP53 is significantly different in HIV-positive cases versus HIV-negative controls. Higher TP53 expression in the HPV-positive patients was associated with poor survival ($p=0.04$) but the numbers in our sample were very small and p -values should be interpreted with caution.

Discussion

The goals of this multi-institutional collaborative study were to investigate the role of hrHPV in head and neck cancer in individuals with HIV infection, and compare the rate of HPV involvement in control HIV-negative individuals with similar head and neck cancers. We assessed frequency of HPV involvement and site of HPV-positive HNSCC in the cases, and then selected controls matched for sex, tumor site, and age at cancer diagnosis from pathology archives. Going into the study we expected that the HIV-positive cohort might be much more susceptible to HPV-induced head and neck cancers and the proportion of HPV-positive tumors would be much higher in the HIV-infected group. This was based on studies demonstrating the higher prevalence and persistence of oral HPV infection in HIV-positive groups compared with a healthy population (12, 13). Furthermore, it was expected that the HIV infected individuals with head and neck cancer would have poorer outcome due to immunosuppression from their underlying HIV infection and associated health risks, including higher rates of smoking, intravenous drug use, complications of AIDS and susceptibility to other infections and inflammation (2, 14–16).

The population for this study was drawn from a collaboration between five head and neck cancer SPORES (Emory, Johns Hopkins, MD Anderson, Pittsburgh, and Michigan). No prior knowledge of HPV status was available. We previously reported (2) the median HIV-HNSCC survival (a cohort that included the same cases in this study) was not appreciably lower than US-HNSCC survival (63% vs. 61%). In that study, we also reported that the median CD4 count in the cohort was 300 cells/ μ L, which is indicative of good control of the

HIV infection in most of the cases. Nevertheless, poorer survival was associated with CD4>100 cells/ μ L. Additionally, tumor sites of larynx and hypopharynx, as well as current tobacco use, were adverse predictors of survival(2). Of the 82 subjects included in the present study, 41 cases and 41 controls, we did not see a significant difference in survival between the two groups.

The rate of HPV involvement in the head and neck cancers did not differ significantly between cases and controls, with 11 HPV-positive tumors among the cases and 10 HPV-positive tumors among the controls. However, the small size of some of the tumor biopsies from the HIV-positive cases did not allow for isolation of sufficient DNA for HPV testing. The tumor sites with HPV involvement included the usual preponderance of oropharynx (10/21); however, HPV16 involvement was also found in squamous cancers of the eye in one case and one control, raising a question of how the virus was acquired at this site. Most HPV infections are thought to be acquired by direct sexual contact, although transfer of infectious virus from one site to another by a autoinoculation by a contaminated finger (17) or by contact with a partner's HPV infected anogenital tissue or fluids. In cattle, transmission of BPV by fly bites (18) is thought to be a vector in ocular squamous cell carcinomas, so HPV transmission by other means cannot be excluded.

The limited size, fixation conditions, and minimal remaining tissue of many of the biopsy specimens precluded isolation of high quality RNA. Thus evidence of viral oncogene activity as a driver of the tumors was assessed by expression of p16 by immunohistochemistry. By this indicator, only one of the HPV-positive tumors (UP1880, p16 score= 1) among the HIV-infected cases was not driven by the HPV16 viral DNA found in the tumor. Among the HPV-positive tumors from HIV-negative controls, one (UM2495) failed to express p16, and two others (UM1349 and UM 1803) expressed only low levels of p16, raising a question about whether the virus is a driver in these cases or if p16 has been completely or partially inactivated by other mechanisms such as methylation or copy loss (19–22).

Three HPV types that were detected and identified by L1 consensus PCR but not included in the PCR-MA assay or HPV IHC test were HPV26, HPV69, and HPV82. The tumors from the HIV-positive cases UP1864, EU1851, and UM2359 containing HPV26, HPV69, and HPV82 viral DNA, respectively, all expressed high levels of p16, suggesting that all three tumors might be driven by these HPV types, resulting in the high p16 expression. These HPV types are considered possibly carcinogenic (carcinogenic group 2B according to the International Agency for Research on Cancer)(23), and they are grouped together in alpha papillomaviruses group 5 with HPV type 51, which is among the definitive carcinogenic types. HPV26 and HPV82 have been found to be associated with invasive cervical cancer, albeit a rare occurrence: a meta-analysis found 25 positive out of 9265 tested (0.27%) for HPV82, and 8 positive out of 6111 tested (0.13%) for HPV26 (24). Another study examined single HPV type-infected cervical cancer tissue biopsies with probable/possible carcinogenic HPV types and found expression of five markers suggestive of HPV transformation (type-specific E6*I HPV spliced viral transcripts, high p16, low RB, low cyclin D1, and low p53 expression) for those harboring HPV26, and four of these markers (type-specific E6*I HPV spliced viral transcripts, high p16, low RB, and low cyclin D1 expression) for HPV82. Only

one of the four HPV82 cervical cancer cases had low TP53 expression (25). The HPV26-positive case in our study demonstrated similar expression to the cervical cases with high p16, low RB, low cyclin D1, and moderate p53 expression, supporting HPV26 as a carcinogenic driver in this tumor. The HPV82-positive case in our study also exhibited the same expression pattern as seen in the previous study: high p16, low RB, low cyclin D1, and high TP53.

TP53 is typically wild type in tumors with HPV as a driver of carcinogenesis (26–28). Generally when *TP53* is wild type, little or no TP53 protein expression is seen in the tumor; however, TP53 protein expression is often high in tumors with mutant *TP53* (22). Among the HPV-positive tumors from HIV-positive cases, TP53 expression was low or moderate in all but one tumor (UM2359, p53 score= 12)). In the HIV-negative control HPV-positive tumors, three tumors expressed high TP53 levels; these were the same tumors that had no or low p16 expression. Taken together, these findings suggest the possibility that mutant *TP53*, not HPV, might be the primary driver of these cancers. While we did not expect to see indications of mutant *TP53* in the HPV-positive cases or controls, a limited number of studies have demonstrated a higher frequency of HPV and mutant *TP53* co-involvement in HNSCC cases than previously reported, which may be dependent on specific tumor site or cocarcinogenic factors such as tobacco, alcohol, betel quid, or areca nut use (29, 30). Furthermore, the commonly used HNSCC HPV16-positive cell line VU-SCC-147, established from a floor of mouth tumor removed from a patient with a history of smoking, harbors a *TP53* (L257R) mutation (29).

RB expression was also low in most of the HPV-positive tumors, which is consistent with HPV viral oncogene expression (31, 32). Only one control HPV-positive tumor expressed moderate RB (UM1349, RB score= 11). Low RB expression is consistent with HPV oncogene expression; however, many HPV-negative tumors also expressed low RB.

Although survival did not differ between HIV-positive cases and HIV-negative controls, HPV-positive status of the tumor was associated with significantly better survival than that of the HPV-negative cases and controls. As the survival among the HIV-positive cases was slightly better than the HIV-negative controls, HIV status did not adversely affect outcome for HPV-positive patients. Curiously, TP53 expression segregated the HIV-positive subjects for survival. Those with the lowest TP53 expression enjoyed the best survival and those with the highest TP53 expression had significantly poorer survival. In contrast, TP53 expression was not associated with survival for HIV-negative controls.

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Figure 1. Staining of HPV-positive/HIV-negative Controls for Expression of p16, TP53, RB, EGFR, and *in situ* HPV Hybridization. IHC positive staining shows a brown signal, *in situ* HPV positive staining shows a purple signal.

HIV	sex	age	BDbx ID	SITE	p16	TP53	RB	Cyclin D1	EGFR	HPV PCR-MA	L1 HPV PCR	In Situ HPV
+	M	54	UP1871	Tongue	16	1	1	1	12	HPV33	NA	negative
+	M	42	EU1851	Alveolar Ridge	16	1	1	1	16	negative	HPV69	negative

Figure 2. Staining of HPV-positive/HIV-positive Cases for Expression of p16, TP53, RB, EGFR, and *in situ* HPV Hybridization. IHC positive staining shows a brown signal, *in situ* HPV positive staining shows a purple signal.

Figure 3. Staining of HPV-negative/HIV-negative Controls for Expression of p16, TP53, RB, EGFR, and *in situ* HPV Hybridization. IHC positive staining shows a brown signal, *in situ* HPV positive staining shows a purple signal.

Figure 4. Staining of HPV-Tumors from HIV-positive Cases or HIV-negative Controls for Expression of p16, TP53, RB, Cyclin D1, and EGFR. IHC positive staining shows a brown signal.

Figure 5.

Overall survival as a function of TP53 expression. A) All patients (n=72). B) HIV-positive cases (n=36). C) HIV-negative controls (n=36). D) HPV-positive patients (n=15).

Immunohistochemical staining scores were divided into three categories, IHC scores 1–4=low expression; scores of 5–11=moderate expression; and scores of 12–16= high expression.

Table 1

Patient information, tumor site, HIV status, biomarker expression, HPV test results, and summary statistics. Samples ordered by HIV-positive case and HIV-negative control matched pairs.

ID #	HIV	Sex	Age	BDbx ID	Tumor Site ^a	p16 ^b	TP53	RB	Cyclin D1	EGFR	HPV PCR-MA	L1 HPV PCR	In situ HPV
1	+	M	58	UM1281	Buccal mucosa	12	8	8	1	6	-	-	-
2	-	M	71	UM1356	Buccal mucosa	1	1	4	1	15	-	-	-
3	+	M	54	UM2359	Mandible	16	12	1	1	14	-	HPV82 ^c	-
4	-	M	53	UM2142	Mandible	1	11	7	2.5	16	-	-	-
5	+	M	55	JH2461	BOT	10.5	5	1	12	1	Invalid	-	-
6	-	M	68	JH2469	BOT	12	12	1	1	6	-	-	-
7	+	M	58	JH2471	BOT	1	2	1	2	1	-	-	NA
8	-	M	45	JH2472	Tonsil	1	16	1	2	1	-	-	-
9	+	M	48	JH2473	FOM	1	1	16	6	9	-	-	-
10	-	M	58	JH2470	Tongue	1	12	16	1	16	-	-	-
11	+	M	58	MD2459	Tongue	9	1	12	2	8	NA	-	-
12	-	M	76	JH2474	Tongue	1	16	14	1	16	-	-	-
13	+	M	49	MD2466	L Mandible	NA	1	NA	NA	NA	-	-	NA
14	-	M	64	UM2168	FOM	16	1	1	1	16	HPV18	-	+
15	+	M	47	MD2468	FOM	1	NA	6	2	8	NA	-	-
16	-	M	56	UM1803	FOM	8.5	16	2.5	1	8.5	HPV16	-	-
17	+	M	57	MD2498	Maxilla	16	1	1	1	4	NA	NA	-
18	-	M	51	UM0956	FOM	1	15	7	3	15	-	-	-
19	+	M	52	UP1875	Larynx	1	5	14	11	3	-	-	-
20	-	M	56	UM1999	Larynx	1	12	1	1	15	-	-	-
21	+	M	42	UP1880	R Tonsil/BOT	1	1	4	1	8	HPV16	-	-
22	-	M	50	UM1407	Tonsil/BOT	1	16	6	12	14	-	-	-
23	+	M	44	UP1864	Hard palate	16	9	2.5	1	1	-	HPV26 ^c	-
24	-	M	29	UM1938	Alveolar ridge	16	6	4	1	8.5	-	-	-
25	+	M	50	UP1878	Tongue	1	1	6.5	1	12	-	-	-

ID #	HIV	Sex	Age	BDbx ID	Tumor Site ^d	p16 ^b	TP53	RB	Cyclin D1	EGFR	HPV PCR-MA	L1 HPV PCR	In situ HPV
26	-	M	59	UM1272	Tongue	1	1	11	5	1	-	-	-
27	+	M	49	UP1877	Vallecula	1	9	1	1	1	<i>Invalid</i>	-	-
28	-	M	64	UM2502	BOT	1	1	1	9	3.5	-	-	-
29	+	M	54	UP1871	Tongue	16	1	1	1	12	HPV33	-	-
30	-	M	49	UM1653	Tongue	16	4	1	1	1	HPV16	-	+
31	+	M	42	UP1869	Larynx	1	16	4	1	10	-	-	-
32	-	M	53	UM1168	Larynx	13	1	7	1	1	-	-	-
33	+	M	47	UP1873	Larynx	1	16	16	4	1	-	-	-
34	-	M	59	UM1349	Larynx	8	16	11	3	10	HPV16	-	-
35	+	M	48	UP1868	Tongue	1	1	4	8	16	-	-	-
36	-	M	46	UM992	Tongue	1	2.5	9	1	1	-	-	-
37	+	M	70	UP1872	Tongue	6	4	1	1	12	-	-	-
38	-	M	63	UM1081	FOM	1	1	7.5	2.5	12	-	-	-
39	+	F	45	EU1836	Pyrriform sinus	16	6	8	1	1	HPV16	-	NA
40	-	F	44	UM913	Pyrriform sinus	1	1	NA	1	1	-	-	-
41	+	M	58	EU1837	Larynx	16	13	1	1	12	-	-	-
42	-	M	?	EU2488	Supraglottis	1	2	6	6	1	<i>Invalid</i>	-	-
43	+	M	52	EU1840	Tonsil	16	6	1	1	16	HPV16	-	-
44	-	M	48	UM2495	Tonsil	1	12	1	1	16	HPV16	-	-
45	+	M	49	EU1841	R Lower lip	12	12	9	9	16	-	-	-
46	-	M	36	UM2255	Tongue	1	6	1	1	8	-	-	-
47	+	M	53	EU1842	Retromolar	1	1	4	1	4	-	-	-
48	-	M	56	UM1182	FOM	1	1	16	6	16	-	-	NA
49	+	M	49	EU1844	Supraglottis	9	16	16	8	6	-	-	-
50	-	M	59	UM1127	Larynx	1	16	11	5	1	-	-	-
51	+	M	45	EU1845	Tonsil	16	6	6	1	12	HPV16	-	NA
52	-	M	48	UM2496	Tonsil	16	11	1	1	15	-	HPV16	+
53	+	M	60	EU1847	Retromolar	3.5	1	1	1	1	-	-	-
54	-	M	47	UM779	Alveolar ridge	1	16	12	16	8	-	-	-

ID #	HIV	Sex	Age	BDbx ID	Tumor Site ^d	p16 ^b	TP53	RB	Cyclin D1	EGFR	HPV PCR-MA	L1 HPV PCR	In situ HPV
55	+	M	47	EU1848	Tonsil	16	1	1	1	16	HPV16		+
56	-	M	45	UM2493	Tonsil	16	1	1	1	1	HPV16		+
57	+	M	54	EU1849	FOM	1	8	16	8	6	-		-
58	-	M	64	UM1360	FOM	1	16	12	1	9	-		-
59	+	M	62	EU1850	BOT	1	16	16	1	1	-		NA
60	-	M	68	UM2497	BOT	12	12	16	6	6	-		-
61	+	M	42	EU1851	Alveolar ridge	16	1	1	1	16	-	HPV69 ^c	-
62	-	M	35	UM2095	Tongue	1	1	6.5	6	11	-		-
63	+	F	52	EU1852	BOT	1	1	3.5	1	1	-		-
64	-	M	67	UM2499	BOT	1	11	2.5	1	16	-		-
65	+	M	46	EU2475	RM canthus	16	8	1	1	1	HPV16		+
66	-	M	45	UM2500	Conjunctiva	16	3	1	1	1	-	HPV16	NA
67	+	M	63	EU1855	Tongue	1	2	6	1	16	-		-
68	-	M	63	UM2130	RL Tongue	1	16	9	1	16	-		-
69	+	M	67	EU1857	Tonsil	1	3.5	11	1	16	-		-
70	-	M	49	UM2501	Tonsil	16	3	1	1	6	HPV16		NA
71	+	M	54	EU1858	Vallecula	16	6	1	1	1	HPV16		-
72	-	M	56	UM2508	Vallecula	16	4	1	1	1	HPV16		+
73	+	M	35	EU1859	FOM	6	1	9	1	4	-		NA
74	-	M	49	UM1683	Tongue	1	16	16	1	12	-		-
75	+	M	47	EU1860	Maxilla	16	12	1	1	16	-		-
76	-	M	56	UM1698	Maxilla	1	4	16	2.5	1	-		-
77	+	M	65	EU1861	Supraglottis	8	1	1	1	16	Invalid		NA
78	-	M	64	UM2034	Supraglottis	1	12	1	4	16	-		-
79	+	M	61	EU1862	Retromolar	1	1	9	2	16	-		-
80	-	M	X	EU2489	Tongue	1	1	10	6	1	-		-
81	+	M	45	EU1863	Tongue	1	8	1	1	4	Invalid		-
82	-	M	40	UM1779	Tongue	1	16	8	3	11	-		-

Patient sample summary statistics		Overall	HIV+	HIV-	p-value
Age	Median (range)	52 (29–76)	52 (35–70)	56 (29–76)	0.32
Sex	Male	79 (96%)	39	40	0.38
	Female	3 (4%)	2	1	
Tumor Site Category	Larynx (larynx, supraglottis)	12 (15%)	6	6	1.00
	Oral Cavity (alveolar ridge, FOM, mandible, maxilla, tongue, retromolar, lip, buccal mucosa, hard palate)	44 (54%)	22	22	
	Oropharynx (BOT, tonsil, vallecula)	22 (27%)	11	11	
	Hypopharynx (pyriform sinus)	2 (2%)	1	1	
	Other (canthus, conjunctiva)	2 (2%)	1	1	

Grey shading= HIV-positive cases

Bold text= HPV-positive by at least one method

Invalid= Inadequate DNA for testing by PCR-MA

NA= Not applicable, test not performed

X= Age unknown

^aL= left; R=right; BOT=base of tongue; FOM=floor of mouth, RM=right medial, RL= right lateral, Retromolar=retromolar trigone

^bIHC scores are generated by intensity of staining (1: no staining, 2: low, 3: moderate, and 4: high) multiplied by the proportion of positive tumor cells (1: <5%, 2: 5–20%, 3: 21–50%, 4: 51–100%)

^cHPV26, HPV69, and HPV82 are not included in the PCR-Mass Array assay

Table 2

HPV-Positive Cases and Controls by Donor HIV Status

HIV-Positive Cases											
HIV	Sex	Age	BDbx ID	Tumor Site ^a	p16 ^b	TP53	RB	Cyclin D1	EGFR	HPV PCR ^c	HPV ISH ^d
+	M	42	UP1880	R Tonsil/BOT	1	1	4	1	8	HPV16	–
+	M	54	UP1871	Tongue	16	1	1	1	12	HPV33	–
+	F	45	EU1836	Pyriiform sinus	16	6	8	1	1	HPV16	NA
+	M	52	EU1840	Tonsil	16	6	1	1	16	HPV16	–
+	M	45	EU1845	Tonsil	16	6	6	1	12	HPV16	NA
+	M	28	EU1851	Alveolar ridge	16	1	1	1	16	HPV69	–
+	M	47	EU1848	Tonsil	16	1	1	1	16	HPV16	+
+	M	46	EU2475	RM Canthus	16	8	1	1	1	HPV16	+
+	M	54	UM2359	Mandible	16	12	1	1	14	HPV82	–
+	M	54	EU1858	Vallecula	16	6	1	1	1	HPV16	–
+	M	44	UP1864	Hard palate	16	9	2.5	1	1	HPV26	–

HIV-Negative Controls											
HIV	Sex	Age	BDbx ID	Tumor Site ^a	p16 ^b	TP53	RB	Cyclin D1	EGFR	HPV PCR ^c	HPV ISH ^d
–	M	64	UM2168	FOM	16	1	1	1	16	HPV18	+
–	M	49	UM1653	Tongue	16	4	1	1	1	HPV16	+
–	M	59	UM1349	Larynx	8	16	11	3	10	HPV16	–
–	M	48	UM2495	Tonsil	1	12	1	1	16	HPV16	–
–	M	48	UM2496	Tonsil	16	11	1	1	15	HPV16	+
–	M	45	UM2493	Tonsil	16	1	1	1	1	HPV16	+
–	M	49	UM2501	Tonsil	16	3	1	1	6	HPV16	NA
–	M	45	UM2500	Conjunctiva	16	3	1	1	1	HPV16	NA
–	M	56	UM1803	FOM	8.5	16	2.5	1	8.5	HPV16	–
–	M	56	UM2508	Vallecula	16	4	1	1	1	HPV16	+

Summary: Mean(std) of biomarker expression levels among HPV+ subjects				
Biomarker	HPV + subjects (n=21)	HIV + (n=11)	HIV – (n=10)	p-value
P16	13.8 (4.9)	14.6 (4.5)	13.0 (5.3)	0.44
TP53	6.1 (4.9)	5.2 (3.8)	7.1 (6.0)	0.39
RB	2.3 (2.7)	2.5 (2.5)	2.2 (3.1)	0.78
Cyclin D1*	1.1 (0.4)	1.0 (0)	1.2 (0.6)	0.34
EGFR	8.3 (6.5)	8.9 (6.7)	7.6 (6.5)	0.64

* A single (HIV –) control had Cyclin D1= 3

^dR=right; BOT=base of tongue; FOM=floor of mouth, RM=right medial

^hIHC scores are generated by intensity of staining (1: no staining, 2: low, 3: moderate, and 4: high) multiplied by the proportion of positive tumor cells (1: <5%, 2: 5–20%, 3: 21–50%, 4: 51–100%)

^cHPV detected and identified either by PCR-MassArray or by L1 PCR and Sanger sequencing

^dHPV detection by HPV in situ hybridization (Ventana INFORM HPV III)

NA=not available