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1

Prognostic biomarkers in HIV positive patients with head and neck squamous cell carcinoma

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2

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3

ABSTRACT

Background

We examined the prognostic value of a panel of biomarkers in SCCHN patients who were HIV positive (HIV+HNC) and HIV negative (HIV-HNC).

Methods

Tissue microarrays were constructed using tumors from 41 disease site- and age-matched HIV+HNC cases and 44 HIV-HNC controls. Expression of tumor biomarkers was assessed by immunohistochemistry and correlations examined with clinical variables.

Results

Expression levels of the studied oncogenic and inflammatory tumor biomarkers were not differentially regulated by HIV status. Among HIV+HNC patients, laryngeal disease site (p=.003) and CD4 count <200 cells/μL (p=.01) were associated with poor prognosis. Multivariate analysis showed that p16 positivity was associated with improved overall survival (p<.001) whereas increased expression of TGF-β was associated with poor clinical outcome (p=.001).

Conclusion

Disease site has significant effect on the expression of biomarkers. Expression of tumor TGF-β could be a valuable addition to the conventional risk stratification equation for improving HNC disease management strategies.

INTRODUCTION

The continued improvement and availability of highly active combined antiretroviral therapy (HAART) has dramatically prolonged survival in people living with human immunodeficiency virus (HIV) infection and AIDS. While the incidence of AIDS-defining malignancies (ADCs) has declined in the post-HAART era, large epidemiological studies provide emerging evidence of increased risk of non-AIDS-defining cancers (NADCs) over the past decade [1,2]. The incidence of squamous cell carcinoma of the head and neck (SCCHN) is four-fold higher in HIV-infected patients than in the general population [3,4]. Smoking and alcohol consumption are known risk factors for the development of head and neck cancer [5,6] in both HIV-positive and HIV-negative patients [7]. In addition, HIV-infected patients are susceptible to infection by oncogenic viruses, which may contribute to the higher rates of SCCHN. The risk of human papillomavirus (HPV)-associated SCCHN was found to be elevated among persons with AIDS and increased with increasing degrees of immunosuppression [8,9].

NADCs, including oral cavity and pharynx cancer, are often associated with younger age at diagnosis of cancer and more aggressive and advanced stages of disease in the HIV-infected patient population than in the HIV-negative population [10-12]. Advanced cirrhosis and poorer outcome has been reported among HIV-infected patients with hepatocellular carcinoma [13]; a higher risk of local recurrence and metastasis was also noted in HIV-infected patients with skin squamous cell carcinoma [14]. Poor survival in HIV-infected SCCHN patients is associated with low CD4 counts, a larynx/hypopharynx primary site and current tobacco use [15]. Thus, concerns over optimal treatment strategies and disease management arise when treating HIV-infected patients with SCCHN, especially smokers and alcohol users who have higher burden of comorbidity and possible coinfection with HPV [16]. The identification of prognostic factors in HIV-infected patients with SCCHN would be pivotal to the development of effective cancer prevention, surveillance and treatment strategies.

Hence, in this study, we examined protein expression of a panel of candidate prognostic biomarkers (NFkB, pAKTS473, pSTAT3Y705, Bcl-2, TGF-β, IL-6 and VEGF-A (VEGF)), known to be associated with oncogenic activities, involved in the complex host-tumor interaction, or to function as inflammatory mediators [17-21], acting either independently or in concerted fashion. Chronic inflammation affects all stages of cancer development [22] and STAT3, NF-κB and IL-6 are key players in mediating the signaling pathways involved in inflammation-induced carcinogenesis, with the tissue microenvironment being the focal point of interaction between the tumor and host immune system [23]. Lung tumor growth in immunodeficient mice promoted by inflammation has been shown to be mediated by IL-6 through the STAT3/MAPK and NFkB pathways, suggesting a strong causal link between immunodeficiency, inflammation and cancer orchestrated by the STAT3 and NFkB pathways [24]. Furthermore, immunosuppression can be worsened by pro-inflammatory factors induced by cigarette

5

smoking [25, 26] in which process the PI3K/AKT/NFkB pathway has been frequently implicated [27]. Like IL-6, TGF-β, an inflammatory cytokine and potent immune suppressor produced by cancer cells, myeloid cells, and T lymphocytes, plays a dual role in tumor suppression and promotion [20]. The Cancer Genome Atlas (TCGA) investigations in SCCHN have revealed that mutation profile and rates vary substantially by HPV infection, anatomic subsite and smoking history [28], which makes the identification of prognostic tumor biomarkers daunting. Thus, we conducted a retrospective study using tissue microarray (TMA) with tumor tissues derived from disease site- and age-matched SCCHN patients who were HIV infected (HIV+HNC) and non-HIV-infected control patients (HIV-HNC). These rare specimens were acquired through the concerted effort of 5 Head and Neck SPORE centers. This exploratory study aimed to examine the prognostic potential of candidate tumor biomarkers.

PATIENTS AND METHODS

Patients and TMA construction

Patients were identified from one of 5 US tertiary care referral centers (Emory University, Johns Hopkins University, M.D. Anderson Cancer Center, University of Michigan and University of Pittsburgh). HIV+HNC patients were diagnosed between 1991-2011; HIV-HNC patients were diagnosed between 1996-2010. The study was approved by the Institutional Review Boards of all participating institutions and was conducted using anonymized specimens.

TMA was designed using tumor tissues derived from HIV+HNC cases and HIV-HNC controls with sufficient viable tumor tissues that allowed anatomic subsite and age matching. De-identified information including demographic and clinical information, documentation of HIV infection, cancer diagnosis, behavior information, CD4 counts, viral load and HAART use at the time of cancer diagnosis were submitted to the study data center as previously described [15]. Specimens were collected per recommended guidelines [29, 30] according to a well-defined protocol via collaboration of the HNC SPORE HIV consortium, and the TMA was centrally constructed and distributed by the University of Michigan. For each biomarker, two TMA slides (4µm thickness) and 1 H&E slide were obtained. **Supplementary Table 1** shows the number of cases used for each biomarker staining and the number of cases with clinical information.

Immunohistochemistry (IHC)

IHC was performed with validated antibodies: pATKS473 (1:100, clone EP2109Y, Epitomics, Burlingame, CA, USA), Bcl-2 (1:50, clone 100, CalBiochem, San Diego, CA, USA), IL-6 (1:500, AbCam, Cambridge, UK), NFkBp65 (1:200, C-20, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), pSTAT3Y705 (1:25, clone D3A7, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), TGF-β (1:50,

6

Santa Cruz Biotechnology, Inc., Dallas, TX, USA), VEGF-A (1:100, clone A-20, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Primary antibody incubation was carried out overnight at 4°C followed by secondary antibody incubation at room temperature. Finally, slides were incubated with 3,3'-diaminobenzidine to visualize staining and counterstained with hematoxylin. A non-malignant non-HNC tissue sample was included as a negative control. Tumors were tested for p16 expression as a marker of oncogenic HPV by IHC using the CINtec p16 Histology kit and protocol (MTM Laboratories, MA, USA). IHC scores ≥12 were considered p16 positive [15]. The results of p16 staining were provided by investigators at the University of Michigan. The whole cell staining of all other biomarkers was assessed regardless of nuclear and/or cytoplasmic localization. Scoring was described previously [15]; briefly, intensity of tumor cells 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 (proportion times intensity) from each tissue core section were averaged for each patient. All specimens were scored by a board-certified pathologist (GS) blinded to tumor categories.

Tumor HPV DNA testing was conducted using PCR MassArray by investigators at the University of Michigan as previously described [15], all specimens with identified high-risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73) were scored as HPV positive.

Statistical analysis

Comparison between HIV+HNC cases and HIV-HNC control patients' characteristics, and the correlation of IHC scores with clinical covariables were conducted using t-test or Wilcoxon rank-sum test where assumption of normal distribution was violated for numerical variables, and chi-square test or Fisher's exact test for categorical variables. A logistic regression model was employed to examine the adjusted association of each variable with HIV status after adjusting for other factors. Pairwise correlations between the seven biomarkers were examined with Pearson or Spearman correlation coefficients. Univariate association of each biomarker with covariates was examined with t-test or Wilcoxon rank-sum test for categorical covariates, and Pearson correlation or Spearman correlation coefficient for numerical covariates, where appropriate.

Survival estimates were calculated for dichotomized biomarkers, binary prognostic factors, HIV status, and other categorical variables with Kaplan-Meier method and compared between two stratified groups using log-rank test [31]. Univariate and multivariable survival analyses were carried out using the Cox proportional hazards model [32]. The proportional hazards assumption was also examined with scaled Schoenfeld residuals [33]. To avoid choosing arbitrary cut-off points in the levels of biomarker expression, continuous variables were used in the model. Multivariable survival analysis was carried out by entering all variables in a Cox proportional hazard model and using a backward variable

selection method with an alpha level of removal of 0.15 while the HIV variable was arbitrarily kept in the model. HAART use at diagnosis and CD4 counts at cancer diagnosis were not included in the model as they were available only for HIV+ patients. January 1st of the year was used where only year of diagnosis or year of death/last contact was available. All analyses were performed using SAS 9.3 (SAS Institute, Inc., Cary, North Carolina) and R package version 3.3.2 (The R Foundation for Statistical Computing) with two-sided tests and a significance level of 0.05.

RESULTS

Patient characteristics

Forty-one (41) cases with sufficient tumor tissues were identified from the original total of 71 HIV+HNC patients who had HIV-related clinical information; 44 cases with sufficient anatomic subsite- and age-matched tumor tissues were identified from the 47 HIV-HNC control patients. Patient characteristics are shown in **Table 1**. We initially examined whether the subset used for the TMA study reflected the characteristics of the original HIV+HNC cohort [15]: the majority of the current subset were male (92.7%) versus 90.0% in the original cohort, 65.9% were on HAART at the time of cancer diagnosis (versus 80.3%), 19.5% had CD4 count below 200 cells/μL (versus 26.8%), 65.9% were current alcohol users (versus 55.3%), and 19.5% were HPV+ (versus 27.9% in the original cohort).

Eight of 41 HIV+HNC cases were HPV-positive as determined by HPV DNA testing (19.5%, 1 HPV18 and 7 HPV16); 5 oropharynx, 1 oral cavity, 1 larynx, and 1 parotid gland. Eight out of 44 cases in the HIV-HNC group had HPV positive disease (18%, 1 HPV33 and 7 HPV16); 5 oropharyngeal, 2 oral cavity, 1 larynx. HPV positive patients were younger (48.3 ± 9.1) than HPV negative patients (52.8 ± 10.3) though the difference was not significant (p=.11). The HIV+HNC group included 1 case with a parotid tumor, and the HIV-HNC group included 1 case of conjunctiva and 1 case of esophageal cancer.

Thirty-two of 41 (78.0%) HIV+HNC patients had CD4 levels examined at the time of cancer diagnosis, 24 patients of these patients (75%) had CD4 counts ≥200 cells/μL. African Americans accounted for 71.4% of patients with low CD4 (<200 cells/μL). Within the HIV+ group, 36 of 41 (87.8%) patients had HAART information available; 27 of these (75%) had taken HAART at the time of cancer diagnosis and 82.6% of patients receiving HAART had high CD4 count (≥200 cells/μL) compared with 57.1% of patients not taking HAART (p=.30, data not shown). CD4 count was not correlated with stage at presentation, but interestingly, was significantly associated with disease site (p=.001); of the 8 patients with low CD4 counts (<200 cells/μL), 4 had laryngeal cancer (50%) compared with 13% (3/24) in patients with higher CD4 counts (≥200 cells/μL, data not shown).

When the HIV+HNC group was stratified by HPV status, HPV+ patients had significantly higher median CD4 counts (n=8, median 567, range 209-872) than HPV-negative patients (n=26, 232, range

5-700, $p=.04$). Expression of biomarkers did not differ by CD4 level or by the use of HAART at cancer diagnosis.

Correlations between biomarker expression, HIV infection and prognostic factors

As the study groups were not matched based on race, there was a significantly greater proportion of African Americans (46.3%) in the HIV-infected group than in the HIV negative group (6.8%, $p<.001$). HIV+HNC patients (N=41) were about 4.5 years younger than HIV-HNC patients (N=44, $p=.04$) and were more frequently current alcohol users (66% versus 23%, $p<.001$) (**Table 1**). There was no significant difference in gender, tumor stage, disease site, HPV status, or smoking history between HIV+HNC and HIV-HNC groups (**Table 1**). There was no significant difference in the expression of each of the 7 biomarkers (pAKT, NFKB, pSTAT3, Bcl-2, TGF-β, IL-6, VEGF) between HIV+HNC and HIV-HNC groups (**Supplementary Table 2**). After stratifying by disease stage, VEGF expression levels were significantly lower in HIV+ than HIV- stage I-II HNC ($p=.01$). pSTAT3 expression levels were significantly lower in HIV+ than HIV- stage III-IV disease ($p=.03$) (**Table 2**).

We also examined the expression of biomarkers by HPV status. Levels of VEGF, IL-6 and NFkB expression were significantly lower in HPV+HNC than in HPV-HNC ($p<0.001$, $p=.04$, $p=.01$, respectively, **Figure 1A**). The other 4 biomarkers (pAKT, pSTAT3, Bcl-2, TGF-β) were not associated with HPV status. pAKT, NFkB, and VEGF were expressed differentially by disease sites, with highest expression in the oral cavity (**Table 3**, $p<.001$, $p<.001$, $p=.02$, respectively). Current alcohol use was strongly associated with HIV positive status compared to former/never use ($p<.001$, Table 1), there was no interaction effect on the expression of any biomarker among HIV, HPV status and alcohol use. Expression of pAKT was significantly lower in current alcohol users than in former or never users (**Table 4**, $p=.02$). White patients had higher pAKT expression than African American patients ($p=.01$, **Table 4**). The level of NFkB was marginally significantly lower in African American than in White patients ($p=.06$).

Correlations among biomarkers

To verify the signals detected by TMA, we examined the expression of key target molecules shared by the NFkB and pSTAT3 pathways, including VEGF, IL-6, pAKT, and Bcl-2 [17]. In both groups, marked associations were observed, of pAKT with NFkB (**Supplementary Table 3**, $p<.001$ in both HIV+HNC and HIV-HNC), NFkB with VEGF ($p=.02$ in HIV+HNC, $p<.001$ in HIV-HNC), and TGF-β with IL-6 ($p=.006$ in HIV+HNC, $p<.001$ in HIV-HNC), whereas an inverse correlation of pSTAT3 with TGF-β was observed only in the HIV+HNC group ($p<.05$), and a correlation of Bcl-2 with pAKT, NFkB, and TGF-β was observed only in the HIV-HNC group. When pAKT, pSTAT3 and NFkB were grouped as an

9

oncogenic signature using combined score, univariate association revealed that this signature was significantly associated with disease site (p<0.001), former and current alcohol use (*p*=.04), and race (*p*=.02), but not with HIV status, HPV status, p16 positivity, age, gender, smoking history, CD4 count or HAART use at cancer diagnosis (data not shown). Using a combined score of IL-6, VEGF and TGF-β as a tumor microenvironment signature, this score was significantly lower in the HPV+ group than in the HPV- group (*p*=.01).

Prognostic value of biomarkers in overall survival

Survival data was available for 37 of 41 HIV+HNC cases (61.7%) and 23 of 44 HIV-HNC controls (38.3%). Median follow-up was 565 days for the HIV+HNC cases and 1095 days for the HIV-HNC controls. Survival analysis was conducted for all groups where both biomarker and survival information were available. When only the HIV+HNC cases with all clinical covariables were considered, univariate analysis showed that laryngeal disease site (*p*=.003) and CD4 count <200 cells/μL (*p*=.01) were associated with poor prognosis of OS whereas HPV co-infection did not have a significant impact on OS (**Supplementary Table 4**). As expected, the HIV-HNC group exhibited typically poor prognosis associated with advanced age and stage (**Supplementary Table 5**). Multivariate analysis with all biomarkers and clinical covariables in all patient groups is summarized in **Table 5**. The best predictive survival model using a Cox proportional hazard model included HIV infection, p16, pAKT, IL-6, and TGF-β. In the best predictive model, HIV infection and p16 were treated as binary variables while pAKT, IL-6, and TGF-β were treated as continuous variables. HIV status did not have a significant impact on OS in all patient groups after adjusting for the significant biomarkers in the best predictive model (**Figure 1B**). Improved OS was associated with positive p16 (*p*<.001) and increased expression of pAKT (HR=0.80, 95%CI 0.60-0.92, *p*=.002) and IL-6 (HR=0.74, 95%CI 0.60-0.92, *p*=.005), whereas increased expression of TGF-β was associated with poor clinical outcome (HR=1.49, 95%CI 1.17-1.89, *p*=.001).

DISCUSSION

This is the first multi-institutional study exploring the prognostic significance of a panel of tumor biomarkers among HIV+HNC and HIV-HNC patients. To reduce bias, comparison between groups was conducted by pairing each tumor specimen based on anatomical site and patient's age whenever possible. We chose a panel of oncogenic (NFkB, pAKT, pSTAT3 and Bcl-2) and inflammatory (TGF-β, IL-6 and VEGF) tumor biomarkers known to play roles independently or cooperatively in tumor growth and progression and tumor-host immune interaction [17-21]. The subset of HIV+HNC patients largely retained the characteristics of the original patient cohort, particularly, consistent with the findings in the

original study [15], we found that low CD4 count (<200 cells/μL) was significantly associated with poor overall survival in the HIV+HNC cases when only clinical information was included (**Supplementary Table 4**).[34] In addition, current alcohol users were more likely to have HIV infection compared with non- or former alcohol users (OR=6.0, 95%CI 1.8-20.0, *p*=.003), consistent with the high prevalence of lifestyle-related cancer risk factors (smoking and alcohol intake) associated NADCs among patients with HIV infection [7, 35, 36]. HIV infection did not have significant impact on patients' overall survival, consistent with the findings of investigators who used specimens derived from similar cohorts [34].

Our study has revealed that TGF-β expression stands out as an independent poor prognosis factor for OS, a better prognostic factor than stage, disease site, HIV and/or HPV infection when controlling for all clinical covariables and the expression levels of the other biomarkers in all patient groups (**Table 2**). TGF-β, an inflammatory cytokine and potent immune suppressor, plays a dual role in cancer development by acting as a tumor suppressor during the early stages and as a tumor promoter during the later stages of disease[20]. One of the mechanisms by which tumor TGF-β may promote tumorigenesis is through acting as a potent immunosuppressor, and/or recruiting T_{reg} cells (CD25+-Foxp3) and myeloid-derived suppressor cells [19], thus decreasing tumor cell recognition and clearing by the innate immune system. In a recent randomized phase II trial of cetuximab with or without sorafenib in recurrent and/or metastatic SCCHN, high plasma TGF-β was found to be associated with inferior progression free survival regardless of study arm [37]. Furthermore, SCCHN patients receiving single-agent cetuximab had increased frequency of CD4+FOXP3+ intratumoral Treg expressing CTLA-4, CD39, and TGF-β, which were associated with suppressed cetuximab-mediated antibody dependent cellular cytotoxicity and poor clinical outcomes [38]. These studies underscore the complexity of pro-inflammatory tumor markers and demand more systematic future approaches to study tumor biomarkers, including host immune status, tumor infiltrating cells, and genomic approaches to identify molecular signatures with prognostic value. In addition to TGF-β being detected as a significant negative prognostic factor, multivariate analysis detected a statistically significant effect of p16 positivity and increased pAKT and IL-6 expression on OS. The strong prognostic effect of positive p16 on superior clinical outcome was detected in all patient groups including oral cavity, larynx and oropharynx disease site. Our findings are consistent with those of a study assessing the prognostic effect of positive p16 by IHC in oral cavity, hypopharynx, and larynx cancers [39]. It has been reported that elevated systemic IL-6 level at baseline was strongly related with all-cause mortality in HIV-infected patients [40] and at 1 year post HAART treatment with non-AIDS-defining events [41]. It remains to be examined whether the levels of host systemic IL-6 and tumor IL-6 convey similar or different profiles regarding host immune status. The slightly beneficial effect of both pAKT and IL-6 expression on OS indicated by the odd ratios (**Table 5**) warrants further investigation.

11

Evidence of a differential effect of HPV on tumor biomarkers in SCCHN has begun to emerge [42]. The current study observed lower expression levels of IL-6, VEGF and NFkB in HPV+HNC than in the HPV-HNC group (**Figure 1A**), furthermore, the strong association of VEGF with NFkB was not affected by HIV infection (**Supplementary Table 3**). A recent biomarker study of HNC tissues from the base of tongue, tonsil and vocal fold revealed that tumor IL-6 assessed by IHC and serum IL-6 were significantly lower in HPV16-positive patients (N=11) than in HPV-negative patients (N=11), but IL-6 expression levels were not correlated with SCCHN location, stage, or level of HPV viral load [43]. A study of hypoxia-related genes in oropharyngeal SCC found that HPV-positive tumors displayed less hypoxia than HPV-negative tumors [44]. A study using high-throughput analyses and the TRANSFAC database reported that HPV-positive tumors had reduced whole cell protein expression and significantly lower nuclear staining of both STAT3 and NFkB by IHC than HPV-negative tumors, marked colocalization and coactivation of both transcription factors was observed by TMA [45]. Taken together, our findings are consistent with the consensus that HPV infection has significant effects on the tumor expression of IL-6, VEGF and NFkB.

We were surprised by a more prominent effect of disease anatomical site (**Table 3**), race and alcohol use (**Table 4**) than HIV infection and CD4 counts on biomarker expression. This is consistent with the notion of distinct molecular signatures according to disease anatomical site and the impact of risk behaviors such as cigarette smoking on tumor genetic characteristics identified by TCGA investigations [28]. The lack of significant effect of HIV infection raises further questions, such as whether a patient's viral load may be a better prognostic factor than HIV infection for disease progression and overall survival, whether HIV infection alone or HPV coinfection may have significant effect on tumor biomarker expression, and whether the anatomical site and/or risk behaviors may have greater impact than HIV infection on tumor molecular characteristics and behaviors. Whether these factors are related to the strong association of the prevalence of HIV+HNC with race, sex, age, CD4 counts, and risk behaviors [1, 2, 12] certainly warrants further investigation.

The limitations of this study include the retrospective and observational nature of the design, limited sample size, missing information of stage and viral load, single pathologist review, and the lack of information on detailed treatment history and progression-free survival which is highly relevant to disease progression.

In summary, tumor biomarkers were not differentially regulated by HIV status and HIV infection did not have significant impact on overall survival of HNC patients. The high expression of TGF-β was significantly associated with poor OS whereas positive p16 status was significantly associated with improved OS. Addition of biomarkers such as TGF-β to the conventional risk stratification equation

12

could improve prognostic and predictive values, and it would be desirable to implement a disease management strategy targeting this growing but largely under investigated patient population.

13

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14

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15

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16

TABLE 1. HNC patient characteristics by HIV status

Covariate	Level	All patients (N=85)	HIV Positive (N=41)	HIV Negative (N=44)	P-value
Age	Mean (± SD)	51.9 (± 10.2)	49.6 (± 9.9)	54.1 (± 10.1)	.04
Gender	Female	5 (5.9)	3 (7.3)	2 (4.6)	.67
	Male	80 (94.1)	38 (92.7)	42 (95.4)	
Race	African American	22 (25.9)	19 (46.3)	3 (6.8)	<.001
	White	42 (49.4)	18 (43.9)	24 (54.5)	
	Unknown	21 (24.7)	4 (9.7)	17 (38.6)	
Stage	I-II	23 (27.0)	14 (34.1)	9 (20.5)	.36
	III-IV	31 (36.5)	15 (36.6)	16 (36.4)	
	Unknown	31 (36.5)	12 (29.3)	19 (43.1)	
Anatomical site	LX	14 (16.5)	7 (17.1)	7 (15.9)	.98
	OC	45 (52.9)	21 (51.2)	24 (54.5)	
	OP	23 (27.1)	12 (29.3)	11 (25)	
	Other*	3 (3.5)	1 (2.4)	2 (4.6)	
HPV	Negative	61 (71.8)	26 (63.4)	35 (79.5)	.60
	Positive	16 (18.8)	8 (19.5)	8 (18.2)	
	Test invalid	8 (9.4)	7 (17.1)	1 (2.3)	
CD4 at cancer diagnosis** (cells/µL)	<200	-	8 (19.5)	-	NA
	≥ 200	-	24 (58.5)	-	
	Unknown	-	9 (22.0)	-	
HAART*** at cancer diagnosis	No	-	9 (21.9)	-	NA
	Yes	-	27 (65.9)	-	
	Unknown	-	5 (12.2)	-	
Alcohol history	Current	37 (43.5)	27 (65.9)	10 (22.7)	<.001
	Former	6 (7.1)	6 (14.6)	0 (0.0)	
	Never	16 (18.8)	3 (7.3)	13 (29.5)	
	Unknown	26 (30.6)	5 (12.2)	21 (47.7)	
Smoking history	Current	42 (49.4)	29 (70.7)	13 (29.5)	.54
	Former	13 (15.3)	6 (14.6)	7 (15.9)	
	Never	2 (2.3)	2 (4.9)	0 (0.0)	
	Unknown	28 (32.9)	4 (9.8)	24 (54.5)	

Data are presented as number of patients (column %), mean (± SD) or median (range).
P-value is calculated by Student's t-test for numerical covariate; and chi-square or Fisher's exact test for categorical variables as appropriate.
*Other anatomical site: 1 case of parotid gland in HIV+HNC group; 1 case of esophageal and 1 case of conjunctiva in HIV-HNC group.
** CD4 counts at the time of cancer diagnosis were identified for the HIV+HNC group only.
***HAART at the time of cancer diagnosis were identified for the HIV+HNC group only.

17

TABLE 2. Biomarker expression among HNC patients stratified
by HIV status and stage

Stage	Biomarker	HIV+HNC N=29 (70.7% of total)	HIV-HNC N=25 (56.9% of total)	P-value*
I-II	p16+	7 (50%)	2 (22.2%)	
	p16-	7 (50%)	7 (77.8%)	.23**
	Bcl-2	8 (2-12)	7 (1-16)	.32
	pAKT	8.14 (3-16)	10.38 (1-16)	.82
	NFkB	6 (3.2-8)	10 (1-14)	.10
	pSTAT3	6 (1-8.75)	2.63 (1-6)	.11
	TGFβ	5 (4-8)	7 (3-14)	.36
	VEGF	4 (2-8)	10 (6-12)	.01
	IL-6	7 (4-16)	9 (6-12.5)	.70
III-IV	p16+	4 (26.7%)	6 (37.5%)	
	p16-	11 (73.3%)	10 (62.5%)	.70
	Bcl-2	6 (1.5-12)	8 (1-12)	.66
	pAKT	6 (1-14)	8 (2.3-16)	.15
	NFkB	7 (1-10)	8 (1.7-12)	.82
	pSTAT3	1.15 (1-5.2)***	5 (1-7.59)	.03
	TGFβ	8 (2-12)	6 (1.5-16)	.43
	VEGF	8 (4.6-12)	8 (1-10)	.17
	IL-6	10.4 (7.5-12)	10.5 (2-16)	.66

Data are presented as number of patients (column %) or median (range),
* P-value is calculated by Wilcoxon rank-sum test or chi-square test.
** The percentage of p16+ or p16- cases over total number of cases with both p16 staining and stage information available was compared between groups.
*** The level of pSTAT3 expression is significantly different by stage in HIV+HNC group (*p*=.02).
No significant differences were detected by stage in HIV-HNC group.

18

TABLE 3. Biomarker expression by anatomic sub-site* in all patient groups

Biomarker	Oral cavity (n=45)	Oropharynx (n=23)	Larynx (n=14)	P-value
Bcl2	8 (1-12)	6 (1-16)	6.5 (3.2-12)	.41
pAKT	12 (1-16)	4.5 (1-16)	7.5 (1-14.4)	<.001
NFkB	8 (4.16-16)	6 (1-12)	5.49 (1.69-9.2)	<.001
pSTAT3	3 (1 - 8)	2.17 (1 - 12)	2.1 (1 - 7.59)	.94
TGF-β	6.95 (1-16)	4 (1.5-12)	6.6 (2-8)	.13
VEGF	8 (1-14)	6 (2-12)	6.13 (2-10.5)	.02
IL-6	10.4 (2-16)	9 (4-12)	10.15 (6.9-12.25)	.55

Data are presented as median (range),
P-value is calculated by Wilcoxon rank-sum test.
*These cases were not included in the analysis due to different histology origin: 1 case of parotid gland in the HIV+HNC group; 1 case of esophageal and 1 case of conjunctiva in the HIV-HNC group.

19

**TABLE 4. Univariate association of seven biomarkers
with history of alcohol consumption or race**

Biomarker	Alcohol consumption*			Race**		
	Current (N=37)	Former or Never (N=22)	P-value	African American (N=22)	White (N=42)	P-value
Bcl-2	6 (1 - 14.4)	8 (3.2 - 16)	.13	6.5 (1.5 - 12)	7.25 (2 - 16)	.65
pAKT	6.58 (1 - 16)	10.44 (1 - 16)	.02	6 (1 - 12)	8 (1 - 16)	.01
NFkB	7 (1 - 14)	8 (2 - 16)	.02	5.98 (1 - 10.5)	8 (1 - 16)	.06
pSTAT3	3.84 (1 - 12)	5 (1 - 7.59)	.59	3.6 (1 - 12)	3.68 (1 - 6)	.60
TGF-β	6 (1 - 16)	7.6 (2 - 14)	.16	5.5 (1 - 12)	6.9 (2 - 16)	.24
VEGF	7.5 (2-12)	8 (1-14)	.07	6.6 (2-12)	8 (1-14)	.51
IL-6	9.9 (4 - 16)	10.25 (2 - 12.25)	.57	9.45 (4 - 12.25)	10.4 (4 - 16)	.27

Data are presented as median (range),
* Unknown cases of alcohol consumption were excluded from the analysis.
** Unknown cases of race were excluded from the analysis.
P-value is calculated by Wilcoxon rank-sum test.
Note: due to the small sample size, never drinkers were combined with former drinkers.

20

TABLE 5. Multivariate overall survival analysis with biomarkers and covariates

Cohort	Variable	Hazard Ratio (95% CI)	P-value
HIV+HNC	pAKT	0.73 (0.53-1.01)	.05
	IL-6	0.58 (0.29-1.14)	.11
	TGF-β	1.68 (0.94-2.99)	.08
All patients	HIV (positive vs. negative)	1.78 (0.61-5.17)	.29
	p16 (positive vs. negative)	0.12 (0.04-0.43)	<.001
	pAKT	0.80 (0.70-0.92)	.002
	IL-6	0.74 (0.60-0.92)	.005
	TGF-β	1.49 (1.17-1.89)	.001

Note: survival data was available for 37 of 41 HIV+HNC cases and 23 of 44 HIV-HNC controls.
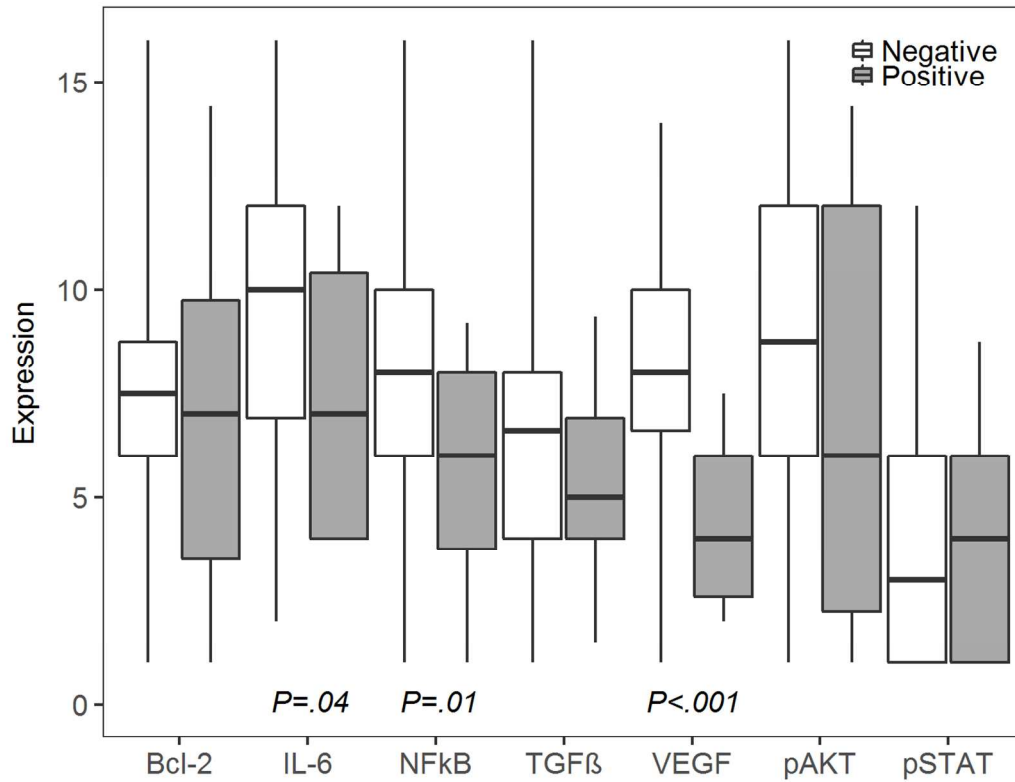
21

FIGURE 1A. Expression of 7 biomarkers by HPV status presented as a boxplot. Expression levels of IL-6, NFkB and VEGF were significantly lower in the HPV-positive group than in the HPV-negative group.
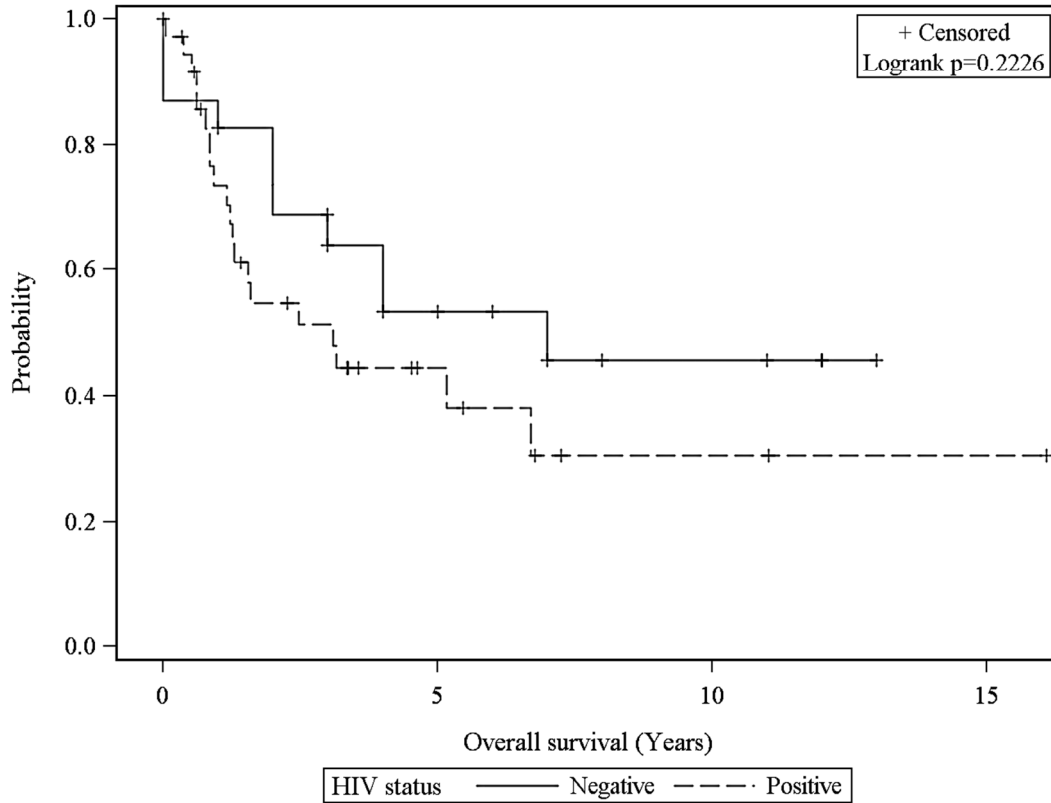
FIGURE 1B. Kaplan-Meier estimates for all patient cohorts by HIV status. Overall survival did not differ among HIV+HNC and HIV-HNC groups.

"Prognostic biomarkers in HIV positive patients with head and neck squamous cell carcinoma". Authors, Hongzheng Zhang, PhD, et al.

Supplemental Tables

Supplemental Table S1: Number of patients with tissues available for each biomarker.

Supplemental Table S2: Expression of biomarkers in HNC patient tumor samples by HIV status.

Supplemental Table S3: Significant correlations of different pairs of biomarkers among all patient groups.

Supplemental Table S4: Univariate overall survival analysis in HIV+HNC cases.

Supplemental Table S5: Multivariate overall survival analysis with biomarkers and covariates in HIV-HNC controls.

TABLE S1. Number of patients with tissues available for each biomarker

Cohort	p16	pAKT	pSTAT3	NFkB	Bcl2	VEGF	TGF-β	IL-6
HIV+HNC (N=41)	41	26	25	25	26	24	27	27
HIV-HNC (N=44)	44	34	36	35	36	35	36	36
HIV+HNC (N=37)*	37	24	23	23	24	22	25	25
HIV-HNC (N=23)*	23	18	19	18	20	18	20	20

*Survival data available.

TABLE S2. Expression of biomarkers in HNC patient tumor samples by HIV status

Biomarker	All patients (N=85)	HIV Positive (N=41)	HIV Negative (N=44)	P-value
p16+	58 (68.2%)	25 (61.0%)	33 (75.0%)	.16
p16-	27 (31.8%)	16 (39.0%)	11 (25.0%)	
BCL-2	7.55 (1 - 16)	6.5 (1.5 - 14.4)	8 (1 - 16)	.23
pAKT	8 (1 - 16)	7.45 (1 - 16)	9.6 (1 - 16)	.13
TGF-β	6.25 (1 - 16)	6.25 (1 - 14)	6.3 (1.5 - 16)	.85
VEGF	8 (1-14)	7.6 (2-14)	8 (1-12)	.71
IL6	10 (2 - 16)	10 (4 - 16)	9.68 (2 - 16)	.57
NFKB	8 (1 - 16)	8 (1 - 16)	8 (1 - 16)	.06
pSTAT3	3 (1 - 12)	3.68 (1 - 12)	2.25 (1 - 7.59)	.42

Data are presented as number of patients (column %) or median (range).
P-value is calculated by Wilcoxon rank-sum test for numerical variables; and chi-square test or
Fisher's exact test for categorical variables, as appropriate.

TABLE S3. Significant correlations of different pairs of biomarkers among all patient groups

Variable1	Variable2	Correlation Coefficient	P-value
HIV+HNC			
pAKT	NFkB	0.75	<.001
TGF-β	IL-6	0.60	<.001
	NFkB	0.45	.02
	pSTAT3	-0.47	.02
VEGF	IL-6	0.50	.01
	NFkB	0.47	.02
HIV-HNC			
pAKT	NFkB	0.68	<.001
	VEGF	0.41	.01
TGF-β	IL-6	0.45	.006
VEGF	NFkB	0.54	<.001
Bcl-2	pAKT	0.40	.02
		0.41	.01
	NFkB	0.37	.03

Note: Refer to TABLE S1 regarding the number of patients with tissues available for each biomarker.
P-value is calculated with Pearson correlation coefficient or Spearman's rank correlation coefficient as appropriate.

TABLE S4. Univariate overall survival analysis in HIV+HNC cases

Variable	Level	N*	Hazard Ratio (95% CI)	P-value
HPV	Positive	7	1.00 (0.33-3.05)	.99
	Negative	25	1 (Reference)	
Site **	OP	11	2.58 (0.90-7.41)	.08
	LX	7	3.88 (1.20-12.53)	.02
	Other	1	2.70 (0.33-22.35)	.36
	OC	18	1 (Reference)	
Gender	Male	34	0.21 (0.05-0.82)	.02
	Female	3	1 (Reference)	
Race	African American	19	4.15 (1.50-11.48)	.006
	White	17	1 (Reference)	
Stage	I-II	13	0.37 (0.13-1.06)	.06
	III-IV	15	1 (Reference)	
HAART at cancer diagnosis	Yes	27	0.39 (0.15-1.01)	.05
	No	9	1 (Reference)	
Alcohol	Current or Former	33	0.76 (0.17-3.39)	.72
	Never	3	1 (Reference)	
Smoking	Current or Former	35	1.19 (0.16-8.99)	.86
	Never	2	1 (Reference)	
CD4 at cancer diagnosis (cells/µL)	≥ 200	23	0.25 (0.08-0.77)	.01
	<200	7	1 (Reference)	
p16	Positive	15	0.87 (0.35-2.20)	.77
	Negative	22	1 (Reference)	
Age		37	0.99 (0.95-1.03)	.59
Bcl-2		24	1.00 (0.86-1.16)	.95
pAKT		24	0.86 (0.74-1.00)	.05
TGF-β		25	1.03 (0.88-1.19)	.74
VEGF		22	1.00 (0.84-1.18)	.98
IL6		25	0.98 (0.82-1.18)	.84
NFKB		23	0.92 (0.78-1.10)	.36
pSTAT3		23	0.91 (0.75-1.11)	.37

*Only the cases with survival information available were included in the analysis.
** Overall p-value is 0.11

TABLE S5. Multivariate overall survival analysis with biomarkers and covariates in HIV-HNC controls

Variable	Hazard Ratio (95% CI)	P-value
Age	1.65 (1.05-2.60)	.03
Stage (I-II vs. III-IV)	0.0 (0.00-0.35)	.03
Bcl-2	6.26 (1.12-34.92)	.04
pAKT	0.30 (0.09-0.96)	.04
NFkB	3.18 (1.03-9.83)	.04
VEGF	0.12 (0.02-0.81)	.03