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Immunologic mediators of outcome for irradiated oropharyngeal carcinoma based on human papillomavirus status

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ABSTRACT

Purpose: To investigate the prognostic value of pre-treatment immune parameters including white blood cell count (WBC) and circulating lymphocyte count (CLC) among patients with oropharyngeal carcinoma treated by radiation therapy.

Methods and Materials: A total of 136 consecutive patients were treated by radiation therapy for locally advanced (stage III/IV) squamous cell carcinoma of the oropharynx with known human papillomavirus (HPV) status. Medical records were reviewed to identify patients with documented pre-treatment laboratory bloodwork. The Kaplan-Meier method and linear regression models were used to evaluate the association between pre-treatment CBC and CLC values with survival endpoints.

Results: One hundred and eleven patients satisfied inclusion criteria. Median age was 62 years (range, 22–91). Eighty-four patients were HPV-positive (76%) and 27 (24%) were HPV-negative. There was no difference in WBC and CLC mean values at baseline between HPV-positive and HV-negative ($p > 0.05$, for both). Trends were detected in the HPV-positive cohort favoring patients with higher CLC, with respect to 2-year local-regional control (93% vs. 82%, $p = 0.06$) and distant control (88% vs. 82%, $p = 0.10$) using the median CLC as cut-off. HPV-positive patients with CLC values in the lowest quartile had inferior local-regional control compared to those in the upper 3 quartiles (69% vs. 89%, $p = 0.01$).

Conclusion: Low pre-treatment CLC was correlated with local-regional recurrence and distant failure among HPV-positive patients. These associations were not observed in the HPV-negative cohort.

Introduction

The association between human papillomavirus (HPV) infection with improved overall survival and disease control has been well-established for patients with squamous cell carcinoma of the oropharynx [1]. While HPV-positive oropharyngeal cancer patients possess epidemiologic and clinical characteristics that contribute to an improved prognosis including fewer medical comorbidities, younger age, decreased tobacco use, and a lower incidence of second primary cancers, these cannot fully explain the abundance of data demonstrating that HPV-positive tumors respond more favorably to therapeutic irradiation than HPV-negative tumors [2–5].

An increasing amount of pre-clinical data has demonstrated that radiosensitivity depends not only on the biological characteristics of cancer cells but also on the tumor microenvironment [6]. For instance, multiple studies have shown that HPV-positive tumors from human cell lines were only curable with radiation in immunocompetent murine models with both CD4+ and CD8+ T cells [7–10]. These have suggested that mediation of radiation response depends largely on the presence of an intact immune system for tumor clearance. The aim of this study was to thus analyze the prognostic significance of pre-treatment hematologic parameters including white blood count (WBC) and circulating lymphocyte count (CLC) in a large cohort of patients with oropharyngeal squamous cell carcinoma treated by radiation therapy and to investigate any potential interactions with HPV status.

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Methods and Materials

Patients

The medical records of 136 consecutive patients with locally advanced (stage III/IV) squamous cell carcinoma of the oropharynx treated with external beam radiation therapy from January 2009 to December 2014 were retrospectively reviewed. We excluded patients with unknown HPV status and/or who had no documentable evidence of laboratory blood work drawn prior to treatment. Patients who presented for palliative treatment and/or with known distant metastases were also excluded. Pre-treatment workup consisted of standard history and physical examination including direct laryngoscopy as well as axial imaging consisting of computed tomography and/or magnetic resonance imaging (MRI). Positron emission tomography (PET) was routinely obtained.

Laboratory evaluation

Blood samples drawn within 12 months before the start of radiation were used to compile a pre-treatment laboratory profile for each patient using the values most proximal to the first fraction. All patients included in this study were previously evaluated for p16 expression as a surrogate for HPV status from tissue obtained from tumor biopsy or surgical resection. Our institutional policy was to perform immunohistochemistry testing for p16 expression following the protocol supplied by the Ventana CINtec p16INK4a Histology Kit #9517 (Ventana Medical Systems, Inc., Tucson, AZ) with the stain considered positive if nuclear and cytoplasmic staining was greater than 70% of the cells. With cases considered equivocal based on p16 staining, in situ hybridization for HPV was performed on the Ventana Ultra auto-stainer using DNA probes and ancillary reagents from Ventana Medical Systems up until July 9, 2014. Subsequent HPV ISH cases were sent to an independent laboratory (Integrated Oncology, Inc., Phoenix, AZ) for testing. The Ventana INFORM® HPV III probe set was used including the low-risk genotypes 6 and 11 and high-risk genotypes 16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66. In situ hybridization for high-risk and low-risk HPV genotypes was considered positive when nuclear-specific staining was detected.

Treatment

At simulation and prior to daily treatment, the head, neck, and shoulders were immobilized in a hyperextended position in a thermoplastic mask supported on a Timo cushion (S-type; Med-Tec, Orange City, IA) mounted on carbon fiber board (S-type; Med-Tec). At the time of CT simulation, the isocenter was placed approximately at the center of gross tumor volume. Axial images with contiguous 3-mm slice thickness without contrast were obtained for planning transferred into a contouring workstation where delineation of target and normal tissue structures were performed.

All patients were treated by intensity-modulated radiotherapy (IMRT). For the patients treated with definitive IMRT, the gross tumor volume (GTV) was specified as the extent of tumor as demonstrated by pre-operative imaging and physical examination including endoscopy. Grossly positive lymph nodes were defined as lymph nodes greater than 1 cm, those with a necrotic center, or having a standardized uptake value (SUV) of greater than 3 on PET. The high-risk CTV (CTV-H) was defined as the GTV plus a margin of 0.5–1 cm to account for microscopic disease spread; the low-risk CTV (CTV-L) included all uninvolved areas in the cervical neck and supraclavicular fossa. For patients treated with IMRT postoperatively, the CTV-H was defined as the surgical tumor bed at risk for harboring residual disease and the ipsilateral neck; the low-risk (CTV-L) in these cases encompassed the uninvolved contralateral neck. Each respective CTV was expanded by 0.3 to 0.5 cm to create a corresponding planning target volume (PTV). Standard prescribed doses to PTV-H in the definitive and post-operative were 66–70 Gy and 60–66 Gy, respectively. Prescribed doses to PTV-L ranged from 54 to 56 Gy.

Follow up and statistical analysis

Patients were asked to return for a follow-up visit 2–3 weeks after completion of treatment and then every 2–3 months for the first year, 4–6 months for the second year, and then annually thereafter. Local control was judged to have been attained if there was no evidence of tumor at the primary site, based on clinical and radiographic findings at follow-up. Regional failure was recorded separately if there was evidence of a cervical or supraclavicular mass distinct from the primary site. Patients who had persistent disease, either clinically or radiographically, after definitive radiation were referred for neck dissection. Failure was counted only if the patient had pathologic evidence of residual disease. Salvage of recurrences was not accounted in the evaluation of local-regional control. Patient follow-up was measured from the first day of radiation therapy and reported up to the date last seen in clinic or up to the date of expiration.

Patients were categorized into separate categories based on HPV status, and descriptive statistics were incorporated with differences between proportions and means compared with a chi-square statistic and t-test, respectively. Actuarial estimates of local-regional control, distant control, overall survival, and cause-specific survival comprising the primary endpoints were calculated using the Kaplan-Meier method. For HPV-positive and HPV-negative patients, separate logistical regression models were constructed to determine variables that influenced the likelihood of local-regional recurrence and distant metastasis. WBC, CLC, and the CLC/WBC ratio was each examined first as continuous variables and then analyzed using the median and quartile values as potential cut-offs for determining statistical significance. To minimize issues of collinearity and maximize power, multivariate analysis was performed and restricted to include only covariates of high clinical interest that could possibly influence decision-making. Variables ultimately selected for inclusion in the multivariate model were age, T-classification, N-classification, smoking history, alcohol history, and Karnfosky Performance Status (KPS). Analysis was performed using SAS version 9.4 (SAS Institute, NC), with statistical significance set using a two-tailed p-value < 0.05. Approval was obtained from the institutional review committee prior to collection of all patient information.

Results

A total of 111 patients satisfied inclusion criteria and comprised the primary study population. Patient and disease characteristics are summarized in Table 1. The median age was 62 (range, 22–91). Eighty-four patients were HPV-positive (76%) and 27 (24%) were HPV-negative. Ninety-five patients (86%) had bloodwork drawn within 30 days of starting radiation therapy. Eighty-nine patients (80%) were treated with primary radiation therapy and 22 patients (20%) were treated by primary surgery. Pathological findings among the latter group included extracapsular extension in 7 patients, close (< 2 mm) margins in 6 patients, and positive margins in 2 patients. Eighty patients (72%) received concurrent chemotherapy, which consisted of single-agent cisplatin or carboplatin (69 patients), cetuximab (8 patients), or cisplatin/cetuximab (3 patients).

At baseline, the HPV-positive patients had significantly improved KPS ($p = 0.04$), decreased tobacco use and history ($p < 0.01$), and more advanced N-classification ($p = 0.02$). HPV-positive patients were also more likely to be never-smokers ($p < 0.001$). There was no difference in the proportion of any of the other patient and disease characteristics between the HPV-positive and HPV-negative cohorts ($p > 0.05$, for all). There was no significant difference in the pre-treatment WBC, CLC, and ratio of WBC/CLC distributions at baseline in

Table 1
Clinical and disease characteristics.

Characteristic	HPV-positive (N = 84)	HPV-negative (N = 27)
Gender		
Male	64	19
Female	20	8
Ethnicity		
White	65	20
Black	9	3
Hispanic	6	4
Asian	4	0
Primary site		
Tonsil	39	10
Base of tongue	39	15
Uvula	6	2
Primary treatment		
Definitive RT	74	15
Surgery and post-operative RT	15	7
T-classification		
T1	25	4
T2	27	5
T3	15	10
T4	17	8
N-classification		
N0	10	7
N1	11	6
N2	51	10
N3	12	4
Tumor grade		
Well-differentiated	14	4
Moderately-differentiated	26	6
Poorly-differentiated	44	17
Smoking history		
None	55	0
Yes, < 10 pack-year	10	2
Yes, 10–40 pack-year	11	17
Yes, > 40 pack-year	8	8
Karnofksy Performance status		
90–100	51	5
80	25	9
70	5	7
< 70	3	6

Abbreviations: RT, radiation therapy; HPV, human papillomavirus

the HPV-positive patients when compared to those of the HPV-negative population ($p > 0.05$, for all). The median CLC was 1.6 (range, 0.7–2.7) and the median WBC was 6.4 (range, 3.4–9.4) for HPV-positive patients. The median CLC was 1.4 (range, 0.72–3.3) and the median WBC was 6.6 (range, 3.1–13.8) for HPV-negative patients.

Mean follow-up was 23 months (range, 5–70 months). For the entire population, the actuarial 2-year rates of local-regional control, distant control, overall survival, and cause-specific survival were 78%, 84%, 83%, and 87%, respectively. With the exception of distant control, significant differences in each of these endpoints existed between HPV-positive and HPV-negative cases. The 2-year rates of local-regional control (86%% vs. 61%, $p = 0.01$), distant control (85% vs. 78%, $p = 0.09$), overall survival (92% vs. 63%, $p = 0.001$), and cause-specific survival (95% vs. 67%, $p = 0.001$) for patients with HPV-positive and HPV-negative tumors, respectively, are illustrated in Fig. 1.

When the HPV-positive cohort was analyzed, there was no correlation on univariate analysis between WBC, CLC, and the CLC/WBC ratio with 2-year rates of local-regional control, distant control, overall survival, and cause-specific survival when each was analyzed as continuous variables. As shown in Fig. 2 and 3, when CLC was analyzed for the HPV-positive cohort using the median value of 1.6, statistical trends were detected favoring patients with higher CLC, with respect to 2-year local-regional control between those below and under the cut-off point (93% vs. 82%, $p = 0.06$) and distant control (88% vs. 82%, $p = 0.10$). As shown in Fig. 4, HPV-positive patients with CLC values in the lowest quartile had significantly inferior local-regional control compared to those in the upper 3 quartiles (69% vs. 89%, $p = 0.01$). No difference in overall survival or cause-specific survival for HPV-positive patients was detected when CLC was analyzed using the median and quartile values as potential cut-offs. There was no correlation identified between CLC/CBC ratio and any of the endpoints when analyzed as both median and intra-quartile values. Logistical regression models confirmed that the absolute CLC was significantly associated with the 2-year local-regional control (OR: 1.02, 95% C.I. 0.98–1.05, $p = 0.01$) and distant control (OR: 1.03, 95% C.I. 1.02–1.05, $p = 0.01$) for the HPV-positive cohort.

With respect to the HPV-negative patients, no correlation was detected between WBC, CLC, and the CLC/WBC ratio with local–regional control, distant control, overall survival, and cause-specific survival when analyzed as continuous variables or using the median and quartile values as cut-off. The 2-year local–regional control between those below and under the median CLC of 1.4 was 63% and 60%, respectively ($p = 0.71$). The corresponding 2-year rates of distant control were 75% and 79%, respectively ($p = 0.43$).

Discussion

Our analysis of pre-treatment laboratory values in patients with oropharyngeal squamous cell carcinoma adds to the growing body of literature supporting the immune system's role in tumor clearance, particularly for patients with HPV-positive disease. Both pre-clinical models and retrospective single-institutional studies have suggested that HPV-positive subjects with head and neck cancer have improved outcomes possibly due to a more robust anti-tumor response mediated by viral antigens [6–10]. The present study, representing a large cohort of patients treated by radiation therapy for oropharyngeal cancer, showed that higher levels of pre-treatment CLC correlated with decreased likelihood of local-regional recurrence and distant metastasis for HPV-positive patients, but not for HPV-negative patients. Due to the limited number of events, we were unable to demonstrate an association between CLC and cancer-specific mortality.

It has been hypothesized that HPV-positive tumor cells express viral antigens that elicit a lymphocytic response that may initiate a tumor rejection pathway and/or an immune surveillance pathway [11–15]. For instance, biopsy specimens of HPV-positive squamous cell tumors have been shown to harbor a greater number of tumor-infiltrating T-lymphocytes than HPV-negative tumors from the head and neck. To illustrate the striking significance, Ward et al. showed that the 3-year overall survival of HPV-positive patients with low levels of tumor-infiltrating lymphocytes was more similar to that of HPV-negative patients than to HPV-positive patients with higher levels of tumor-infiltrating lymphocytes in their specimens among 270 oropharyngeal cancer patients treated by a variety of approaches [12]. Indeed, among the HPV-positive patients, subjects with increased tumor-infiltrating lymphocytes had the best outcomes with a 3-year survival of 96% compared to those with low levels of tumor-infiltrating lymphocytes with a 3-year survival of 59%. While it has been suggested that tumor-infiltrating lymphocytes could possibly be used as a tool to stratify HPV-positive patients with oropharyngeal cancer into prognostic sub-groups which may help guide definitive treatment, our findings suggest that simple laboratory values, which are inexpensively and routinely obtained, could also be used as a prognostic measure in this population.

Although the concentration of peripheral T cells in the circulation does not directly reflect that at the local tumor site, patients with higher pre-treatment lymphocyte levels have been shown to have better prognosis in several other cancer sites, including those of the gastrointestinal tract, lung, and cervix [16,17]. Within the latter, patients without progressive nodal disease have been shown to have higher levels of circulating T cells reactive to HPV specific E6- and E7-encoded antigens [17]. In another study analyzing 57 HPV-positive head and neck cancer patients, HPV16 specific anti-E6/E7 serum antibodies predicted for improved disease-specific and recurrence-free survival [18]. E6 and E7 specific CD4+ and CD8 T cells have also been isolated

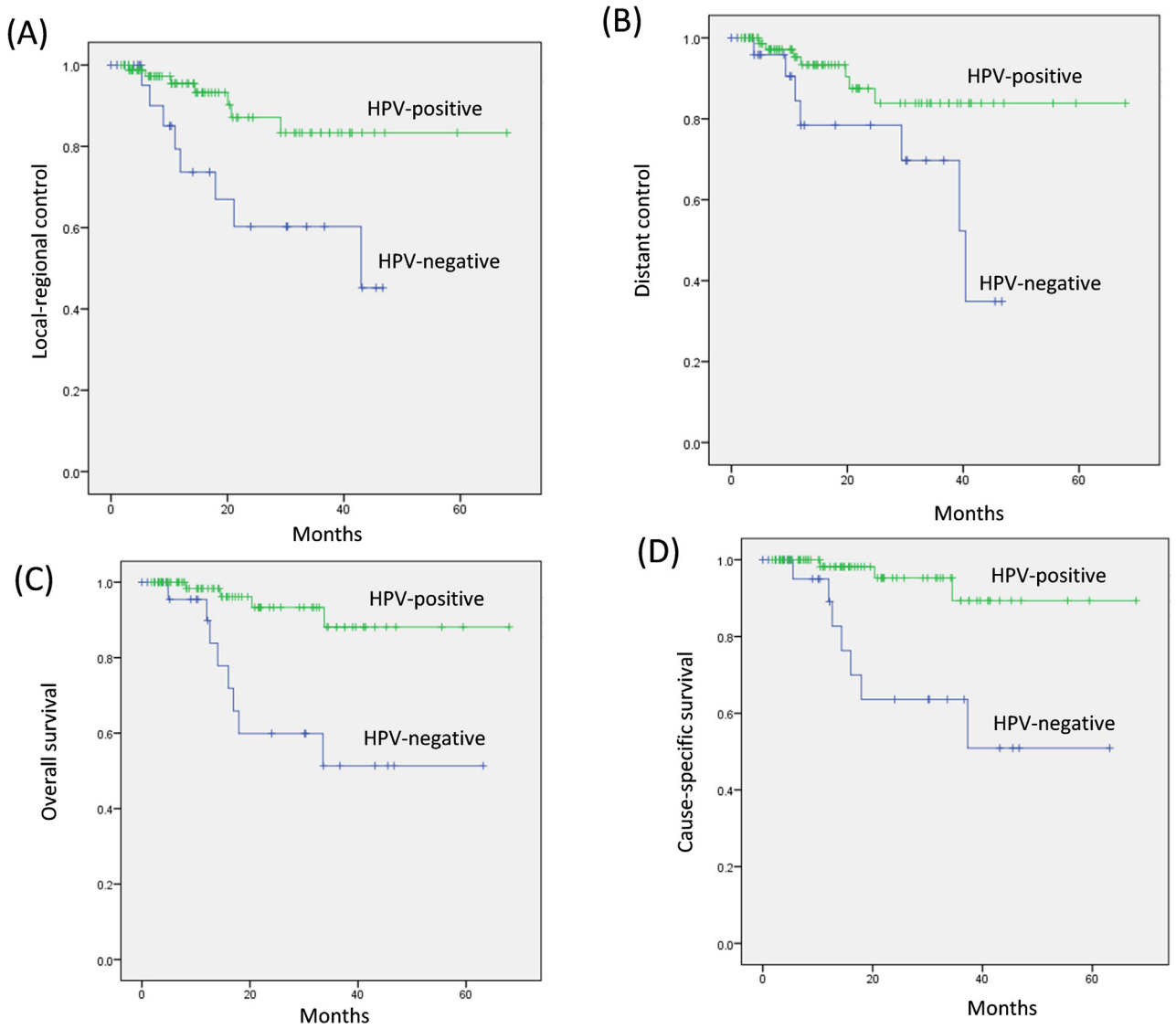

Fig. 1. (A) Overall survival; (B) local-regional control; (C) distant control; and (D) cause-specific survival for the entire patient population stratified by HPV-status.

in HPV-positive head and neck squamous cell carcinoma in both the circulation and at the level of the local tumor microenvironment [19].

Our findings are consistent with others supporting the prognostic significance of pre-treatment CLC in HPV-positive patients and indeed suggest that the immune system plays a role in mediating radiation response to HPV-positive squamous cell cancer of the head and neck. Wansom et al. showed that higher pre-treatment percentage of circulating CD8+ cells predicted for response to induction therapy and complete response to chemoradiation among 47 HPV-positive oropharyngeal patients [13]. Huang et al. recently reported the largest study analyzing circulating leukocytes in 510 HPV-positive and 192 HPV negative patients with oropharyngeal squamous cell carcinoma. They found that HPV-positive patients with higher CLC had improved survival and decreased risk of disease recurrence independent of smoking, the disease extent at diagnosis, or treatment modality. Similar to the results of the present study, there were no significant associations between immune parameters and outcome among the HPV-negative cohort [20].

A limitation of our study related to the lack of consistency with p16 evaluation and the possibility of misclassification. While pathologists generally score tumors as positive on the basis of the current standard of strong and diffuse nuclear and cytoplasmic staining in greater than 70% of the tumor, inter-observer variability in the analysis and interpretation of p16 status is possible. Although p16 expression has been shown to be a reliable surrogate for tumor HPV status with high concordance, it is not 100% accurate. Given that the false positive rate has been demonstrated to range from 2 to 7%, the possibility of inadvertently and unknowingly including patients with HPV-negative disease in the study needs to be considered [21]. Notably, it has been demonstrated that such patients, despite being p16 positive, do not retain the favorable prognostic benefits associated with those who are truly HPV-positive [22].

Other confounding factors must be acknowledged in the interpretation of this data. All pre-treatment laboratory values were used regardless of the patients' other pre-existing conditions or acute disease. Patients with chronically high or low counts were included in addition to patients who may have had active infections or inflammatory states affecting the immune system. We also relied on lymphocyte counts without a subset panel of CD4+ or CD8+. Future prospective studies are needed using larger cohorts of patients to control for medical comorbidities and to ensure that more specific lymphocyte subset panels are ordered. Additionally, we did not specifically analyze temporal changes in WBC or CLC counts over the course of therapy and were thus unable to determine whether fluctuations in values may have influenced outcome. This was limited further by the inclusion of some blood values that were obtained months prior to the initiation of radiation

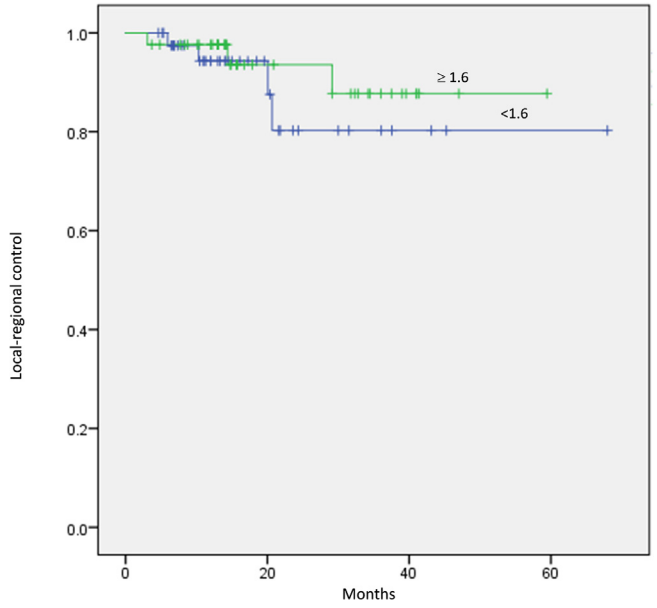

Fig. 2. Local-regional control for HPV-positive oropharyngeal carcinoma patients treated by radiation therapy stratified by the median CLC value.

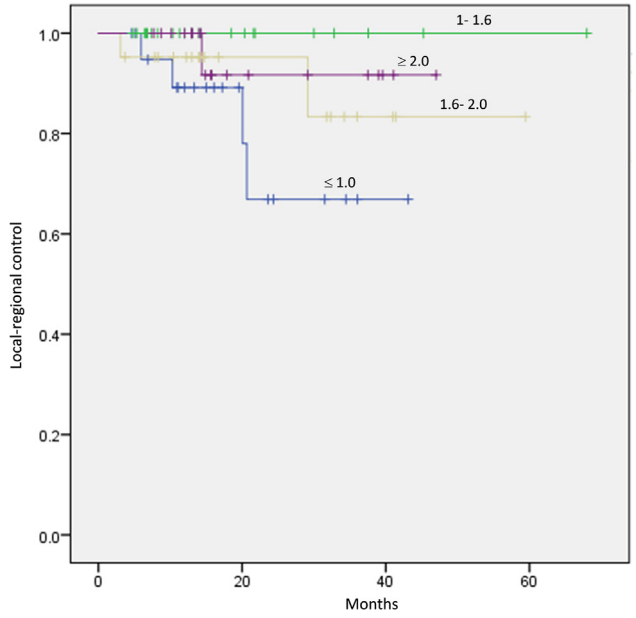

Fig. 3. Local-regional control for HPV-positive oropharyngeal carcinoma patients treated by radiation therapy stratified by quartiles in CLC value.

therapy.

Lastly, it must be recognized that biological factors not related to the immune system have also been suggested to contribute to the differential response of head and neck tumors to radiation therapy. Indeed, biologically resistant tumors, even in the setting of HPV-positivity could possibly be explained by the harboring of an increased proportion of cancer stem cells and/or hypoxic elements. For instance, Linge et al. showed that high levels of hypoxia-induced gene signatures and over-expression of the cancer stem cell markers CD44, MET, and SLC3A2 were correlated with tumor recurrence after chemoradiation and may be responsible for the differential response to treatment [23]. In another recently published study by Vlashi et al., it was demonstrated that HPV-positive head and neck squamous cell tumors have a lower frequency of intrinsic cancer stem cells and also have a less robust ability to dedifferentiate into resistant cells than HPV-negative tumors

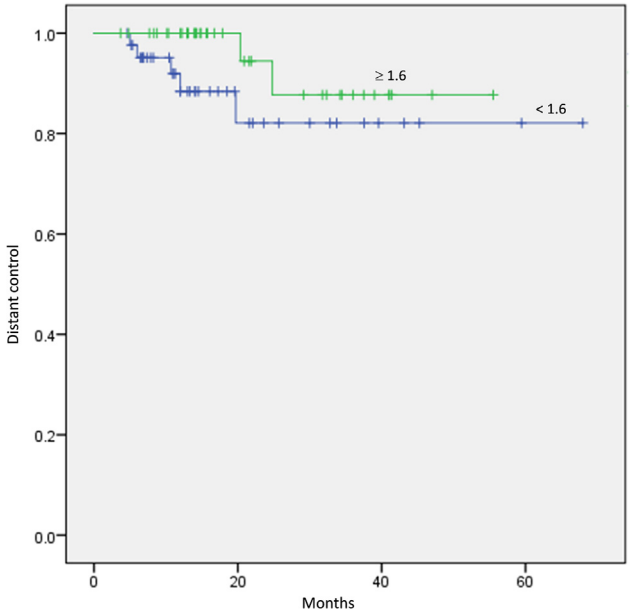

Fig. 4. Distant control for HPV-positive oropharyngeal carcinoma patients treated by radiation therapy stratified by the median CLC value.

after beginning of radiation therapy [24].

In conclusion, we have provided data showing that an intact immune system is important to mediate radiation response for HPV-positive, but not HPV-negative tumors, arising from the oropharynx. More specifically, we demonstrated that elevated CLCs as measured in the peripheral blood in the HPV-positive patients correlate with improved outcomes. This information may be useful to help stratify patients into risk groups predicting for failure with treatment de-intensification approaches.

Conflicts of interest

The authors indicate no disclosure of potential conflicts of interest.

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None

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