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CHAPTER 6

HLA class II Expression on Tumor Cells and Low Numbers of Tumor-Associated Macrophages Predict Clinical Outcome in Oropharyngeal Cancer

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ABSTRACT

Background: Human papilloma virus-positive (HPV) oropharyngeal squamous cell carcinoma (OPSCC) is a highly immunogenic tumor and differences in tumor microenvironment might contribute to the improved survival of HPV-positive OPSCC patient. A comprehensive multivariate analyses with clinical and immune variables (HLAI/II, PD-L1, PD1, T cells and macrophages) was performed in 142 OPSCC patients. We found an inverse correlation between the expression of HLA class II molecules on tumor cells and CD68+CD163+ tumor-associated macrophages (TAMs). High HLA-DP/DQ/DR expression and low number of TAMs were associated with longer disease-specific survival (DSS) and disease-free survival (DFS). Furthermore, a new population of CD8+FoxP3+ T cells was correlated with shorter DFS in multivariate analysis.

In conclusion, in this study we identified new prognostic markers for oropharyngeal cancer patients, which can be used for selecting patients that can benefit from immunotherapy.

INTRODUCTION

Head and neck cancer is the 6th most common cancer worldwide and about 90% of the head and neck cancers are squamous cell carcinomas (HNSCC)[1]. The most important etiological factors of HNSCCs are the genetic predisposition, the use of tobacco and/or alcohol and the infection with high-risk Human Papilloma Virus subtypes, most notably HPV16 [2]. HPV-positivity is predominantly associated with Oropharyngeal Squamous Cell Carcinoma (OPSCC). In these patients, HPV status, smoking history, tumor (T) and lymph node (N) stage represent strong prognostic markers for treatment outcome [3].

HPV-positive OPSCCs have longer disease-free survival (DFS) and disease-specific survival (DSS) compared to HPV-negative OPSCCs [4]. There is strong indication that the tumor microenvironment plays a pivotal role in the pathogenesis and clinical behavior of various malignancies [5] and that the increased immune reactivity of HPV-positive OPSCCs might contribute to a favorable clinical outcome [6-8]. While several studies previously addressed the role of specific subsets of immune cells in OPSCC development and progression [9-12], there is still a need for comprehensive analyses of the heterogeneity and complexity of the tumor microenvironment.

In the tumor microenvironment, various subsets of immune cells interact together to either promote or suppress tumor cells growth [13]. Tumor cells expressing the human leukocyte antigen I complex (HLA-I) are recognized by CD8+ T cells promoting T-cell specific tumor cytotoxicity [14]. Furthermore, expression of HLA-II on antigen-presenting cells (APCs) promotes the CD4+ T-helper-mediated activation of B cells as well as cytotoxic CD8+ T cells [15]. Importantly, numbers of CD8+ tumor-infiltrating T cells have been recently described as markers for improved prognosis in oropharyngeal cancer [16].

In contrast, several mechanisms were proposed to promote tumor growth. Tumor cells, indeed, can escape killing by expressing the programmed death ligand 1 (PD-L1), which, upon binding to the PD1 receptor on T-cells, leads to suppression of T-cell activation and cytotoxicity [17, 18]. However, PD-L1 expression is not only limited to tumor cell as the presence of PD-L1+ tumor-associated macrophages (TAMs) was shown to be associated to functionally anergic CD4+ T cells in HNSCC.

Furthermore, increased numbers of CD4+FoxP3+ regulatory T-cells were associated with an unfavorable prognosis [19-21], however, results were controversial as other studies observed opposite results [22, 23]. The complexity of the CD4+FoxP3+ population was also elucidated in colorectal cancer, where instability of FoxP3 was associated with less immunosuppressive T-cell phenotypes [24].

In addition, CD68+ tumor-associated macrophages (TAMs), commonly identified by the expression of CD163, CD206 or CD204 [25], also sustain the development and progression of many tumors including oropharyngeal cancer, by suppressing the cytotoxic activity of CD8+ T-cells, and promoting angiogenesis and tumor cell migration [26-28].

In this retrospective study, we aim to obtain a comprehensive 'immune signature' of the HPV-negative and HPV-positive OPSCCs using immunohistochemistry (IHC) and

immunofluorescence (IF) techniques for HLA expression, the presence of innate- and adaptive immune cells and explore their correlation with clinical outcome. Ultimately, with the implementation of univariate and multivariate analysis, we aim to find independent prognostic factors for DFS and DSS of OPSCC patients.

MATERIALS AND METHODS

Patient and tumor characteristics

This study was undertaken with the written consent of all patients. The study was performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands. 142 patients diagnosed with OPSCC treated at the Dutch Cancer Institute Antoni van Leeuwenhoek hospital in Amsterdam, the Netherlands, between 1998 and 2007 were included in this study. Two hundred patients were initially included in our cohort. Selection was based on treatment (RT or ChemoRadioTherapy). Out of 200 patients, 30 could not be included in the study due to the limitation of the material and 28 samples could not be used for automatic scoring of immunofluorescence staining due to the low quality of the tissue. Included were patients diagnosed with a T1-4N1-3M0 OPSCC (7th AJCC edition) treated with RT or CRT in a curative setting. Archival diagnostic FFPE tumor specimens taken at the time of diagnosis were retrieved from the Core Facility Molecular Pathology (CFMP).

Clinical data were collected retrospectively by reviewing patients' medical files. Disease-free survival (DFS) was calculated as time from date of diagnosis and time to local or regional recurrence, distant metastasis or death. Disease-specific survival (DSS) was calculated as time from date of diagnosis and time of death due to the disease (e.g. evidence of recurrence, metastasis, patient refused treatment, no more treatments available or compliance of the disease). Overall survival (OS) included any cause of death. Death due to other causes included: no evidence of disease, failures, other disease or unknown reasons. Minimal follow up time was 3 years.

Treatment

Prior to definitive radiotherapy, an excision biopsy was performed in 13 patients by tonsillectomy (n=11), uvula resection (n=1) and anterior pharyngeal wall excision (n=1). Three patients received a selective- and 27 patients a (modified) radical neck dissection. Consecutively, 51 (36%) patients were treated with radiotherapy alone, 1 patient was treated with concurrent cetuximab (400 mg/m^2 loading dose, 250 mg/m^2 weekly). All other patients were treated with platinum-based concurrent chemoradiotherapy. Multiple dose regimens have been administered both intravenous and intra-arterial, e.g. low dose (6 mg/m^2 daily or 40mg/m^2 weekly, n=26), high dose cisplatin i.v. 100mg/m^2 on day 1, 21 and 43 (n=43) and 150 mg/m^2 i.a. weekly in the first 4 weeks of treatment (n=21). Patients were treated with 3D conformal radiotherapy between 1998 and 2005. Intensity modulated radiotherapy (IMRT) was gradually introduced in 2006. Radiation treatment consisted of an elective irradiation dose of 46 Gy in 23 fractions (sequential) or 54.25 Gy in 35 fractions (concomitant) followed by a boost to the primary tumor and the involved nodes resulting in a total dose of 70 Gy.

Immunohistochemical staining

Standard immunohistochemistry (IHC) was applied on whole slide tissue sections taken from archival formalin-fixed, paraffin embedded (FFPE) tumor samples. IHC was performed on a BenchMark Ultra autostainer (Ventana Medical Systems, Mountain View, CA, US). FFPE sections were cut at 3 mm, heated at 75°C for 28 minutes and deparaffinized in the instrument with EZ prep solution. Heat-induced antigen retrieval was carried out using Cell Conditioning 1 for 32 minutes at 95°C or 64 minutes at 95°C.

The antibodies used for staining are shown in Table S1. Incubation was performed at 37°C for 32 minutes for all the antibodies except PD-L1, which was incubated for 64 minutes at room temperature. Bound antibodies were detected using the Omap anti-Rb HRP or Omap anti-Ms HRP for 12 minutes at 37°C, after which the ChromoMap DAB Kit was applied. Slides were counterstained with Hematoxylin and Bluing Reagent. For p16, signal amplification was applied using the Optiview Amplification Kit. Bound antibody was detected using the OptiView DAB Detection Kit. Slides were counterstained with Hematoxylin II and Bluing Reagent. All reagents were purchased from Ventana Medical Systems. Antibodies used for IHC were: HLA-HCA2, HLA-HC10, b2-microglobulin, HLA-DRA, HLA-DP/DQ/DR, PD1, PD-L1, P53 and P16. Antibodies used immunofluorescence were CD4, CD8, CD68, FoxP3, CD163 and panCK. Details can be found in Supplementary Table S1.

Multiplex immunofluorescence staining

Paraffin sections were cut at 3 mm and heated for 30 minutes at 69°C and subsequently deparaffinized and rehydrated using a Multistainer (Leica, ST5020). Afterwards, slides were fixed using Neutrally Buffered Formalin (NBF) for 20 minutes. After rinsing in distilled water, antigen retrieval was performed using AR9 solution (Perkin Elmer, AR900). Antibodies used for the multiplex immunofluorescence (IF) are listed in Table S1. All antibodies were incubated for 30 minutes at room temperature except CD4 and CD8, which were incubated for 2 hours and 1 hours respectively. Opal Polymer HRP Ms+Rb (Perkin Elmer, ARH1001EA, 10 min. at RT) was used as secondary antibody. Visualization of antibody binding was performed using Opal520, Opal540, Opal570, Opal620, Opal650 or Opal690. Stripping of the antibody complex in between staining cycles was performed using microwave treatment for 15 minutes at 100°C in AR6 (Perkin Elmer, AR600) or AR9 buffer solution as appropriate. Slides were counterstained with DAPI (Perkin Elmer, FP1490) and rinsed with distilled water and mounted with ProLong Diamond Antifade Mounting Medium (Molecular Probes, P36970). The antibodies used for staining are listed in Supplementary Table S1.

Human Papilloma Virus detection

IHC detection of p16^{ink4a} and p53 was used for HPV status assessment as proposed previously [29], P16 was scored as positive or negative with a cut-off of intense diffuse nuclear or cytoplasmic staining of > 70%, while p53 staining was scored as "wild-type" expression or "mutation pattern" [30]. As FFPE material was old, no further HPV16 molecular testing could be performed due to the low quality of DNA.

Scoring system for immunohistochemical staining

HLA-I (HCA2, HC10, b2-microglobulin), HLA-II (DRA, DP/DP/DR) and PD-L1 expression were scored manually by two researchers (B.C. and K.T.) together with a dedicated head and neck pathologist (S.W.). All were blinded to patient clinical status and treatment outcome. For PD-1 scoring an automated scoring was performed (see below).

Molecules of the HLA-I complex were scored on tumor cells only, while HLA-DRA and HLA-DP/DQ/DR were scored on tumor cells and stromal cells surrounding the tumor (further referred as 'tumor' and 'stroma'). PD-L1 was scored on tumor cells in the center of the tumor (PD-L1 tumor), at the invasive margins of the tumor (PD-L1 margins) and in the tumor surrounding stromal cells (PD-L1 stroma). PD1 was scored on tumor surrounding stromal cells (PD1 stroma). The format of the output results was expressed as categorical data.

HLA-I and II

HLA-I and II scoring was performed as previously described [31]. Briefly, HLA status was scored in two ways: 1. The number of positive cells (%) and 2. The staining intensity. The number of positive cells was categorized as: 0 = <1%, 1 = 1-5%, 2 = 6-25%, 3 = 26-50%, 4 = 51-75% and 5 = >75%. The intensity of the staining was categorized as 1 = absent, 2 = weak and 3 = strong. The two scores of the number of positive cells and intensity of the staining were summed up and integrated into four categories: 1. Negative (1), 2. "weak expression" (2-4), 3. "moderate expression" (5-6) and 4. "high expression" (7-8). Of note: For HLA-I, weak and moderate expression were combined and further defined as 'downregulated'. In addition, a "negative" HLA-I staining is further referred to as "loss" [32]. For HLA-II and b2-microglobulin (b2M) scoring was divided in "negative" (negative and weak) and "positive" (moderate and high).

PD-L1 and PD1

PD-L1 expression was scored as number of positive cells in either tumor, margins or tumor-associated stroma (with a cut off value of 5%) [17]. PD1 staining was used for optimization of the scoring system using the Vectra® 3.0 automated imaging system (PerkinElmer, Hopkinton, MA), and InForm® software version 2.2 (PerkinElmer, Hopkinton, MA). Representative analysis was performed on 11% of the total stromal area in 20x magnification. Regions of interest were selected by an experienced pathologist (S.W.) and an algorithm was created to identify nuclei in the tumor or in the surrounding stroma separately. Results obtained were percentage of positive cells in the tumor-associated stroma compared to the total area. The median value was used as a cut off for positive or negative cases. For 15 random sections, the data obtained from the score of 11% of the tissue was compared with the data obtained from the score of the entire tissue and no differences were observed in terms of percentage of positive cells (data not shown).

Quantification of the multiplex staining

Whole slide scans were taken at 4x magnification using Vectra® 3.0 automated imaging system (PerkinElmer, Hopkinton, MA). Afterwards, stromal or tumorous regions were selected by an experienced pathologist (S.W.). Selected regions were scanned at 20x magnification and analyzed using InForm® software version 2.2 (PerkinElmer, Hopkinton, MA). An algorithm was created to allow batch analysis of all distinct phenotypes. Obtained results were verified (S.W.). Data are expressed as number of positive cells per surface area (mm^2) in the tumor area or in the tumor-associated stromal area. Results were represented as continuous data. Tumor area was identified with the help of the pathologist and segmented in tails (containing tumor or in proximity of the tumor). Segmentation of the tissue in 'tumor' and 'tumor-associated stroma' was performed in each tail and markers were scored in both regions (data not shown).

Statistical analysis

Patients and tumor characteristics were compared between HPV groups using Wilcoxon matched-pairs test or Chi-square test. To evaluate the impact of clinical, immunohistochemical and immunofluorescence measurements on progression, univariate analysis and two types of multivariate analyses were used: Cox proportional hazards regression model (with pre-selected variables with p<0.1 from univariate analysis),as well as competing risks models based upon subdistribution functions [33]. In order to correct for differences between treatment arms, models were fitted using treatment stratification. Analyses were run in R using the packages 'survival' and 'cmprsk'. In all cases, a significance level of 5% was used to consider tests as statistically significant. Multivariate analysis with clinical and immunohistochemistry variables, included: HPV status, gender, smoking, T classification, HLA-HC10, HLA-HCA2, β2-microglobulin, HLA-DRA (Stroma and Tumor), HLA-DR/DP/DQ (Stroma and Tumor), PD-L1 (stroma, tumor and margins), PD1 (Stroma and Tumor). Multivariate analysis with clinical and immunofluorescence variables included: HPV, gender, smoking, T classification, CD163 (Stroma and Tumor), CD4 (Stroma and Tumor), CD4+FoxP3+ (Stroma and Tumor), CD68 (Stroma and Tumor), CD68+CD163+ (Stroma and Tumor), CD8 (Stroma and Tumor), CD8+FoxP3+ (Stroma and Tumor).

Spearman test was used for correlation analysis (competing risks model). Interaction terms were added to the regression model to understand the relationships among selected variables.

RESULTS

Patients and tumor characteristics

A total of 142 oropharyngeal cancer patients were included in this study (Table 1), of whom 82 (57.7%) had HPV-negative tumors and 60 (42.3%) had HPV-positive tumors. For both groups, the tumor was predominantly found at the base of the tongue and tonsil (combined: 67.1% for HPV-negative tumors and 96.7% for HPV-positive tumors). The proportion of men was significantly higher in the HPV-positive group compared to HPV-negative, 85% and 68.3% respectively (p=0.02). T classification at time of diagnosis was higher in patients with HPV-negative tumors (p<0.01) as T3-4 stage tumor was determined in 64.6% of the HPV-negative and in 31.7% of the HPV-positive tumors. Tumors were differently distributed in the 5 different locations (base of the tongue, vallecular, tonsil, posterior wall and soft palate) (p<0.01) in

HPV-negative and HPV-positive tumors, however, none of the different tumor locations were significantly correlated with survival (data not shown).

For both groups, the majority of the patients were still smoking at time of diagnosis (85.4% for HPV-negative and 55% for HPV-positive). Both groups were treated similarly with radiotherapy alone or chemo-radiotherapy (p=0.22). HPV-negative tumors were more frequently associated with metastases to distant organs than HPV-positive tumors (p = 0.01).As expected, number of disease-related deaths were significantly higher in HPV-negative OSPCC patients compared to HPV-positive (p<0.01).

Median follow up time for alive patients in the entire cohort (No. patients 44) was 82.0 months (standard deviation 'SD' =29.9 months) and 81.9 months (SD=26.4) for patients with HPV-positive tumors (No. patients 32) and 96.7 months (SD=38.2) for patients with HPV-negative tumors (No. patients 12) . Two patients were lost due to follow up.

Distribution of HLA-I, HLA-II, PD-L1 and PD1 in view of Human Papilloma Virus status

IHC staining for p53, p16, HLA-I, HLA-II, PD-L1 and PD1 was successfully performed in 99-100% of all tumor specimens. Representative figures of IHC staining are shown in Figure 1A and B. Table 2 summarizes tumor- and stroma cells expression per marker in the whole group and in the HPV-positive and HPV-negative subgroups.

Downregulation or complete loss of the two HLA-I molecules, i.e. HLA-B/C and HLA-A, was seen in the vast majority of the tumors (combined loss or downregulation was 75.9% and 71.7%, respectively), while b2M expression was completely absent in 16.7% of the tumors. No significant differences in expression of HLA-A, B/C molecules were observed between the HPV-negative and positive tumors. Expression of HLA-DRA or HLA-DP/DQ/DR on tumor cells was found in a minority of the patients (14.1 and 31.9% respectively), while expression of HLA-DRA or HLA- DP/DQ/DR on stromal cells was found in the majority of stromal cells (57.4 and 78.7% respectively). HLA-DRA expression on tumor cells was significantly higher in the HPV-positive tumors compared to HPV-negative tumors (p=0.03), and a trend was observed for HLA- DP/DQ/DR (p=0.06). On stromal cells, HLA-DRA or HLA-DP/DQ/DR expression did not differ in the HPV-positive and HPV-negative group (p=0.12 and p=0.29, respectively).

PD-L1 expression on tumor cells, at the tumor margins or in the tumor-surrounding stroma was found in 24.8%, 17.6% and 28.4% of the tumors, respectively. PD-L1 expression at the invasive tumor margins or in the tumor-surrounding stroma was found to be significantly higher in HPV-positive compared to HPV-negative tumors (p=0.05 and p=0.02 respectively). Expression of PD-1 was not significantly different between HPV-positive and HPV-negative tumors (p=0.09). Altogether, these data suggest that HPV-positive OPSCC is characterized by higher expression of HLA-II in tumor cells and higher expression of PD-L1 in the surrounding stroma.

Table 1. Patient, tumor and treatment characteristics. Clinico-pathological characteristics of the entire cohort of patients and patients with HPV-positive and HPV-negative tumor separately.

		Entire OPSCCs cohort		HPV negative OPSCCs		HPV positive OPSCCs		
		No. of patients	%	No. of patients	%	No. of patients	%	P-value
Patients	Number	142		82	57.7	60	42.3	
Age	Median	58.5		60		55.5		
	Mean	58.4 (10.3)		59.57 (9.5)		56.8 (11.2)		0.26
Gender	1: Male	107	75.4	56	68.3	51	85	
	2: Female	35	24.6	26	31.7	9	15	0.02
Site	Base of tongue	46	32.4	19	23.2	27	45	
	Vallecula	5	3.5	3	3.7	2	3.3	
	Tonsil	67	47.2	36	43.9	31	51.7	<0.01
	Posterior wall	9	6.3	9	11	0		
	Soft palate	15	10.6	15	18.3	0		
T classification	1	29	20.4	9	11	20	33.3	
	2	41	28.9	20	24.4	21	35	<0.01
	3	34	23.9	25	30.5	9	15	
	4	38	26.8	28	34.1	10	16.7	
N classification	0	27	19	18	22	9	15	
	1	23	16.2	15	18.3	8	13.3	0.46
	2	79	55.6	41	50	38	63.3	
	3	13	9.2	8	9.8	5	8.3	
Smoking status	1: Currently smoking	103	72.5	70	85.4	33	55	
	2: Quit < 5 years	9	6.3	7	8.5	2	3.3	<0.01
	3: Quit > 5 years	17	12	4	4.9	13	21.7	
	4: Never smoked	13	9.2	1	1.2	12	20	
Treatment	1: Radiotherapy	51	35.9	26	31.7	25	41.7	
	2: Chemoradiotherapy	91	64.1	56	68.3	35	58.3	0.22
Loco/regional Recurrence	Yes	18	12.6	13	15.8	5	8.3	
	No	124	87.3	69	84.1	55	91.6	0.18
Distant metastasis	Yes	31	21.8	24	29.3	7	11.7	
	No	111	78.2	58	70.7	53	88.3	0.01
Follow-up (alive)	Median	82.0 (29.9)		96.7 (38.2)		81.9 (26.4)		
	Range	38.8-150.2		45.4-150.2		38.8-139.0		

Distribution of myeloid cells and lymphocytes in view of Human Papilloma Virus status

Multiplex immunofluorescence staining was successfully performed in 89-94% of all tumor specimens. Representative figures of IF stainings and phenotypic annotation are shown in Figure 1C and 1D. Distribution of myeloid cells and lymphocytes markers is shown in Figure 2. Quantification of the multiplex staining is shown in supplementary Table S2. Interestingly,
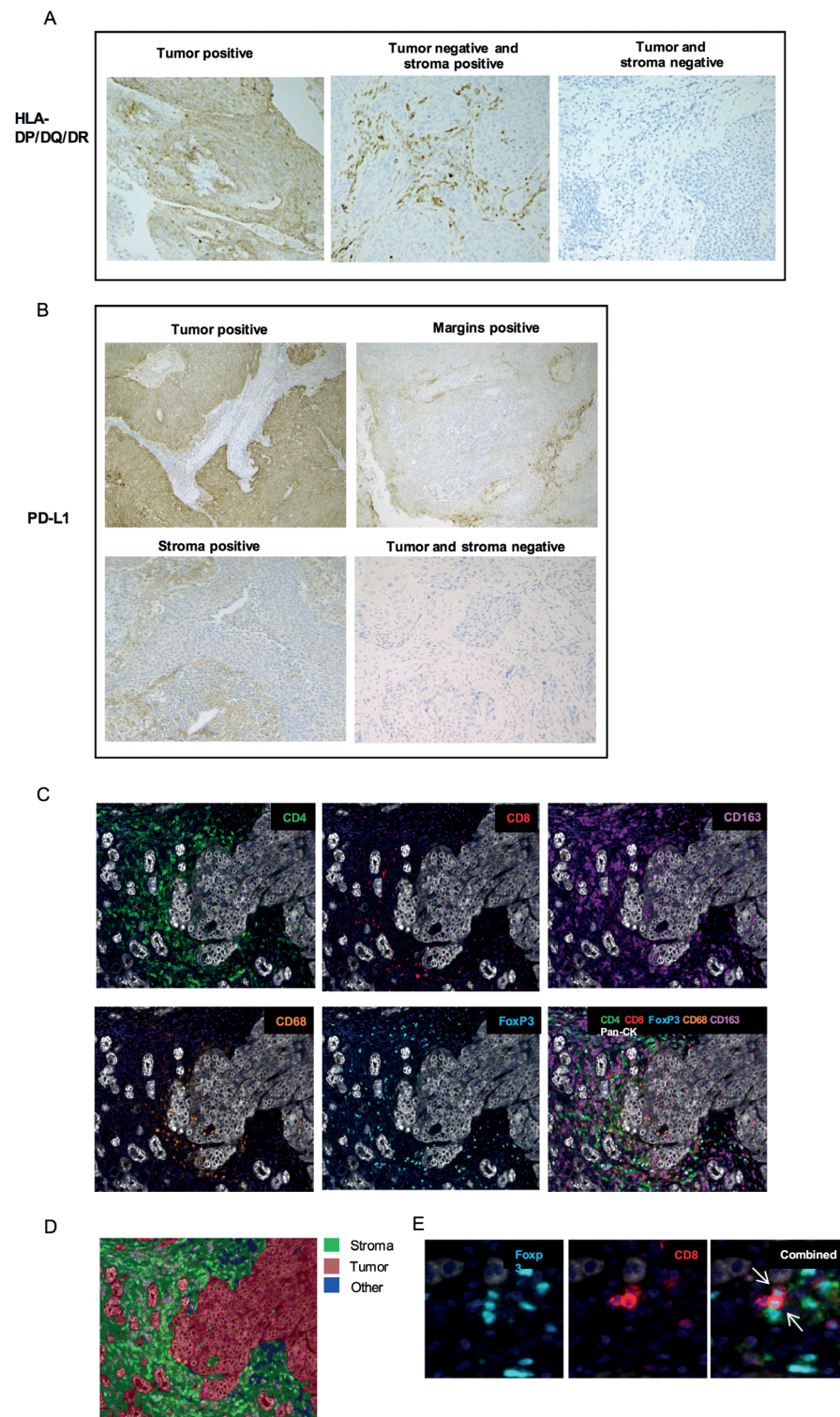
we found a considerably higher number of single positive CD163+ cells compared to double positive CD68+CD163+ TAMs or single positive CD68+ cells in the stroma (median of cells/mm^2 20.7, 4.6 and 2.9, respectively), while numbers in the tumor compartment were comparable (median of cells/mm^2 4.1, 3.1 and 2.2, respectively). No significant differences were observed in the numbers of any of the macrophage cell sub-populations between HPV-negative and HPV-positive tumors, neither in the tumor nor in the stroma.

The number of CD4+ cells and CD8+ cells was much higher In the HPV-positive tumors compared to HPV-negative tumors, both in the tumor and stromal compartment (p<0.001 in the tumor and p<0.01 in the surrounding stroma for CD4 and p<0.01, for both CD8 in the tumor and in the surrounding stroma), while very low numbers of CD4+ and CD8+ T cells were found in HPV negative tumors. As well known, lymphatic tissue contains large number of lymphocytes that could bias the quantification of tumor-associated T lymphocytes, however, evaluation of tumor-associated T lymphocytes could be performed as tumor cells were clearly distinguishable from other cells (Supplementary Figure S1A) 4A. As shown in Supplementary Figure S1B, segmentation of the tissue was performed in tails that either contained or were in proximity of the tumor. Tumorous and non-tumorous areas were than identified (Supplementary Figure S1C). Thus, only infiltrating immune cells found either inside or in proximity of the tumor and not far from the tumor were scored ('tumor' and 'stroma') (Supplementary Figure S1D).

Also, the number of CD4+Foxp3+ cells was significantly higher in the tumor compartment of HPV-positive compared HPV-negative tumors (p=0.03). Interestingly, we also found low numbers of CD8+Foxp3+ cells both in the stromal and tumor compartment (median of cells/mm^2 1.1 and 1.2, respectively) (representative figure of the staining is shown in Figure 1E). Also, their numbers did not differ between HPV-negative and HPV-positive tumors.

Correlation between immune and clinical variables

In order to explore associations between all variables included in this study and their correlation with clinical outcome, we computed Spearman correlations between clinical variables (HPV status, T classification, gender, smoking history and treatment) and immune variables (all the staining performed in this study), displaying results as a heat map in Figure 3A. As expected, the number of CD4+ and CD8+ T cells were correlated with each other both in the stroma and in the tumor (r: 0.70 in the stroma and r: 0.61 in the tumor).

Interestingly, we also found that the number of double positive in the stroma CD68+CD163+, commonly identified as pro-tumor macrophages [34], was negatively correlated with expression

Table 2. Expression of HLA-I, HLA-II, PD-L1 and PD1 in tumor, tumor margins and stroma in view of HPV status. Quantification of immunohistochemical staining scored in the tumor, on the margins and in the surrounding stroma. Tot: total number of patients, Loss: loss of expression, Down: downregulation of expression, Pos: positive expression, Neg: negative expression, T: tumor, S: stroma, M: tumor margins.

| Staining	Scoring	Entire OPSCCs cohort		HPV negative OPSCCs		HPV positive OPSCCs		
		No. of patients	%	No. of patients	%	No. of patients	%	p value▲
HLA-HC10 T	Tot	141		82		59		0.19
	Loss	12	8.5	4	4.9	8	13.6	
	Down	95	67.4	57	69.5	38	64.4	
	Pos	34	24.1	21	25.6	13	22.0	
HLA-HCA2 T	Tot	138						0.16
	Loss	24	17.4	9	11.5	15	25.0	
	Down	75	54.4	46	59.0	29	48.3	
	Pos	39	28.3	23	29.5	16	26.7	
B2M T	Tot	143		82		60		0.08
	Neg	24	16.8	10	12.2	14	23.3	
	Pos	119	83.2	72	87.8	46	76.7	
HLA-DRA T	Tot	142		82		60		**0.03**
	Neg	122	85.9	75	91.5	47	78.3	
	Pos	20	14.1	7	8.5	13	21.7	
HLA-DR/DP/DQ T	Tot	141		82		59		0.06
	Neg	96	68.1	61	74.4	35	59.3	
	Pos	45	31.9	21	25.6	24	40.7	
HLA-DRA S	Tot	141		81		60		0.12
	Neg	60	42.6	39	48.2	21	35.0	
	Pos	81	57.5	42	51.9	39	65.0	
HLA-DR/DP/DQ S	Tot	141		82		59		0.29
	Neg	30	21.3	20	24.4	10	17.0	
	Pos	111	78.7	62	75.6	49	83.1	
PD-L1 T	Tot	141		81		60		0.22
	Neg	106	75.2	64	79.0	42	70.0	
	Pos	35	24.8	17	21.0	18	30.0	
PD-L1 M	Tot	142		82		60		**0.05**
	Neg	117	82.4	72	87.8	45	75.0	
	Pos	25	17.6	10	12.2	15	25.0	
PD-L1 S	Tot	141		82		59		**0.02**
	Neg	101	71.6	65	79.3	36	61.0	
	Pos	40	28.4	17	20.7	23	39.0	
PD1 S	Tot	142		82		60		0.09
	Neg	64	45.1	42	51.2	22	36.7	
	Pos	78	54.9	40	48.8	38	63.3	
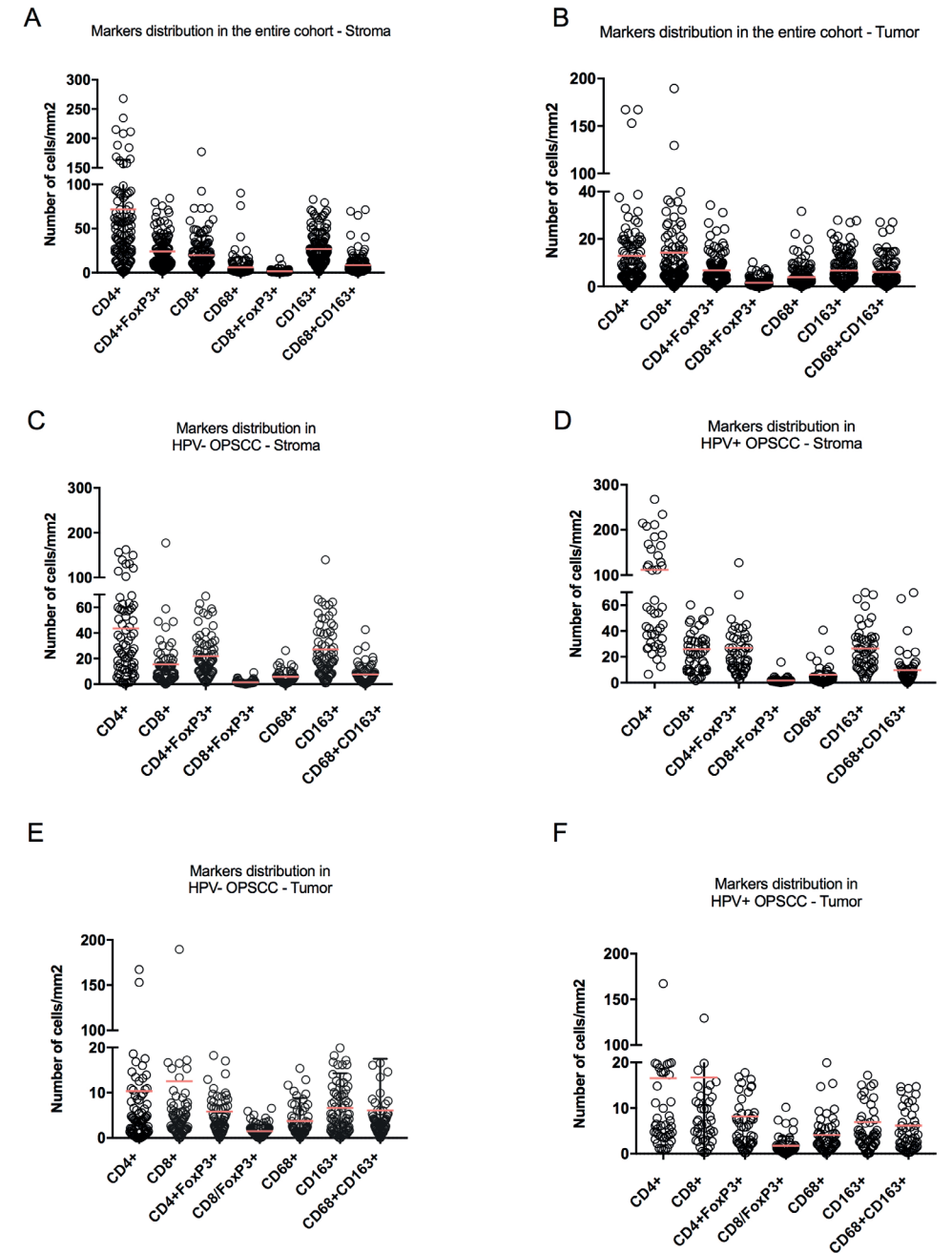
Figure 2. Distribution of myeloid cells and lymphocytes in view of HPV status. Quantification of the multiplex immunofluorescence staining scored in the tumor and in the surrounding stroma in the entire cohort (A-B) and in HPV- negative (C-D) and positive (E-F) tumors separately.
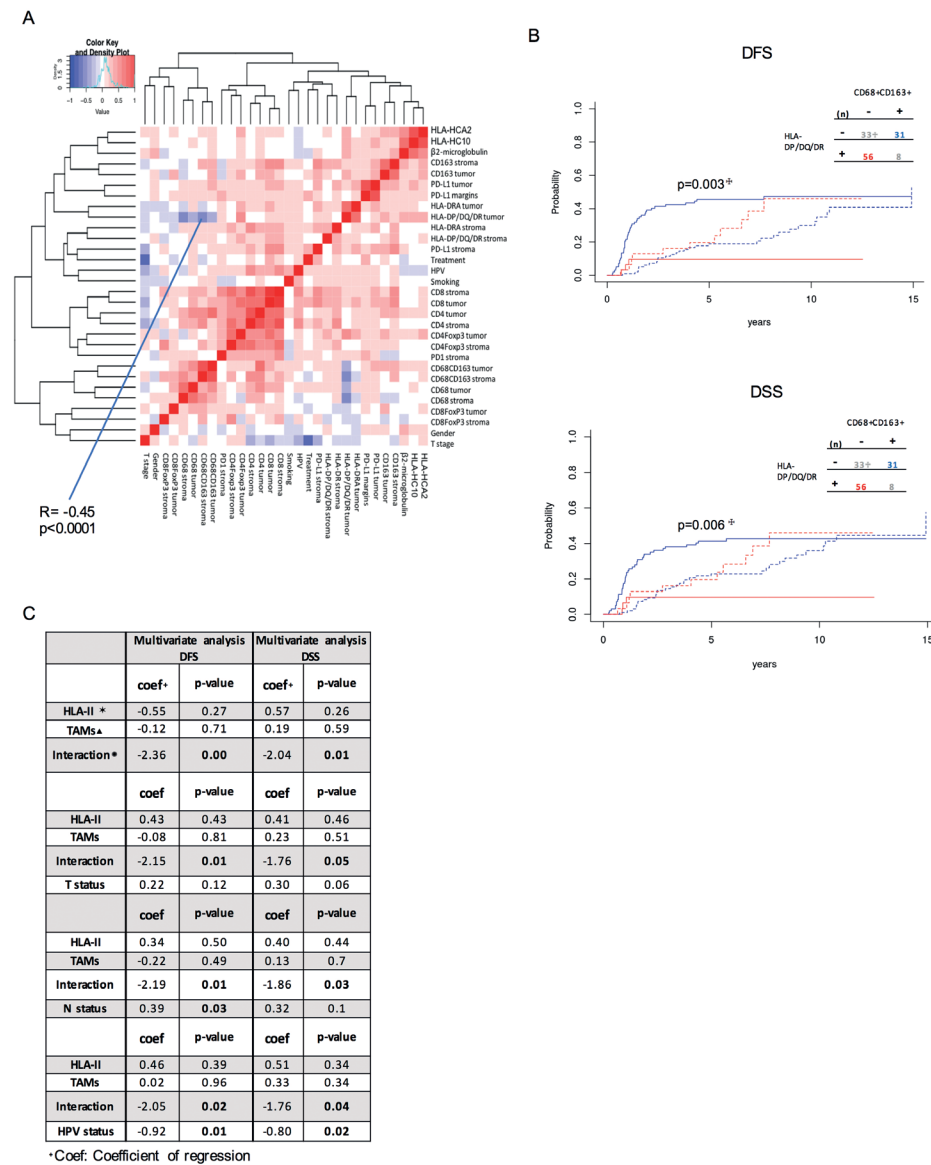
A

R= -0.45
p<0.0001

B

C

	Multivariate analysis DFS		Multivariate analysis DSS	
	coef⁺	p-value	coef⁺	p-value
HLA-II *	-0.55	0.27	0.57	0.26
TAMs ▲	-0.12	0.71	0.19	0.59
Interaction ●	-2.36	**0.00**	-2.04	**0.01**
	coef	p-value	coef	p-value
HLA-II	0.43	0.43	0.41	0.46
TAMs	-0.08	0.81	0.23	0.51
Interaction	-2.15	**0.01**	-1.76	**0.05**
T status	0.22	0.12	0.30	0.06
	coef	p-value	coef	p-value
HLA-II	0.34	0.50	0.40	0.44
TAMs	-0.22	0.49	0.13	0.7
Interaction	-2.19	**0.01**	-1.86	**0.03**
N status	0.39	**0.03**	0.32	0.1
	coef	p-value	coef	p-value
HLA-II	0.46	0.39	0.51	0.34
TAMs	0.02	0.96	0.33	0.34
Interaction	-2.05	**0.02**	-1.76	**0.04**
HPV status	-0.92	**0.01**	-0.80	**0.02**

⁺Coef: Coefficient of regression

Figure 3. HLA-DP/DQ/DR expression in inversely correlated with CD68+CD163 numbers and the interaction is a strong predictor of survival. A) Heat map shows the degree of correlation (Spearman) between the clinical and immunological variables included in the study. B) Cumulative incidence analysis for DFS and DSS of patients with high HLA-DP/DQ/DR expression on tumor cells and low numbers of CD68+CD163+ TAMs in the surrounding stroma (red) or the other way around (blue). ‡P value is calculated between straight lines. Segmented lines represent competing risks. †(n) represents the number of patients. C) Multivariate analyses show the predictive power of the interaction variable for DFS and DSS. ●Interaction between the HLA-DP/DQ/DR and CD68+CD163+ variables. *HLA-II = HLA-DP/DQ/DR on tumor cells, ▲TAMs = CD68+CD163+ numbers in tumor-associated stroma, Interaction = interaction variable between HLA-II and TAMs. Competing risks and treatment stratification are included in the analysis.

of HLA-DP/DQ/DR on tumor cells (Spearman r: -0.45 and p<0.0001). The median value of the number of CD68+CD163+ cells was used to identify samples with high or low number of CD68+CD163+ cells in the stromal compartment. As shown in Figure 3B, patients with high HLA-DP/DQ/DR expression on tumor cells and low numbers of CD68+CD163+ TAMs in the surrounding stroma were strongly associated with a significant decreased in cumulative incidence for DFS and DSS (p= 0.003 and p=0.006, respectively). Importantly, equal expression of both markers (low-low or high-high) did not significantly improved DFS (Supplementary Figure S2).

We then performed a number of multivariate analyses using four of the most important clinical prognostic factors for OPSCC, HPV status, tumor and lymph node status, together with the HLA-DP/DQ/DR, TAMs and the new variable generated by their interaction (Figure 3C). Interestingly, we found that the interaction between these two immunological variables was stronger than T status and N status in predicting DFS and DSS, however, HPV status remained the most significant predictive factor.

Indeed, in multivariate regression model including clinical variables (HPV status, gender, T classification, smoking history and treatment) only, HPV status showed the strongest effect on DFS and DSS (p<0.001 for both) (Supplementary Table S4). Patients with HPV-positive tumors were associated with significantly less disease-specific deaths, loco-regional recurrence or distant metastasis compared to HPV-negative tumors (Supplementary Figure S3). Importantly, we also found that treatment (radiotherapy alone or chemo-radiotherapy), which did not differ between HPV-negative and positive tumors, significantly affected both DFS and DSS (p=0.01 and p=0.04 respectively). Patients receiving chemo-radiotherapy performed better compared to patients receiving radiotherapy alone (Supplementary Figure S2). Therefore, here we used a treatment-based stratification model for multivariate COX regression analysis in order include treatment variable in our analysis but preventing it to bias the results.

Prognostic Importance of Clinical and Immunological Variables

Univariate analysis of all the variables included in this study was performed to find indicative biomarkers with an effect on OS, DFS and DSS and subsequently tested for statistical power in multivariate analyses. As expected patients with HPV-positive tumor were associated with significantly longer OS, DFS and DSS (Table 3). Also, female patients were associated with longer OS, and DFS survival. Patients with higher N status classification were associated with significantly shorter OS, DFS and DSS (p=0.012, HR=3.27, p= 0.023, HR=4.61 and p=0.020, HR=3.15, respectively).

High expression of HLA-DP/DQ/DR and HLA-DRA in tumor cells was correlated with significantly longer OS (p=0.026, HR= 0.58 and p<0.01, HR=0.45, respectively) and DFS (p=0.009, HR= 0.33 and p=0.05, HR=0.55, respectively). Furthermore, HLA-DP/DQ/DR expression in tumor cells was also correlated with significantly longer DFS (p=0.021, HR=0.37). Surprisingly, none of the molecules of the HLA-I complex were associated with OS, DFS or DSS. PD-L1 expression scored in the stromal compartment was associated with significantly longer

OS, DFS and DSS (p=0.025, HR=0.48; p=0.056, HR=0.46 and p=0.04, HR=0.61, respectively). Furthermore, PD-1 expression in stromal cells was associated with significantly longer DFS (p=0.033, HR=0.51). Numbers of CD4+ and CD4+FoxP3+ T lymphocytes in the stromal compartment were associated with significantly longer OS (p=0.017, HR=0.99 and p=0.016, HR=0.97, respectively). Also, numbers of CD8+T lymphocytes were associated with significantly longer OS (p=0.036, HR=0.98).

Interestingly, we found that increased numbers of CD68+CD163+ TAMs in the tumor compartment was significantly correlated with reduced OS, DFS and DSS (p= 0.036, HR=1.02, p= 0.009, HR=1.04 and p=0.002, HR=1.03, respectively). Furthermore, high numbers of CD68+CD163+ TAMs in the stromal compartment was associated with shorter DSS (p=0.040, HR=1.01).

To test the statistical power of the variables in multivariate analysis we fit a Cox proportional hazards regression model including variables with p<0.1 from univariate analysis. As shown in in Table 3, statistical power of HLA-DP/DQ/DR expression in tumor cells was validated in multivariate analysis as it was significantly associated with longer DFS and DSS survival (p=0.029, HR=0.39 and p=0.015, HR=0.36, respectively), but not for OS. Furthermore, high expression of HLA-DRA and high numbers of CD4+FoxP3+ T lymphocytes in the stromal compartment was associated with significantly longer OS (p=0.003, HR=0.48 and p=0.024, HR=0.97). As expected HPV status was an independent prognostic factor for OS, DFS and DSS. These data suggest that expression of HLA-DP/DQ/DR, HLA-DRA and CD4+FoxP+ T lymphocytes are independent prognostic markers of survival of OPSCC patients.

Ultimately, we performed an additional multivariate regression model using subdistribution functions in the competing risks and treatment stratified model including immunological and clinical parameters. This analysis aimed to explore the complex network of interactions among immune components of the tumor microenvironment of OPSCC. In this model, no pre-selection based on univariate analysis was performed, instead, all immunological and clinical variables were included. However, due to the very large number of clinical- and immune variables and different methodology of scoring, immunohistochemical data (HLA-HCA2, HLA-HC10, HLA-DRA, HLA-DP/DQ/DR, PD-L1, PD1) were analyzed separately from the immunofluorescence data (CD4, CD8, FoxP3, CD68, CD163). As shown in Table 4, we confirmed that high expression of HLA-DP/DQ/DR in tumor cells was correlated with significantly longer DFS, DSS and OS (p=0.03, HR=0.39; p=0.02, HR=0.36 and p=0.03, HR=0.59, respectively. Furthermore, higher PD-L1 expression in the tumor-stroma borders or stromal compartment was associated with significantly longer OS (p= 0.04, HR=0.55 and p=0.04, HR=0.60, respectively). Additionally, we showed that high numbers of CD68+CD163+ TAMs in the tumor-surrounding stroma was significantly associated with shorter DFS, DSS and OS (p=0.04, HR=1.03; p=0.01 HR=1.04 and p=0.00, HR=1.02, respectively). Finally, we found that numbers of CD8+FoxP3+ T lymphocytes found in tumor compartment were associated with significantly shorter DFS (p=0.04. HR=1.39). Importantly, key clinical variables, including HPV status and N classification were significant only in relation to OS and not in DFS and DSS, suggesting that their statistical power is reduced when multiple immune components of the tumor microenvironment are taken in account in the analysis.

In conclusion, these data suggest that HLA-DP/DQ/DR, CD163+CD68+ TAMs, PD-L1 and CD8+FoxP3+ T lymphocytes are independent prognostic markers of survival of OPSCC patients in our immunology-based multivariate analysis.

DISCUSSION

The tumor microenvironment is a key contributor to the development and progression of many tumors [35]. In the last decade, implementation of scoring systems for immunological biomarkers found in the tumor or in the surrounding stroma has become a key tool to help to predict prognosis and response to therapy in many cancer types [36]. In OPSCC tumors, HPV status is one of the key prognostic variable for survival, however, new and stronger predictors of survival might be identified exploring the interaction between immune cells and tumor cells in the tumor microenvironment. Previous studies investigated the role of the microenvironment in head and neck tumors with contradictory results [10]. Here, we obtained a comprehensive 'immune signature' of HPV-negative and HPV-positive OPSCCs and we found new potential prognostic markers for survival of OPSCC patients. More specifically, expression of HLA-DP/DQ/DR on tumor cells, the numbers of CD68+CD163+ TAMs in the tumor-associated stroma and the numbers of CD8+FoxP+ cells in the tumor, were established in our multivariate analyses as independent prognostic markers of survival in oropharyngeal cancer.

The tumor immune microenvironment can either promote or suppress tumor growth depending on the number and phenotype of immune cells. Accessibility to tumor antigens also play a key role in the immune surveillance. Indeed, often, tumor cells downregulate expression of HLA-I, thus evading CD8+ T-mediated tumor-specific immune response [37]. Nevertheless, in contrast with previous studies where loss of HLA-I was more frequently found in HPV-negative OPSCC [38], we did not find differences in expression level of HLA-I between HPV-positive and HPV-negative tumors. However, the scoring system used in these studies was different from the one used in this study as 'fraction' and 'intensity' of the staining of the different HLA-I markers were not combined and were used as separate variables for univariate and multivariate analysis [38, 39]. In addition, in one of these studies only two subgroups were generated in both 'fraction' and 'intensity' of the staining [39]. Therefore, we think that results might be the consequence of different scoring systems and we believe that a standard method of scoring should be used.

Importantly, in our study we found an indirect correlation between the expression of the HLA-II molecule, HLA-DP/DQ/DR on tumor cells and the number of CD68+CD163+ TAMs in the tumor-associated stroma of OPSCC patients. Expression of HLA-II on epithelial cells has been described for colorectal cancer, ovarian cancer and melanoma, however, the role in head and neck cancer still needs to be elucidated [40-44]. Interestingly, recent studies in melanoma suggested that high expression of HLA-DR/DP/DQ in tumor cells is associated with improved response rates and clinical benefit of PD-1/PD-L1 targeted therapy [41]. We

Table 3. Univariate and multivariate analysis including immunological and clinical variables. Univariate analysis of immunological and clinical variables in relation to DFS, DSS and OS was performed. T: tumor, S: stroma, M: tumor margins. Treatment stratification was included in the analysis. Variables with p<0,1 in univariate analysis were included in multivariate analysis.

Univariate regression model - clinical and immunological variables

Variable	DFS HR	DFS p value	DFS 95%CI	DSS HR	DSS p value	DSS 95%CI	OS HR	OS p value	OS 95%CI
HPV (+ Vs -)	0,205	**0,002**	0,096 0,439	0,339	**0,009**	0,150 0,766	0,361	**0,000**	0,210 0,620
Gender (F Vs M)	0,471	**0,052**	0,220 1,007	0,472	0,082	0,202 1,101	0,435	**0,003**	0,249 0,760
Smoke (yes Vs non-smoker or stopped)	1,271	0,147	0,919 1,757	1,114	0,830	0,405 3,065	1,192	0,623	0,417 2,366
T class. (3-4 Vs 2-1)	1,185	0,379	0,812 1,730	1,677	0,361	0,366 2,086	1,946	0,087	0,907 4,176
N class (3-2 Vs 1-0)	3,400	0,060	0,950 12,164	4,615	0,023	1,232 17,286	3,279	0,012	1,262 7,161
HLA_B/C T	0,747	0,270	0,301 1,265	0,857	0,570	0,501 1,465	1,145	0,500	0,770 1,702
HLA_B/C T	0,942	0,800	0,868 1,501	1,209	0,410	0,768 1,901	1,165	0,370	0,836 1,623
B2M T	0,979	0,960	0,874 2,532	1,435	0,440	0,574 3,592	1,555	0,120	0,896 2,698
HLA-DRA T	0,272	0,080	0,080 0,894	0,301	0,110	0,070 1,294	0,564	0,068	0,306 1,042
HLA-DP/DQ/DR T	0,336	**0,009**	0,265 0,561	0,377	**0,021**	0,165 0,861	0,580	**0,026**	0,359 0,937
HLA-DRA S	0,554	**0,051**	0,473 0,975	0,690	0,240	0,373 1,275	0,459	**0,000**	0,298 0,707
HLA-DP/DQ/DR S	0,939	0,860	0,990 1,005	1,016	0,970	0,490 2,105	0,727	0,190	0,451 1,171
PD-L1 T	0,464	0,085	0,275 1,289	0,651	0,310	0,285 1,489	0,766	0,290	0,468 1,254
PD-L1 M	0,546	0,190	0,446 1,142	0,616	0,300	0,246 1,542	0,573	0,056	0,323 1,014
PD-L1 S	0,408	**0,025**	0,309 0,818	0,461	0,056	0,209 1,018	0,615	**0,044**	0,384 0,986
PD1 S	0,512	**0,033**	0,446 1,062	0,679	0,220	0,366 1,262	0,738	0,150	0,490 1,112
CD163+ S	0,989	0,190	0,974 1,005	0,989	0,200	0,972 1,006	0,994	0,350	0,983 1,006
CD4+ S	0,997	0,290	0,992 1,002	0,998	0,520	0,994 1,003	0,994	**0,017**	0,988 0,999
CD4+FoxP3+ S	0,982	0,200	0,955 1,010	0,987	0,330	0,960 1,014	0,976	**0,016**	0,957 0,995
CD68+ S	1,010	0,420	0,985 1,036	1,010	0,450	0,984 1,038	1,004	0,750	0,978 1,031
CD68+CD163+ S	1,014	0,110	0,997 1,031	1,018	**0,040**	1,001 1,035	1,005	0,650	0,985 1,025
CD8+ S	0,991	0,620	0,956 1,027	0,997	0,840	0,963 1,031	0,981	**0,036**	0,964 0,999
CD8+FoxP3+ S	0,974	0,730	0,840 1,129	0,983	0,820	0,846 1,142	0,918	0,200	0,805 1,046
CD163+ T	0,978	0,510	0,915 1,046	0,977	0,520	0,910 1,049	0,990	0,570	0,955 1,026
CD4+ T	1,017	**0,009**	1,004 1,030	1,020	**0,002**	1,007 1,033	1,001	0,920	0,975 1,029
CD4+FoxP3+ T	0,986	0,740	0,911 1,068	0,997	0,930	0,927 1,072	0,972	0,330	0,917 1,030
CD68+ T	1,030	0,370	0,966 1,100	1,036	0,300	0,969 1,107	1,021	0,470	0,964 1,082
CD68+CD163+ T	1,033	**0,002**	1,012 1,055	1,040	**0,009**	1,021 1,059	1,028	**0,036**	1,002 1,055
CD8+ T	1,008	0,180	0,996 1,021	1,010	0,079	0,999 1,022	0,999	0,940	0,982 1,017
CD8+FoxP3+ T	1,064	0,380	0,926 1,223	1,084	0,250	0,946 1,241	0,940	0,340	0,829 1,066

Table 3. (continued)

Multivariate regression model with competing risks and treatment stratification

Disease-free survival

Variable	HR	p value	95%CI
HPV (+ Vs -)	0,204	**0,004**	0,095 0,432
Gender (M Vs F)	1,042	0,835	0,920 1,207
N class (3-2 Vs 1-0)	1,21	**0,01**	1,020 7,264
HLA-DRA T	0,655	0,590	0,144 2,997
HLA-DP/DQ/DR T	0,390	**0,029**	0,167 0,908
HLA-DRA S	0,624	0,130	0,342 1,142
PD-L1 T	0,655	0,350	0,268 1,602
PD-L1 S	0,614	0,250	0,268 1,399
PD1 S	0,653	0,200	0,342 1,247
CD4+ T	1,012	0,260	0,991 1,034
CD68+CD163+ T	1,017	0,400	0,978 1,056

Disease-specific survival

Variable	HR	p value	95%CI
HPV (+ Vs -)	0,432	**0,016**	0,121 0,722
Gender (M Vs F)	0,700	0,350	0,520 1,289
N class (3-2 Vs 1-0)	3,231	0,056	1,120 7,365
HLA-DP/DQ/DR T	0,362	**0,015**	0,144 2,997
PD-L1 S	0,679	0,220	0,167 0,908
CD68+CD163+ S	1,014	0,120	0,996 1,032
CD4+ T	1,015	0,240	0,990 1,041
CD68+CD163+ T	1,016	0,500	0,971 1,063
CD8+ T	0,940	0,062	

Overall survival

Variable	HR	p value	95%CI
HPV (+ Vs -)	0,506	**0,015**	0,291 0,873
Gender (M Vs F)	0,819	0,430	0,610 1,349
N class (3-2 Vs 1-0)	7,053	**0,003**	1,120 12,225
T class. (3-4 Vs 2-1)	1,730	0,066	0,970 2,939
HLA-DRA T	1,155	0,700	0,562 2,359
HLA-DP/DQ/DR T	0,647	0,160	0,349 1,195
HLA-DRA S	0,487	**0,003**	0,301 0,787
PD-L1 M	0,680	0,240	0,360 1,308
PD-L1 S	0,921	0,780	0,543 1,639
CD4+ S	1,000	0,890	0,996 1,005
CD4+FoxP3+ S	0,974	**0,024**	0,952 0,996
CD8+ S	1,037	0,064	0,998 1,077
CD68+CD163+ T	1,030	0,083	0,996 1,065

Table 4. Subdistribution multivariate regression model. Multivariate analysis of immunological and clinical variables in relation to DFS, DSS and OS was performed. T: tumor, S: stroma, M: tumor margins. Treatment stratification was included in the analysis.

Multivariate regression model with competing risks and treatment stratification - IHC variables

Variable	Disease-free survival			Disease-specific survival			Overall survival		
	HR	p value	95%CI	HR	p value	95%CI	HR	p value	95%CI
HPV (+ Vs -)	0.397	0.110	0.128 1.228	0.580	0.290	0.210 1.602	0.420	**0.003**	0.235 0.750
Gender (F Vs M)	0.443	0.070	0.183 1.069	0.437	0.074	0.176 1.082	0.481	**0.016**	0.265 0.874
Smoke (yes Vs non-smoker or stopped)	2.688	0.110	0.799 9.044	1.734	0.470	0.387 7.764	1.483	0.450	0.531 4.141
T class. (3-4 Vs 2-1)	0.374	0.140	0.100 1.396	0.619	0.460	0.173 2.207	1.758	0.096	0.905 3.413
N class (3-2 Vs 1-0)	1.385	0.155	0.884 2.171	3.010	0.272	0.422 21.481	3.279	**0.012**	1.297 8.292
HLA_B/C T	0.54	0.16	0.232 1.264	0.46	0.10	0.185 1.155	1.02	0.92	0.680 1.530
HLA_B/C T	1.46	0.19	0.829 2.566	2.05	0.09	1.130 3.724	1.07	0.71	0.758 1.498
B2M T	1.22	0.72	0.419 3.552	1.39	0.59	0.424 4.576	1.35	0.28	0.781 2.341
HLA-DRA T	0.61	0.57	0.110 3.401	0.58	0.54	0.104 3.240	0.58	0,09	0.313 1.087
HLA-DP/DQ/DR T	0.39	**0.03**	0.168 0.895	0.36	**0.02**	0.159 0.821	0.59	**0.03**	0.359 0.959
HLA-DRA S	0.82	0.56	0.421 1.593	0.97	0.94	0.482 1.971	0.45	**0.00**	0.286 0.698
HLA-DP/DQ/DR S	1.51	0.30	0.696 3.268	1.47	0.36	0.638 3.404	0.79	0.35	0.479 1.295
PD-L1 T	0.61	0.47	0.164 2.293	0.94	0.93	0.243 3.655	0.75	0.27	0.450 1.254
PD-L1 M	1.12	0.89	0.235 5.301	0.92	0.93	0.175 4.889	0.55	**0.04**	0.301 0.994
PD-L1 S	0.53	0.20	0.202 1.408	0.47	0.17	0.161 1.390	0.60	**0.04**	0.370 0.977
PD1 S	0.92	0.83	0.413 2.035	1.20	0.64	0.550 2.626	0.76	0,21	0.496 1.158

Multivariate regression model with competing risks and treatment stratification - IF variables

Variable	Disease-free survival			Disease-specific survival			Overall survival		
	HR	p value	95%CI	HR	p value	95%CI	HR	p value	95%CI
HPV (+ Vs -)	0.257	0.009	0.060 1.263	0.456	0.350	0.089 2.345	0.294	**0.007**	0.121 0.714
Gender (F Vs M)	0.523	0.301	0.153 1.796	0.705	0.620	0.178 2.794	0.288	**0.008**	0.115 0.719
Smoke (yes Vs non-smoker or stopped)	0.797	0.770	0.177 3.591	1.368	0.720	0.254 7.377	0.593	0.400	0.177 1.984
T class. (3-4 Vs 2-1)	1.644	0.570	0.300 9.007	2.253	0.390	0.347 14.624	2.686	**0.040**	1.046 6.897

Table 4. (continued)

Variable	Disease-free survival			Disease-specific survival			Overall survival		
	HR	p value	95%CI	HR	p value	95%CI	HR	p value	95%CI
N class (3-2 Vs 1-0)	9.974	**0.035**	1.177 84.568	12.222	0.056	0.938 159.168	7.053	**0.003**	1.937 25.678
CD163+ S	0.98	0.17	0.974 1.005	0.97	0.12	0.933 1.012	0.997	0.66	0.99 1.012
CD4+ S	1.00	0.48	0.992 1.002	1.00	0.53	0.994 1.012	0.998	0.62	0.932 1.006
CD4+FoxP3+ S	1.00	0.89	0.955 1.010	0.99	0.76	0.934 1.048	0.967	0.07	0.952 1.003
CD68+ S	0.98	0.60	0.985 1.036	0.97	0.44	0.893 1.047	0.987	0.46	1.01 1.023
CD68+CD163+ S	1.03	**0.04**	0.997 1.031	1.04	**0.01**	1.008 1.073	1.029	**0.00**	0.952 1.003
CD8+ S	0.94	0.09	0.956 1.027	0.96	0.30	0.875 1.003	0.996	0.85	0.787 1.042
CD8+FoxP3+ S	0.80	0.14	0.840 1.129	0.80	0.28	0.529 1.169	0.961	0.69	0.905 1.173
CD163+ T	0.93	0.11	0.915 1.046	0.91	**0.02**	0.835 0.966	0.954	0.08	0.984 1.005
CD4+ T	1.05	0.06	1.004 1.030	1.05	0.11	0.988 1.119	1.016	0.33	0.974 1.05
CD4+FoxP3+ T	0.91	0.21	0.911 1.068	0.93	0.37	0.762 1.059	1.046	0.22	0.976 1.123
CD68+ T	1.07	0.54	0.966 1.100	1.07	0.55	0.844 1.397	1.079	0.14	0.949 1.193
CD68+CD163+ T	0.96	0.44	1.012 1.055	0.99	0.79	0.898 1.046	0.993	0.76	0.979 1.039
CD8+ T	1.05	0.06	0.996 1.021	1.04	0.16	0.977 1.109	1.007	0.64	0.942 1.035
CD8+FoxP3+ T	1.39	0.09	0.926 1.223	1.33	0.27	0.803 2.382	1.135	0.18	1.259 1.368

therefore think that our study could optimize the selection of patients that would benefit from immunotherapy treatment. For instance, Nivolumab is an inhibitor of the immunocheckpoint PD-1 expressed on exhausted T cells and is currently used to treat metastatic OPSCC patients. Based on our results, we speculate that patients with high HLA-II expression on tumor cells and low numbers of TAMs would represent a selective population of the patients that would benefit from Nivolumab treatment. Indeed, we showed that both high HLA-DP/DQ/DR expression and low numbers of TAMs are required to observe a beneficial effect in survival, as equal expression of both markers did not significantly affect DFS or DSS. Also, infiltration of tumor-associated macrophages are known to affect treatment responses in several tumor types [45] and pre-clinical and clinical studies using anti-CSFR1 Ab (macrophage Colony Stimulating Factor Receptor 1) have been performed [46-48]. We, therefore, speculate that immunotherapy treatment targeting tumor-associated macrophages, might be beneficial especially for patients with high expression of HLA-DP/DQ/DR on tumor cells.

One of the possible reasons why patients with high HLA-DP/DQ/DR and low TAMs numbers are associated with improved survival is that HLA-DP/DQ/DR expression on tumor cells leads to recruitment of CD4+ T-cells for Ag recognition, which leads to increased IFN-γ production in the tumor microenvironment. In turn, IFN-γ is thought to reduce the generation of TAMs [49], therefore inhibiting the macrophage-mediated tumor progression. It is known that expression of HLA-II is increased by CD8- and CD4-mediated IFN-γ production [50, 51]. Importantly, in this study, we showed that HPV-positive tumors have higher numbers of CD4+ and CD8+ cells both in the tumor and in the surrounding stroma, which might explain the higher expression of HLA-II, both HLA-DRA and HLA-DP/DQ/DR, on tumor cells compared to HPV-negative tumors. As previously observed [52], a positive correlation between PD-L1 expression on immune cells and longer OS was found.

Finally, in our study, we identified a relatively new subset of double positive CD8+FoxP3+ cells in OPSCC. Interestingly, recent works identified CD8+FoxP3+ T cells as a potent immunosuppressive population of T-cells in vitro and in vivo [53-55], however, their role in cancer is poorly described and nothing is known in relation to OPSCC. For the first time in oropharyngeal cancer, we showed that despite low in numbers, increased infiltration of CD8+FoxP3+ T-cells in the tumor compartment is an independent prognostic factor for shorter DFS in our comprehensive multivariate analysis. As the role of double positive CD8+FoxP3+ T-cells is poorly understood in cancer [55], we think additional functional assays should be performed in order to elucidate the possible immunosuppressive effect of this specific subset of cells and find new cell-specific pathways targetable with immunotherapy.

For future studies, we aim to conduct a similar multi-parameters analysis in a bigger cohort of patients to further support the relevance of our findings. Studying the spatial interaction between markers as proximity between cells will also be of additional value [56]. In addition, differences in phenotype and functionality of immune cells found within or in proximity of the tumor and immune cells found far from the tumor could be explored. Also, functional and single cell sequencing analysis could be performed in order to explore the potential immunosuppressive phenotype of the newly described CD8+FoxP3+ T cells.

In conclusion, we think that similar comprehensive multivariate analysis should be performed in this and other cancer types to assess the potential of survival prediction of various immunological and non-immunological factors to mimic their 3-dimentional context in the tumor microenvironment.

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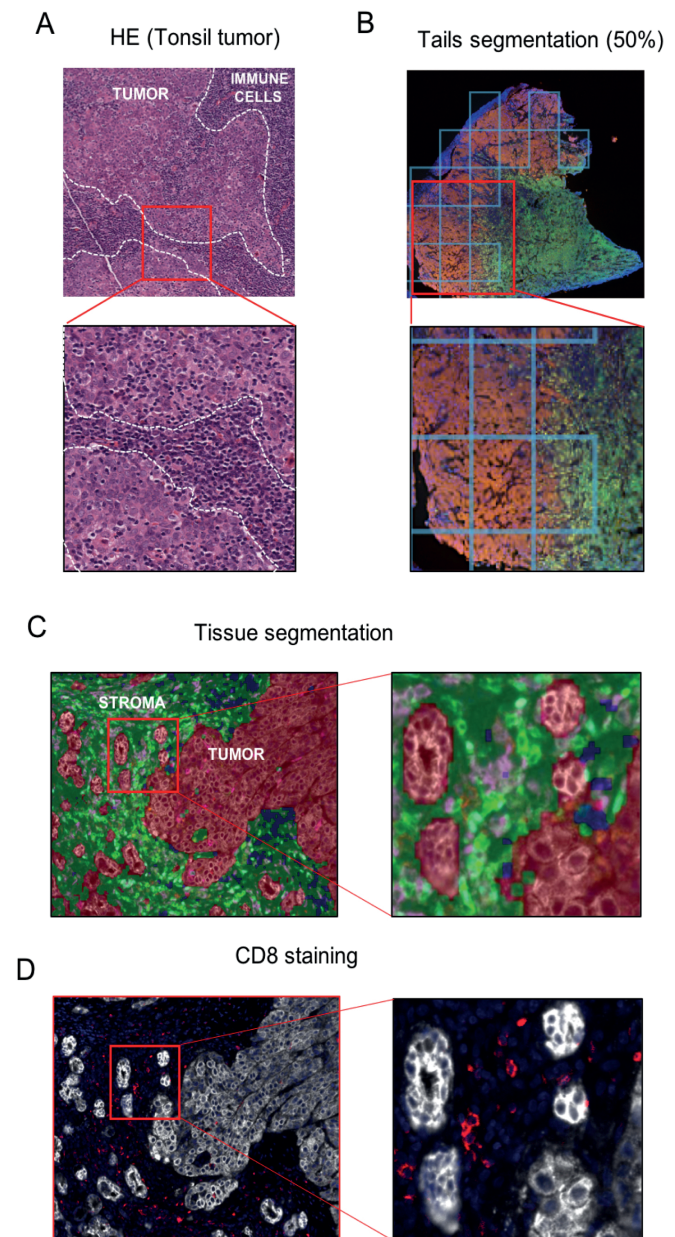
SUPPLEMENTARY INFORMATION

Figure S1. Identification and quantification of tumor-associated T lymphocytes in tonsil. A) Hematoxylin-eosin (HE) staining staning of tonsillar tumor specimen. Tumor area was delimited by the segmented line. B) Tails segmentation of tumor-containing area. 50% of random tails were used for the analysis. C) Pre-selected tails were segmented into 'tumor' or 'stroma' area. D) CD8+ lymphocytes found within or in proximity of tumor area were scored as tumor-associated lymphocytes or stroma-associated lymphocytes, respectively.
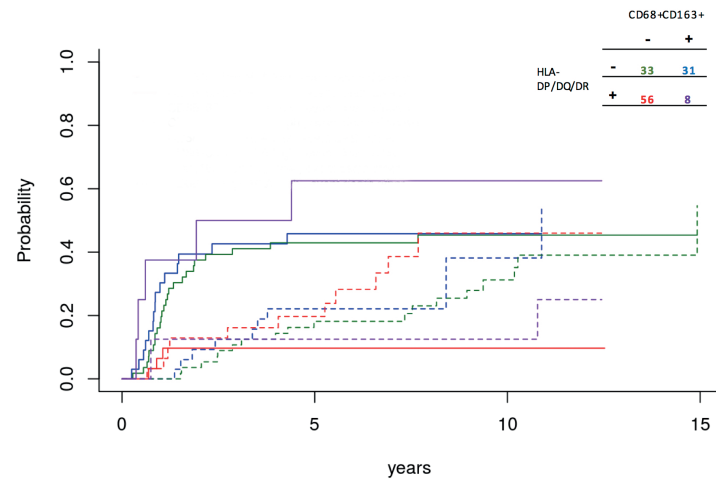
Figure S2. Effect of HLA-DP/DQ/DR expression and numbers of CD68+CD163+ TAMs on survival. Cumulative incidence analysis was performed with HLA-DP/DQ/DR expression and numbers of TAMs. Importantly, only patients with high HLA-DP/DQ/DR expression and low number of TAMs showed significantly improved DFS and DSS.

Supplementary Table 1. List of all the antibodies used in this study.

Antibodies used for immunohistochemistry	Clone	Dilution	Supplier
HLA-I	HC10	1:20000	Noric-MUbio
HLA-I	HCA2	1:5000	Noric-MUbio
β2-microglobulin	A0072	1:12000	Dako. Agilent Technologies
HLA II - (DR/DP/DQ)	CR3/43	1:1000	Dako. Agilent Technologies
HLA-II (DRA)	TAL.1B5	1:500	Dako. Agilent Technologies
PD1	NAT105	1:400	AbCam
PD-L1	E1L3N	1:200	Cell Signaling Technologies
p53	DO-7	1:7000	Dako. Agilent Technologies
p16	JC8	1:800	Santa Cruz Biotechnology

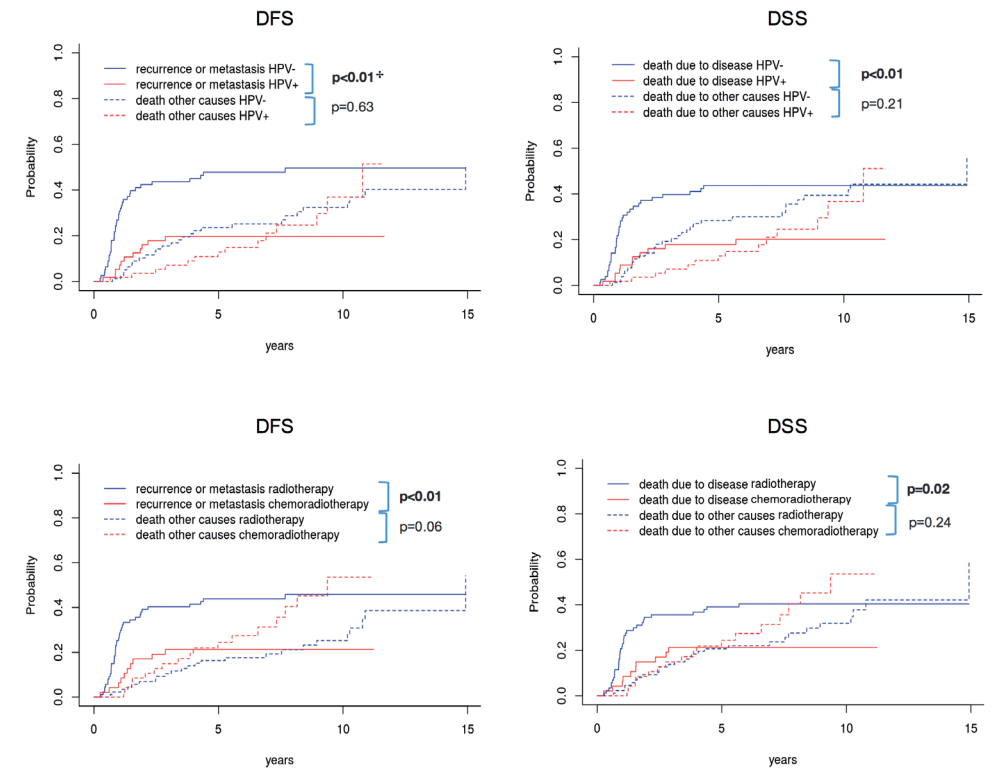| Antibodies used for immunofluorescence			
CD4	Ep204	1:100	PerkinElmer
CD8	C8/144B	1:2000	PerkinElmer
CD68	KP1	1:3000	PerkinElmer
FoxP3	236A/E7	1:1000	Abcam
CD163	10D6	1:1000	Leica Biosystems
panCK	AE1/AE3	1:2000	Thermo Fisher Scientific

Figure S3. Impact of HPV status and treatment on disease free survival and disease progression. 2A) Effect of HPV status on the cumulative incidence or loco-regional recurrence, metastasis (DFS) or disease-specific death (DSS). 2B) Effect of different treatment regimens in the cumulative incidence or loco-regional recurrence, metastasis (DFS) or disease-specific death (DSS). ✚ P value is calculated between straight lines or segmented lines.

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Supplementary Table 2. Quantification of the number of myeloid cells and lymphocytes in tumor, tumor margins and stroma in view of HPV status. Quantification of the multiplex immunofluorescence staining scored in the tumor and in the surrounding stroma. Median represents the median of number of cells/mm².

Stroma	CD163+	CD4+	CD4+FoxP3+	CD68+	CD68+CD163+	CD8+	CD8+FoxP3+
Patients	134	133	134	129	129	133	127
All patients median/SD*	20.7/21.7	44.6/91.6	17.8/20.1	2.9/11.3	4.6/11.9	13.3/22.3	1.1/1.8
HPV neg. Median/SD	19.6/24.3	29.5/41.3	17.5/17.7	3.2/10.9	4.6/10.4	10.6/23.0	1.0/1.4
HPV pos. Median/SD	23.8/17.5	71.6/123.6	21.6/22.9	2.6/11.9	5.1/13.9	22.7/19.7	1.3/2.2
HPV neg/HPV pos p value*	0.28	<0.0001	0.09	0.7	0.6	<0.01	0.5

Tumor	CD163+	CD4+	CD4+FoxP3+	CD68+	CD68+CD163+	CD8+	CD8+FoxP3+
Patients	134	133	134	129	129	133	127
All patients median/SD	4.1/7.4	5.3/25.4	3.8/8.6	2.2/4.6	3.1/9.9	5.4/30.6	1.2/1.7
HPV neg. Median/SD	4.2/7.7	4.0/25.6	3.1/8.9	2.1/5.0	2.9/11.4	4.0/36.0	1.2/1.4
HPV pos. Median/SD	4.1/7.0	7.8/25.1	6.0/8.1	2.9/4.0	3.5/7.2	10.0/20.8	1.2/2.1
HPV neg/HPV pos p value*	0.65	<0.01	0.03	0.43	0.16	<0.01	0.31