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Original article

Influence of tumor-associated macrophages and HLA class I expression according to HPV status in head and neck cancer patients receiving chemo/bioradiotherapy

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ABSTRACT

Background and purpose: To investigate the prognostic value of tumor-associated macrophages (TAM) and HLA class I expression according to HPV status in patients with head and neck squamous cell carcinoma treated with definitive radiotherapy combining cisplatin (CRT) or cetuximab (BRT).

Material and methods: Ninety-five patients were enrolled. The density of CD68+ cells and CD68+ CD163+ cells (further referred as M2) in the intraepithelial and the stromal compartments, respectively, as well as HLA class I expression in tumor cells, were evaluated semi-quantitatively. Correlations between biomarker expression and treatment outcomes were analyzed.

Results: Multivariate analysis showed that the intraepithelial macrophage density (IEMD) was prognostic for favorable progression-free survival (PFS) and there was a non-significant trend for improved overall survival (OS). HLA class I down-regulation was not an independent prognostic factor. Subgroup analysis showed that in p16+ population, patients with high IEMD had improved 5-year PFS vs. patients with low IEMD (81.2% vs. 25.0%, $p < 0.001$), while in p16− population, no difference was observed. Similarly, when stratified by primary tumor site, IEMD showed prognostic value in oropharyngeal cancer patients (OPC) but not non-OPC patients. Five-year PFS of patients with low stromal M2 macrophage density treated with CRT was significantly improved vs. those with BRT (54.5% vs. 36.1%, $p = 0.03$), while in tumors with high M2, there was no significant difference (50.3% vs. 42.9%, $p = 0.67$).

Conclusions: The prognostic role of TAM phenotype and distribution depends on HPV status and might predict treatment response. They prompt further validation in prospective studies.

Head and neck squamous cell carcinoma (HNSCC) is among the most frequent human malignancies worldwide. HNSCC patients have benefited from recent advances in radiotherapy, chemotherapy, immunotherapy and surgical techniques [1–3]. Despite considerable progress in our understanding of the pathogenesis, genetics, and pathology of head and neck cancers, the prediction of the clinical outcome for individual cases remains difficult. The incidence of human papillomavirus (HPV)-associated head and neck cancers is rising rapidly and HPV status is now the strongest prognostic factor [4,5]. In recent years, with a renaissance in immune therapies for cancer, substantial attempts have been made in identifying new immuno-biological markers in combination with HPV to develop more accurate risk stratification for HNSCC patients.

The immune system is a major player in the cancer cell/tumor micro-environment (TME) crosstalk. Macrophages, which constitute a major component of the immune-reaction in tumors, are derived from monocytes and exhibit two polarization states in response to different micro-environmental signals [6]. CD4+ T lymphocytes helper 1 (Th1) cells produce interferon-gamma, interleukin-2, and tumor necrosis factor-beta which activate M1 macrophages and are responsible for cell-mediated immunity

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and phagocyte-dependent protective responses as well as tumor cytotoxicity. On the other hand, Th2 cells produce various interleukins that alternatively activate M2 macrophages which contribute to tumor progression and invasion [7]. The in situ identification of macrophage subtypes is possible, through specific markers. CD68 is commonly used as a general macrophage marker for both M1 and M2 subtypes [8]; CD68 corresponds to a lysosomal antigen and presents a cytoplasmic distribution. CD163 is commonly used as a first-line marker for M2 macrophages [9]; it corresponds to a membrane protein belonging to the scavenger receptor family. High density of CD163-positive macrophages in tumor stroma has been shown to be significantly correlated with a poor outcome in patients with oral squamous cell carcinoma (OSCC) [10,11]. So far, the prognostic and even predictive value of macrophages in HNSCC patients treated with definitive chemo-radiotherapy (CRT) or bio-radiotherapy (BRT) with cetuximab remains unclear. In addition, the relationship between HPV status and macrophages in these patients has not been sufficiently analyzed.

Tumor escape remains a major obstacle for cancer treatment. One mechanism of the immune escape by cancer development is the downregulation of tumor antigen processing and presentation because of abnormalities in the human leukocyte antigen (HLA) class I antigen processing machinery [12]. HLA class I molecules are the major antigen-presenting molecule for cytotoxic T lymphocytes and therefore play a pivotal role in the recognition and elimination of abnormal cells, including tumor cells [13]. Downregulation of HLA class I molecules is frequently observed in many tumor types including HNSCC and has been reported to be a poor prognostic marker in some studies [14,15]. Previous study findings indicated that tumor HLA-I expression is strongly correlated with the distribution of macrophages in tumor tissue [16].

In an effort to define the role of macrophages and HLA class I molecules in HNSCC, the goals of our study were (i) to evaluate the characterization of HLA class I molecules and tumor-associated macrophages (TAMs) in patients with HNSCC; (ii) to investigate the relation between TAMs and HLA class 1 expression and the outcome of patients with HNSCC treated with concurrent CRT with cisplatin or BRT with cetuximab, according to their HPV status.

Patients and methods

Patients and tissues

Patients were selected from the former study cohort of 265 patients with histologically confirmed HNSCC, diagnosed between 2006 and 2012 in our institute (CRT: $n = 194$, BRT: $n = 71$) [17,18]. Patients with stage III–IVb disease according to American Joint Committee on Cancer (AJCC)/International Union for Cancer Control (UICC) TNM classification 2010, received total radiation dose of 70 Gy, $\geq$2 cycles of concurrent cisplatin or $\geq$3 cycles of concurrent cetuximab were selected from the initial cohort. A matched control subset of patients treated with CRT vs. BRT (2:1 ratio) was then identified. Matching criteria were T stage and N stage. If the matching criteria could not be fulfilled, the case was excluded in the study. Thus 120 eligible patients were included for test of biomarkers. Finally, 95 patients who had evaluable immunohistochemical (IHC) results were included in the analysis. Supplementary Fig. 1 is the diagram of patient selection for the study.

Institutional research ethics board approval was obtained. Written informed consent was obtained from included patients. All patients received either one of the following two treatments: definitive radiotherapy (RT) concomitantly with cisplatin (100 mg/m^2 every 3 weeks on days 1, 22, and 43) or cetuximab (initial loading dose of 400 mg/m^2 one week prior to RT, followed by weekly injection at 250 mg/m^2 during RT). Patients received either three-dimensional conformal radiotherapy (3D-CRT) or intensity-modulated radiotherapy (IMRT). External beam definitive RT was delivered with a total dose of 70 Gy to the gross tumor volume (GTV) in 35 fractions (range 30–35 fractions) at 5 fractions per week, with median overall treatment time of 49 days (range 39–70 days). A dose of 60 Gy and 50–54 Gy were delivered to the intermediate- and low-risk clinical target volume (CTV). The CTVs were each expanded using 3–5 mm margins to generate their respective planning target volumes (PTV). Patient assessments in follow-up were previously described [17].

Immunohistochemistry

IHC was performed on 3-μm thick sections from paraffin-embedded pretreatment biopsy material which had been fixed in either formalin ($N = 51$) or alcohol-formalin-acetic acid (AFA) ($N = 44$, patients treated before 2010). All pretreatment biopsy sections were reviewed by two experienced pathologists (OC, IG). HPV status was determined through p16 immunohistochemistry (CINtec P16 INK4A, Ventana, Tucson, AZ). Nuclear staining for p16 in more than 75% of cells was considered indicative of HPV positive status [19,20]; cytoplasmic only staining for p16 was considered indicative of HPV negative status.

Macrophage detection was achieved using double chromogenic staining performed on a Ventana Discovery Ultra platform with CD68 and CD163 monoclonal antibodies. First, CD163 staining was performed using a monoclonal mouse antibody (clone 10D6, Diagnostic BioSystems, Pleasanton, CA) at a dilution of 1:200, an UltraMAP HRP detection kit and diaminobenzidine (DAB) as a chromogen. After a denaturation step, CD68 staining was performed using a monoclonal mouse antibody (PG-M1, Dako, Glostrup, Denmark) at a dilution of 1:200, an UltraMAP HRP detection kit and Discovery purple as a chromogen. As a result, CD163+ cells were stained in DAB (brown) and CD68+ cells were stained in purple. Human lymph node and tonsil sections were used as external positive controls for CD68 and CD163, respectively. Negative controls were obtained by omitting the primary antibody. HLA class I detection was performed on a Ventana BenchMark Ultra platform, using a monoclonal mouse anti-pan-HLA Class I antibody (clone EMR8-5, D226-3, MBL International, Woburn, MA, USA) at a dilution of 1:1600, an UltraView detection kit and diaminobenzidine (DAB) as a chromogen. Human colon carcinoma tissue sections were used as positive control for HLA class I. Negative controls were obtained by omitting the primary antibody.

The level of CD163+ and CD68+ staining were scored semi-quantitatively in the stroma and inside epithelial tumor nests. In the stroma, the percentage of stromal area covered by positive cells was evaluated. CD68+ cell density was indicative of the total macrophage population while CD68+ CD163+ were identified as TAMs with M2 phenotype. For intraepithelial infiltration, a semi-quantitative scale was used (from 0 to 3+; where 0: absence of stained cells; 1+: isolated stained cells, 2+: small aggregates of stained cells; 3+: large aggregates or sheets of stained cells) (Fig. 1A, B).

HLA class I membrane staining in tumor cells was evaluated semi-quantitatively using the "histochemistry score" (H score) method. H score is obtained by the formula: $3 \times$ % of strongly staining cells (3+ intensity) + $2 \times$ % of moderately staining cells (2+ intensity) + $1 \times$ % of weakly staining cells (1+ intensity), in the range of 0–300 (Fig. 1C,D).

All analyses were performed independently by two pathologists (I. G. and J. A.), blinded to the clinical and follow-up data. Discordances were resolved by consensus review.

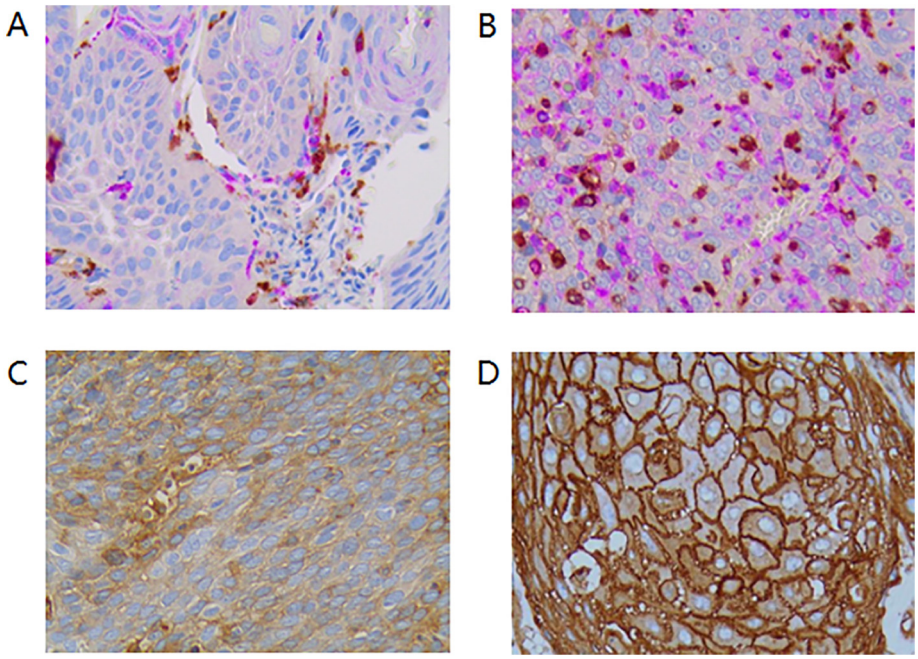

Fig. 1. Representative images of immunohistochemical double staining using CD68 and CD163 antibody (400×) showing intraepithelial infiltration by macrophages. CD163-positive cells are stained in brown and CD68-positive cells are stained in purple. (A) 1+; (B) 3+. Representative images of immunohistochemical of HLA class I (400×). Positive cells are stained brown. (C): negative; (D): 3+.

Statistics

Biomarker patterns in different subgroups of pretreatment characteristics were compared using the chi-square test. Survival rates were calculated with the Kaplan–Meier method and compared using the log-rank test. Survival times were defined as the time from the beginning of radiotherapy until either the time of first event or the date that the patient was last known to be alive (censored). Events were death from any cause for OS, death or tumor progression for progression-free survival (PFS), locoregional recurrence for loco-regional control (LRC), and distant metastasis for distant control (DC). The Cox proportional hazard model was used for multiple regression analysis. Two-sided p values < 0.05 were considered to indicate a significant difference. All statistics were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) and R software version 3.2.4.

Results

Patient characteristics

The clinical and pathological characteristics of the patients included in this study are summarized in Table 1. The age of the study population ranged from 38 to 81 years (median, 60 years). Most (80%) patients were male. Sixty-four (67%) patients were never/former smokers and 31 (33%) were current smokers. With regard to HPV status, 27 (28%) patients had p16+ tumors and 68 (72%) had p16− tumors. All patients with p16+ tumors were oropharyngeal cancer (OPC) patients. Information on p16 status according to sub-sites is provided in Supplementary Table 1. As for treatment, 59 (62%) patients received CRT and 36 (38%) received BRT. Overall treatment time of 39 (41%) patients was ≥50 days.

Survival and relapse

The median follow-up time was 50.3 months (range 3.7–103.9 months). Of the 95 patients, 41 patients (43.2%) died. Thirty-eight (40.0%) patients had disease progression: 17 had locoregional recurrences (LR), 11 had distant metastases (DM), and 10 had both LR and DM. The 5-year actuarial OS, PFS, LRC and DC for the entire population were 58.9%, 47.4%, 70.5% and 75.6%, respectively.

TAM density and HLA class-I expression

The percentage of stromal area covered by CD68+ macrophages (total macrophage population, further referred as M) ranged from 5% to 50% with a median at 15%. The percentage of stromal area covered by CD68+CD163+ macrophages (further referred as M2) ranged from 1% to 45% with a median at 12%. The median value of the M2/M ratio was 0.8 (range, 0.10–0.98) in the whole population. The median percentage of M and M2 in the tumor stroma was used to define the corresponding low (<median) and high (≥median) cell density.

For intraepithelial macrophage density (IEMD), the distribution of each score category was as follows: 0, 3 patients; 1+, 39 patients; 2+, 41 patients; and 3+, 12 patients. For simple statistical analysis, the patients were divided into low IEMD (0 and 1+) and high IEMD (2+ and 3+).

The H score of HLA class I expression ranged from 0 to 300; the median H score was 30. Median H score were used to define low expression (<median) and high expression (≥median) of HLA class I.

Correlation with pretreatment variables

We first analyzed the correlation between TAM density or HLA-I expression and the clinicopathologic characteristics. IEMD differed significantly according to primary tumor location, N stage and HPV status. The M2/M ratio in the stroma and HLA class-I expression differed significantly according to HPV/p16 status but not with T stage or smoking status (Table 1). Positive HPV status was significantly associated with high IEMD ($p < 0.001$) and low HLA class-I expression ($p = 0.003$); while negative HPV status was associated with high M2/M ratio in the stroma ($p = 0.002$).

Table 1
Correlation of expression of biomarkers with clinicopathological parameters in the whole population ($N = 95$).

Characteristics	N (%)	IEMD			%M in stroma			M2/M in stroma			%M2 in stroma			HLA class I		
		Low	High	p	Low	High	p	Low	High	p	Low	High	p	Low	High	p
Age (years)				0.72			0.50			0.68			0.98			0.07
<65	74 (78)	32	42		27	47		28	46		35	39		33	41	
>=65	21 (22)	10	11		6	15		9	12		10	11		14	7	
Gender				0.47			0.45			0.75			1.0			0.84
Male	76 (80)	35	41		25	51		29	47		36	40		38	38	
Female	19 (20)	7	12		8	11		8	11		9	10		9	10	
Smoking status				0.31			0.14			0.63			0.89			0.88
Never/Former	64 (67)	26	38		19	45		26	38		30	34		32	32	
Current	31 (33)	16	15		14	17		11	20		15	16		15	16	
PS (ECOG)				0.98			**0.04**			0.55			0.14			0.77
0	70 (74)	31	39		20	50		26	44		30	40		34	36	
≥1	25 (26)	11	14		13	12		11	14		15	10		13	12	
Charlson index				0.06			0.42			0.63			0.89			0.56
0–1	64 (67)	24	40		24	40		26	38		30	34		33	31	
≥2	31 (33)	18	13		9	22		11	20		15	16		14	17	
Location				**0.03**			0.96			0.52			0.35			0.94
Oropharynx	63 (66)	23	40		22	41		26	37		32	31		31	32	
Non-oropharynx	32 (34)	19	13		11	21		11	21		13	19		16	16	
T classification				0.06			0.16			0.11			0.60			0.09
T1-3	74 (78)	29	45		23	51		32	42		34	40		40	34	
T4	21 (22)	13	8		10	11		5	16		11	10		7	14	
N classification				**0.04**			0.15			0.14			0.55			0.60
N0-1	45 (47)	25	20		19	26		14	31		23	22		21	24	
N2-3	50 (53)	17	33		14	36		23	27		22	27		26	24	
Overall stage				0.68			0.33			0.59			0.70			0.61
III	34 (36)	16	18		14	20		12	22		17	17		18	16	
IV	61 (64)	26	35		19	42		25	36		28	33		29	32	
HPV/p16 status				**<0.001**			0.86			**0.002**			0.06			**0.003**
p16+	27 (28)	4	23		9	18		17	10		17	10		20	7	
p16−	68 (72)	38	30		24	44		20	48		28	40		27	41	
Concurrent regimen				0.09			0.51			0.66			0.41			0.08
CRT	59 (62)	22	37		19	40		24	35		26	33		25	34	
BRT	36 (38)	20	16		14	22		13	23		19	17		22	14	

PS: performance status; ECOG: Eastern Cooperative Oncology Group; HPV: Human Papillomavirus; CRT: chemoradiotherapy with concurrent cisplatin; BRT: bioradiotherapy with concurrent cetuximab; IEMD: intraepithelial macrophage density.
p values < 0.05 are presented in bold type.

Table 2
Correlation between biomarkers.

	Intraepithelial M r_s (p)	%M in stroma r_s (p)	%M2 in stroma r_s (p)
Intraepithelial M	/	/	/
%M in stroma	0.21 (0.04)	/	/
%M2 in stroma	0.06 (0.55)	0.05 (0.65)	/
HLA class I	−0.03 (0.75)	−0.14 (0.18)	0.12 (0.25)

There was a weak positive correlation between IEMD and the stromal density of macrophages ($r = 0.21$, $p = 0.04$). Tumors with high IEMD were more likely to have high macrophage density in their stroma. No other significant correlation was observed among the biomarkers (Table 2).

Characterization of TAM density and HLA class-I expression in OPC patients treated with CRT

In a more homogenous sub-population of OPC patients treated with CRT ($N = 38$), the percentage of M in stroma ranged from 5% to 50% with a median at 15%. The percentage of M2 in stroma ranged from 2% to 45% with a median at 9.25%. The median value of the M2/M ratio was 0.8 (range, 0.20–0.95).

For IEMD, the distribution of each score category was as follows: 0, 2 patients; 1+, 7 patients; 2+, 19 patients; and 3+, 10 patients.

The H score of HLA class I expression ranged from 0 to 300; the median H score was 35.

The characterization of TAM density and HLA-I expression and their correlations with clinicopathologic characteristics were shown in Supplementary Table 2. Similar to the result of the entire population, positive HPV status was significantly associated with low M2/M ratio in the stroma ($p = 0.01$) and low HLA class-I expression ($p = 0.003$). There was a positive correlation between stromal M2 and stromal M density ($r = 0.73$, $p = 0.001$). No other significant correlation was observed among the biomarkers in this sub-population (Supplementary Table 3).

Correlation with prognosis

Univariate analyses (UVA) and multivariate analysis (MVA) were performed to evaluate factors potentially associated with OS and PFS. MVA showed that T stage, smoking status, treatment duration, and p16 status were significantly correlated with OS, while T stage, smoking status, concurrent regimen, treatment duration, PS and IEMD were significantly correlated with PFS (Table 3).

Additionally, subgroup analysis showed that IEMD may play different roles in p16+ vs. p16− tumors. In p16+ tumors, high IEMD was significantly associated with favorable PFS compared with low IEMD (5-year PFS 81.2% vs. 25.0%, $p < 0.001$) (Fig. 2B), and there was a trend for improved OS (5-year OS 95.7% vs. 66.7%, $p = 0.09$). In contrast, in patients with p16− tumors, no difference of survival rates was observed according to high or low IEMD (5-year OS: 48.3% vs. 46.3%, $p = 0.60$; PFS: 39.0% vs. 37.3%, $p = 0.59$) (Fig. 2A).

Similarly, when stratified by primary tumor site, IEMD showed different prognostic value in OPC vs. non-OPC patients. In OPC,

Table 3
Univariate and multivariate analyses of overall survival (OS) and progression-free survival (PFS) of the entire population ($N = 95$).

Characteristics	OS				PFS			
	UVA		MVA		UVA		MVA	
	HR	*P*	HR (95% CI)	*P*	HR	*P*	HR (95%CI)	*P*
T4 classification (T1-3)	3.3	<0.001	2.6 (1.2–6.0)	**0.02**	3.8	<0.001	3.2(1.6–6.4)	**0.001**
N2-3 classification (N0-1)	1.1	0.73	2.1(0.9–4.7)	0.07	1.3	0.35	1.8(0.9–3.6)	0.09
Never/Former smoker (Current)	0.6	0.08	0.4 (0.2–0.8)	**0.007**	0.6	0.08	0.4(0.2–0.8)	**0.01**
BRT (CRT)	1.7	0.11	1.7 (0.9–3.5)	0.11	1.7	0.06	1.9(1.0–3.5)	**0.049**
Treatment duration ≥ 50 days (<50 days)	1.8	0.05	3.5 (1.7–7.2)	**<0.001**	1.6	0.08	2.6 (1.4–4.9)	**0.004**
PS = 0 (PS ≥ 1)	0.5	0.046	0.6 (0.3–1.2)	0.15	0.4	0.001	0.5(0.2–0.99)	**0.046**
p16+ (p16−)	0.2	0.003	0.3 (0.1–0.9)	**0.04**	0.4	0.01	0.6(0.1–1.4)	0.21
M in stroma low (high)	1.6	0.15	1.3(0.6–2.8)	0.43	1.2	0.45		
M2/M in stroma low (high)	0.7	0.36			0.9	0.67		
M2 in stroma low (high)	1.0	0.90			1.0	0.99		
Intraepithelial M low (high)	2.0	0.03	2.2(0.98–4.8)	0.06	2.0	0.01	2.0(1.02–4.1)	**0.04**
HLA-I low (high)	0.5	0.07	0.6 (0.3–1.2)	0.17	0.7	0.17	0.8(0.4–1.5)	0.46

CRT: chemoradiotherapy with concurrent cisplatin; BRT: bioradiotherapy with concurrent cetuximab; PS: performance status.
p values < 0.05 are presented in bold type.

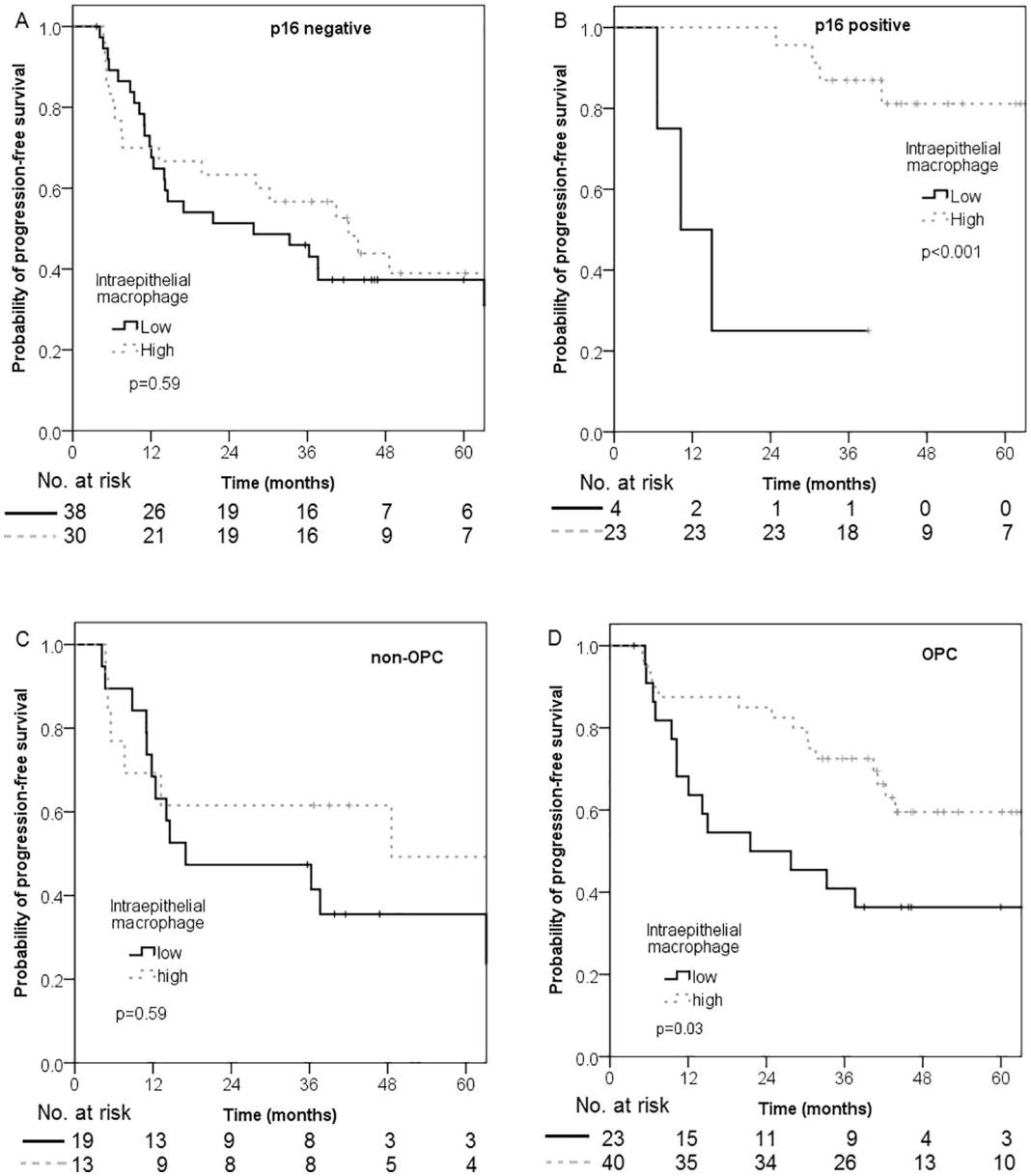

Fig. 2. Kaplan–Meier curves of progression-free survival for patients stratified for low and high expression of intraepithelial CD68+ macrophage in patients with (A) p16− disease; (B) p16+ disease; (C) non-oropharyngeal cancer; (D) oropharyngeal cancer.

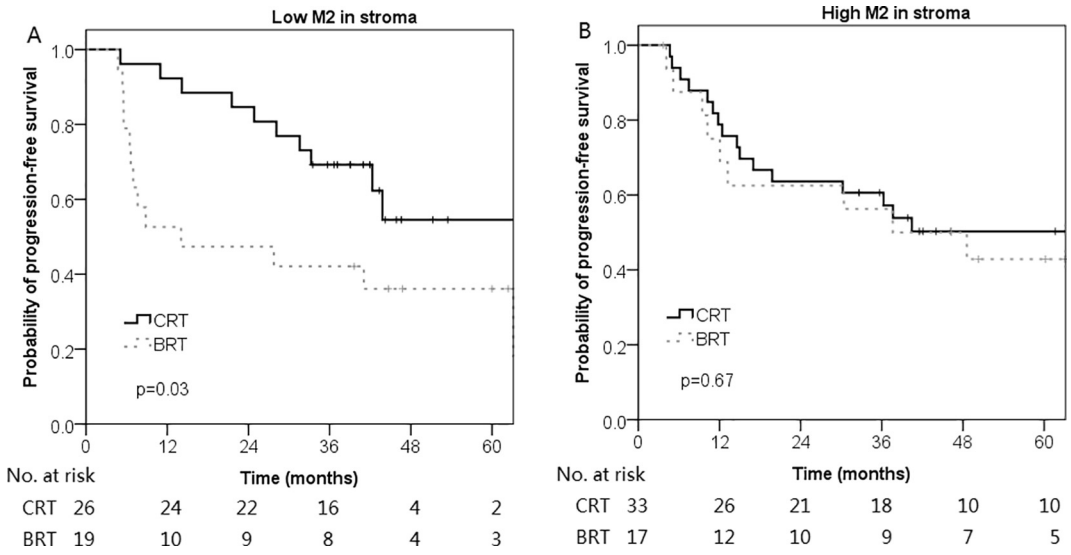

Fig. 3. Kaplan–Meier curves of progression-free survival according to (A) low and (B) high M2 expression in tumor stroma in patients receiving concurrent chemoradiotherapy (CRT) or bioradiotherapy (BRT).

high IEMD was significantly associated with favorable PFS compared with low IEMD (5-year PFS 59.5% vs. 36.4%, $p = 0.03$) (Fig. 2D), and there was also a trend for improved OS (5-year OS 72.8% vs. 49.6%, $p = 0.08$). In contrast, in patients with non-OPC, no difference of survival rates was observed according to high or low IEMD (5-year OS: 48.3% vs. 46.3%, $p = 0.60$; PFS: 39.0% vs. 37.3%, $p = 0.59$) (Fig. 2C).

Subgroup analysis according to treatment option

Five-year PFS of patients with low M2 density in tumor stroma treated with CRT was significantly improved vs. those treated with BRT (54.5% vs. 36.1%, $p = 0.03$). In contrast there was no significantly difference in 5-year PFS for patients with high M2 density in tumor stroma between the two treatment options (50.3% vs 42.9%, $p = 0.67$) (Fig. 3).

Five-year PFS was not significantly different according to treatment option (CRT vs. BRT) in patients with tumors showing either low (35.4% vs. 36.8%, $p = 0.48$) or high IEMD (64.0% vs. 42.2%, $p = 0.11$). Similarly, there was no significant difference in 5-year PFS between the two treatment options (CRT vs. BRT) according to HLA class I expression, either low (67.5% vs. 38.6%, $p = 0.07$) or high (43.0% vs. 38.5%, $p = 0.25$).

Discussion

Tumor microenvironment consists of various host components including stromal cells, blood vessels and inflammatory infiltrates. It plays an important role in carcinogenesis, tumor progression and response to therapeutic interventions [21]. In the present study, although macrophage density and HLA class I expression were not identified as independent prognostic factors by multivariate analysis, subgroup analysis showed that intraepithelial macrophage density may play different roles in HPV+ as compared to HPV- patients.

The ratio between CD68+ CD163+ cells (predominantly M2 macrophages) and CD68+ cells (total macrophage population) is indicative of the degree of M2 polarization. In our study, the median M2/M ratio in the stroma was 0.8, indicating that a large proportion of polarized macrophages in the stroma showed M2 phenotype instead of the classically activated M1 phenotype. In line to our findings, previous studies also found in OSCC the dominance of M2 polarization [10,11].

The relationship between TAM infiltration and prognosis has been reported in a wide spectrum of epithelial and non-epithelial malignancies [22]. Most studies reported that high TAM density is correlated with a poor prognosis; however, other results are controversial [23–25]. These apparently conflicting results may be explained by the inclusion of different tumor types or, the use of different treatments, and by technical differences in the markers used to identify TAM and their different phenotypes or in the use of different cut-off values for the definition of low/high expression [22].

In HNSCC, only a few reports have been published so far and the prognostic relevance of macrophage polarization on tumor outcome is still debated [10,26]. Previous studies have shown that irradiation has a strong impact on tumor microenvironments, leading to an increase in avascular hypoxia and in the promotion of M2 polarization in the context of tumor hypoxia [27].

In 106 patients with HNSCC who received definitive CRT, baseline CD163 density (corresponding to the sum of the individual density scores from the three tumor compartments: intraepithelial compartment, stroma and tumor periphery) was associated with an unfavorable clinical outcome [28]. However, the prognostic significance of TAMs varied with tumor compartments. While high stromal CD68 and CD163 densities were significantly associated with worse survivals, differences in CD68 or CD163 density in the intraepithelial compartment and the tumor periphery were not found to have a prognostic impact [28]. Another study in patients with HNSCC treated with radical surgery or definitive chemoradiotherapy with or without induction/adjuvant therapy failed to show any prognostic significance for CD163+ macrophage density in any compartment [29]. In the present study, our result that high density of intraepithelial macrophages correlated with favorable prognosis was somewhat unexpected. This correlation was also verified in HPV+ patients. However, in the HPV− patient subgroup, there was no difference of survival or tumor control rates between low and high intraepithelial macrophage density. Furthermore, it was observed that high stromal M2/M ratio (71% vs. 37%, $p = 0.002$) and low IEMD (56% vs. 15%, $p < 0.001$) was higher in HPV+ than in HPV− patients, which suggests different immunological patterns in patients according to their HPV status. The number of published reports on the prognostic impact

of TAMs with regard to HPV status in HNSCC is limited and their conclusions remain unclear [28,30]. Although the precise underlying mechanisms are not yet clear, it is generally assumed that macrophages may change their polarization in response to some immunological stimuli, such as growth factors (e.g., CSF-1 and GM-CSF) and cytokines (eg. T-cell secreted IFNγ), microbes (HPV), etc., from immunosuppressive M2 macrophages into immunostimulatory cells [31]. Our previous study showed that p16+ tumors are more heavily infiltrated by CD8+ and FoxP3+ TIL than p16− tumors [32]. In HPV+ HNSCC, PD-L1 expression on both tumor cells and CD68+ TAM has been shown to predominate along lymphocyte fronts, whereas the majority of CD8+ TILs express high levels of PD-1, the inhibitory PD-L1 receptor [33]. Therefore, it is speculated that patients with T-cell rich HPV+ tumors are likely to be more M1 polarized and to have a better immune surveillance. On the other hand, HPV− tumors may require immune stimulation to overcome the relative anti-inflammatory micro-environment. Moreover, TAMs were able to release immunostimulatory cytokines, such as IL-12 which stimulates both the proliferation and cytotoxicity of T cells and NK cells [25,34]. The discrepant role and underling regulatory mechanisms of TAMs in the different compartments of tumor microenvironment need to be further studied. The relationship between TAMs with HPV-associated tumor is currently not well understood, and merits future researches.

Accumulating evidence indicates that the efficacy of chemotherapy and targeted therapy relies on macrophage function directly or indirectly [25,35]. Cetuximab-based BRT is one of the treatment options for HNSCC. As evidence comparing CRT versus BRT for treating locally advanced HNSCC is emerging, the optimal sub-population benefiting from concurrent cetuximab or cisplatin is yet to be defined. Alternatively, in the present study, M2 in stroma did not correlated with prognosis but high stromal M2 did seem to associate with improved efficacy in patients treated with BRT. In high stromal M2 tumors, 5-year PFS was not significantly different between CRT and BRT subgroups, while in low stromal M2 tumors, 5-year PFS of patients treated with CRT was significantly better. The mechanism beneath this observation is still unknown and needs to be further explored. However, the immunologic effect of cetuximab has been previously described. In addition to inhibition of epidermal growth factor receptor (EGFR) tyrosine phosphorylation, another mechanism of action of cetuximab is activating NK cell-mediated antibody dependent cell-mediated cytotoxicity (ADCC), increasing DC maturation and tumor antigen processing, and resulting in an adaptive tumor-specific immune response [36]. Circulating CD11b+ CD14+ HLA-DRhi monocytes of cetuximab responders were observed to display attenuated M2 polarization, with decreased CD163+ expression and IL-10 transcripts after single-agent cetuximab treatment in locally advanced HNSCC [37]. Thus tumors with high stromal M2 could be a potential optimal sub-population to be treated with BRT, which would lead to more tailored and personalized care.

The expression of HLA molecules in HNSCC was also assessed in this study. Downregulation of APM components and HLA class I antigen presentation is a common mechanism of immune evasion shared by many cancers [11]. Our findings failed to demonstrate that HLA class I expression level was an independent prognostic marker for HNSCC patients treated with CRT or BRT, a finding that contradicts with some of the previous reports showing HLA class I loss to be an unfavorable factor in breast [38], non-small cell lung cancer [39], ovarian cancer [40], and oropharyngeal squamous cell carcinoma [41]. This conflict may be partly explained by the variability in methodologies among the reports, which makes the interpretation of the prognostic value of each marker quite difficult. On the other hand, the lack of prognostic impact in our study may be correlated with ADCC during cetuximab compensating for the negative effects of HLA class I loss [36]. Moreover, EGFR blockade triggered STAT1 activation and HLA upregulation. Reversal of HLA class I downregulation was more prominent in clinical responders to cetuximab therapy, supporting an important role for adaptive immunity in cetuximab antitumor activity [42]. There are also studies showing HLA class I expression was related to a high number of regulatory T cell infiltration in HPV-induced vulvar neoplasia [43]. Future studies testing HLA class I expression together with TAM and TIL densities and their inter-correlation with patients' survival is warranted.

Our study has several limitations. As it is a retrospective study with limited sample size, multiple testings might lead to a high risk of false positive results. Moreover, due to the small numbers of patients and events but the comparatively numerous variables included in the multivariate analysis, it should be pointed out that the results of MVA might be underpowered and need to be interpreted with caution and considered as preliminary results. Another limitation is patient heterogeneity (age, Charlson Comorbidity Index, primary tumor sites, p16 status, RT duration and treatment selection bias). It is also worth noting that in subgroup analysis according to treatment option, the number of patients/events was even smaller, hence the exploratory results on the predictive potential of TAMs warrant further study and need external validation.

To conclude, intraepithelial macrophage density was prognostic for favorable PFS whistle HLA class I down-regulation was not an independent prognostic factor. However, HPV/p16 positive and negative tumors had significant different patterns of macrophage distribution and HLA class-I expression. Subgroup analysis showed that intraepithelial macrophage density may play different roles according to HPV status. BRT was found to have equivalent efficacy as CRT in tumors with high stromal M2 density. This might help in selecting suitable patients for concurrent cetuximab instead of cisplatin treatment. Considering the retrospective and single-institution design of the present study, these data should be further validated in an independent cohort.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.radonc.2018.08.013.

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