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Cancer stem cell enrichment marker CD98: A prognostic factor for survival in patients with human papillomavirus-positive oropharyngeal cancer

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Abstract ***Purpose:*** Several hypotheses have been proposed to explain the relatively good prognosis of patients with a human papillomavirus (HPV)-positive oropharyngeal squamous cell carcinoma (OPSCC) and one of these is a higher sensitivity to (chemo)radiation. Previous studies have suggested that treatment failure in OPSCC patients is caused by resistance of cancer stem cells (CSCs). The purpose of this study was to evaluate the association between the number of CSCs and prognosis in HPV-positive OPSCC patients.
Experimental design: All OPSCC patients ($n = 711$) treated between 2000 and 2006 in two Dutch university hospitals were included. Presence of HPV in a tumour tissue specimen was tested by p16-immunostaining followed by HPV DNA GP5+/6+polymerase chain reaction (PCR). The presence and intensity of tumour CSC markers CD44 and CD98 were determined by immunohistochemistry and semiquantitative scoring was performed. Overall survival (OS) and progression-free survival (PFS) rates were compared between patients with low and high CD44/CD98 expression in relation to HPV status.
Results: HPV-positive tumours showed a lower percentage of cells with CD44 and CD98 expression than HPV-negative tumours ($p < 0.001$, χ^2-test). Within the group of patients with

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HPV-positive OPSCC, a high percentage of CD98-positive tumour cells was associated with a significantly worse 5-year OS and PFS (OS: 36.4% and PFS: 27.3%) compared to patients with a low percentage of CD98-positive cells (OS: 71.9% and PFS: 70.5%, respectively) ($p < 0.001$). ***Conclusions:*** HPV-positive OPSCCs harbour fewer cells expressing the CSC enrichment markers CD44 and CD98. Furthermore, OS and PFS were significantly worse for patients with HPV-positive OPSCC with a high percentage of CD98-positive cells.

1. Introduction

Infection with high-risk human papillomavirus (HPV) is aetiologically linked to the development of head and neck squamous cell carcinomas (HNSCCs), particularly those carcinomas that arise in the oropharyngeal region. HPV-positive oropharyngeal squamous cell carcinomas (OPSCCs) are characterised by an epidemiologic, demographical and clinical profile that deviates from that of HPV-negative OPSCCs [1,2]. The most important difference is related to prognosis, which is markedly better for patients with an HPV-positive tumour compared to those with an HPV-negative tumour. Several hypotheses have been proposed to explain the improved outcome for patients with HPV-positive OPSCC, including an increased sensitivity to radiation and chemotherapy, differences in the role of the host immune system and the absence of field cancerisation [3–6]. In this study, we further evaluated the role of cancer stem cells (CSCs) in HPV-positive OPSCCs.

Previous studies suggest that treatment failure in HNSCC patients might be the consequence of therapy resistance of cancer stem cells (CSCs) [7]. CSCs represent a small subpopulation of tumour cells that maintain tumour growth by fuelling the expansion of the malignant cell population infinitely [8]. CSCs can be distinguished from the bulk of the tumour based on differential expression of protein markers on the cell membrane. A large body of evidence indicates that HNSCC cells expressing high levels of the CD44 antigen possess CSC properties. $CD44^{high}$ HNSCC cells have been shown to initiate tumour growth in mice much more efficiently than $CD44^{low}$ cells, indicating that CSCs are enriched in the $CD44^{high}$ subpopulation of HNSCC [9]. Moreover, a high expression of CD44 seems associated with a poor prognosis in patients with HNSCC [10].

Recently, we examined CD98 as a novel, putative CSC enrichment marker in HNSCC and showed that $CD98^{high}$ cells, in contrast to $CD98^{low}$ cells, are able to generate tumours in immune-deficient mice [11]. Studies in a multitude of cancer types showed a higher CD98 expression in progressive and metastatic tumours, which relates to a poor prognosis [12–17].

Recently, it was shown that a small subpopulation of cells with CSC properties could also be isolated from an HPV-positive head and neck cancer cell line [18]. As patients with an HPV-positive OPSCC respond better to treatment and have a more favourable prognosis compared to patients with an HPV-negative OPSCC, we hypothesised that HPV-positive OPSCCs, might have relatively low levels of CSCs. To test this hypothesis, we performed CD44 and CD98 immunostaining on a cohort of OPSCC patients with known HPV status [19]. Furthermore, we evaluated whether CD44 and CD98 expression could be of potential relevance for predicting treatment outcome in patients with HPV-positive OPSCC.

2. Materials and methods

2.1. Patients and tumour samples

To evaluate CD44/CD98 immunostaining and the relation to HPV, a test cohort was composed, which included 88 fresh-frozen, pre-treatment OPSCC samples of patients treated in the period 2008–2011. Eligible samples included histopathologically confirmed invasive squamous cell carcinoma of the oropharynx (International classification of diseases for Oncology, [ICD-10] codes C019, C051, C052, C090–C099 and C100–C109). These OPSCCs were previously tested for HPV using an HPV E6 mRNA reverse-transcriptase polymerase chain reaction (RT-PCR) [19].

To further study the association between survival and CD44/CD98 expression, all patients ($n = 711$) treated between 2000 and 2006 at two Dutch university hospitals were included. Patients were identified through the Dutch Cancer Registries. Patient characteristics and clinical outcome were obtained from the patient files. HPV detection was performed using pre-treatment formalin-fixed, paraffin-embedded (FFPE) biopsies [20]. A sample was scored HPV-positive based on a positive $p16^{INK4A}$-immunohistochemistry (p16-IHC) and a subsequent positive GP5+/6+ HPV DNA PCR, according to a previously validated algorithm [19]. Approval for this retrospective study was obtained from the Institutional Review Board and the study adheres to the guidelines for proper secondary use of human tissue specimen (www.federa.org).

2.2. Immunohistochemical staining of CD44 and CD98

Formalin-fixed paraffin-embedded sections of HNSCC tumour biopsies were deparaffinised and subjected to Tris/EDTA (10 mM/1 mM, pH 9.0) antigen

retrieval. Primary antibodies U36 (anti-CD44v6, developed at our laboratory [21]) and anti-CD98 (clone H-300, Santa Cruz Biotechnology), were diluted in PBS containing 2% goat serum and incubated overnight at 4 °C. Afterwards, the BrightVision +Poly-HRP-Anti Ms/Rb/Rt IgG kit (Immunologic BV, Duiven, The Netherlands) was employed according to the description of the manufacturer. The staining was developed with diaminobenzidine and H_2O_2 as chromogen. Sections were counterstained with haematoxylin and cover slipped with Kaiser's glycerine. To correct for the differences in dimensions of the biopsies, decisions on CD44 and CD98 scores were based on a single area that contained viable tissue with over 70% HNSCC cells.

2.3. Evaluation of immunohistochemical staining

CD44 and CD98 expression were evaluated by staining intensity and by percentage of positive cells. Percentage of CD44-positive cells was evaluated semi-quantitatively, and classified according to the percentage of malignant cells: 1 (⩽10% of tumour cells stained), 2 (11–50% stained), 3 (51–75% stained) or 4 (>75% stained) [10]. Percentage of CD98-positive cells was classified differently, in accordance to previous reports, as overall staining was lower: 1 (⩽10% of tumour cells stained), 2 (11-25% stained), 3 (26–50% stained) and 4 (>50% stained) [15,16]. The intensity of CD44 and CD98 staining was scored separately and evaluated as absent (0), weak (1), moderate (2) or strong (3) [10].

Three investigators independently classified the percentage of positive cells and staining intensity in all cases, without prior knowledge of the clinical data. The data are presented as the mean of the three observations.

2.4. Statistical analyses

Probability values of <0.05 indicated a statistically significant difference. A Mann-Whitney U Test was performed to compute the CD44/CD98 expression differences between HPV-positive and HPV-negative OPSCCs. Differences in patient characteristics between HPV-positive and HPV-negative cases were assessed using the Pearson χ^2-test or Student's *t*-test. Bonferroni correction was used to compare subgroups for specific variables. The end-points were overall survival (OS) and progression-free survival (PFS). OS was defined as the time from date of incidence (defined as the date on which the squamous cell carcinoma was histologically confirmed) to death (any cause). PFS was defined as the time period from date of incidence to death or the first documented relapse, which was categorised as local-regional recurrence or distant metastases. Patients who developed a second primary tumour were censored at the incidence date of that tumour. Survival rates were estimated by means of the Kaplan-Meier method and associations were analysed with the log-rank test. Multivariate analyses were performed using a forward selection procedure (p was set at <0.05 to enter the model) in the Cox proportional hazards model to identify independent prognostic factors. Intraclass correlation coefficients (with 95% confidence intervals [CI]) were computed to determine interobserver variability of the three investigators for percentages of CD44 and CD98 positive cells.

3. Results

3.1. CD44/CD98 expression in the test cohort

CD44 and CD98-immunostaining were first performed on a test cohort to detect a potential difference between HPV-positive and HPV-negative cases and to evaluate the chosen cut-off values. CD44 staining intensity and percentage of CD44-positive cells were significantly higher in HPV-negative tumours ($n = 63$) compared to HPV-positive tumours ($n = 25$) ($p = 0.03$ and $p = 0.002$, respectively), as calculated by the Mann-Whitney U test. CD98 staining intensity and percentage of CD98-positive cells were also significantly higher ($p < 0.001$ for both) for HPV-negative tumours compared to HPV-positive tumours. Distribution curves for percentage of CD44/CD98-positive cells and CD44/CD98 intensity for this test cohort are depicted in the Supplementary data (S1).

3.2. Patient and tumour characteristics

Next, we investigated the relation between CD44/CD98 expression and survival in tumour biopsies obtained from a large, consecutive cohort of 711 OPSCC patients. Patient and tumour characteristics are depicted in Table 1. As published before, patients with HPV-positive tumours had less advanced tumour stages than patients with HPV-negative tumours, but a more advanced nodal stage. A higher HPV-prevalence was found in squamous cell carcinomas in the base of tongue and the tonsils compared to the other oropharyngeal subsites ($p < 0.001$, with Bonferroni correction) [20].

With regard to differentiation, the majority of all HPV-positive tumours (71.3%) were poorly differentiated, while the majority of the HPV-negative tumours (72.5%) were moderately differentiated. The intensity and percentage of CD44 and CD98 positive cells were not statistically different in poorly differentiated tumours compared to well and moderately differentiated tumours. This concerned HPV-positive as well as HPV-negative tumours. Distribution curves for percentage of CD44/CD98-positive cells and CD44/CD98 intensity for the whole patient group are depicted in Fig. 1.

Table 1
Patient and tumour characteristics.

	HPV-positive OPSCC Number (percentages)	HPV-negative OPSCC Number (percentages)	*p*-Value*	HPV-positive OPSCC/CD98 high Number (percentages)	*p*-Value**	All cases Number (percentages)
No. of cases	150 (21.1%)	561 (78.9%)		11		711
Age at diagnosis						
Mean	61.0	60.6	$p = 0.722^{\dagger}$	57.3	$p = 0.31^{\dagger}$	60.7
Median	58.8	59.4		55.6		59.3
Gender			$p = 0.374^{\ddagger}$		$p = 0.93^{\ddagger}$	
Male	106 (70.7%)	375 (66.8%)		8 (72.7%)		481 (67.7%)
Female	44 (29.3%)	186 (33.2%)		3 (27.3%)		230 (32.3%)
Oropharyngeal Sub-site			$p < 0.001^{\ddagger}$		$p = 0.23^{\ddagger}$	
Tonsil	88 (58.7%)	226 (40.3%)		5 (45.5%)		314 (44.2%)
Base of tongue	48 (32.0%)	138 (24.6%)		4 (36.4%)		186 (26.2%)
Soft palate + uvula	9 (6.0%)	101 (18.0%)		2 (18.2%)		110 (15.5%)
Oropharynx nos	5 (3.3%)	96 (17.1%)		0		101 (14.2%)
Smoking					$p = 0.37^{\ddagger}$	
Never	44 (29.3%)	17 (3.0%)	$p < 0.001^{\ddagger}$	1 (9.1%)		61 (8.6%)
Moderate (1–24 pack years)	39 (26.0%)	70 (12.5%)		5 (45.5%)		109 (15.3%)
Heavy (>24 pack years)	66 (44.0%)	468 (83.4%)		5 (45.5%)		534 (75.1%)
Unknown	1 (0.7%)	6 (1.1%)		0		7 (1.0%)
T-stage			$p = 0.001^{\ddagger}$		$p = 0.93^{\ddagger}$	
T1	32 (21.3%)	68 (12.1%)		3 (27.3%)		100 (14.1%)
T2	45 (30.0%)	168 (29.9%)		3 (27.3%)		213 (30.0%)
T3	53 (35.3%)	184 (32.8%)		4 (36.4%)		237 (33.3%)
T4	20 (13.3%)	139 (24.8%)		1 (9.1%)		159 (22.4%)
Tx	0	2 (0.4%)		0		2 (0.3%)
N-stage			$p < 0.001^{\ddagger}$		$p = 0.08^{\ddagger}$	
N0	21 (14.0%)	233 (41.5%)		4 (36.4%)		254 (35.7%)
N1	21 (14.0%)	79 (14.1%)		1 (9.1%)		100 (14.1%)
N2	97 (64.7%)	218 (38.9%)		4 (36.4%)		315 (44.3%)
N3	11 (7.3%)	29 (5.2%)		2 (18.2%)		40 (5.6%)
Nx	0	2 (0.4%)		0		2 (0.3%)
Tumour differentiation			$p < 0.001^{\ddagger}$		$p = 0.26^{\ddagger}$	
Well	2 (1.3%)	30 (5.3%)		0		32 (4.5%)
Moderate	41 (27.3%)	407 (72.5%)		5 (45.5%)		448 (63.0%)
Poor	107 (71.3%)	124 (22.1%)		6 (54.5%)		231 (32.5%)
CD44 intensity			$p < 0.001^{\ddagger}$		$p = 0.07^{\ddagger}$	
Absent (0)	5 (3.3%)	1 (0.2%)		1 (9.1%)		6 (0.8%)
Weak (1)	12 (8.0%)	4 (0.7%)		0		16 (2.3%)
Moderate (2)	62 (41.3%)	111 (19.8%)		1 (9.1%)		173 (24.3%)
Strong (3)	71 (47.3%)	445 (79.3%)		9 (81.8%)		516 (72.6%)
CD44 expression			$p < 0.001^{\ddagger}$		$p = 0.66^{\ddagger}$	
⩽10%	9 (6.0%)	4 (0.7%)		1 (9.1%)		13 (1.8%)
11–50%	37 (24.7%)	25 (4.5%)		1 (9.1%)		62 (8.7%)
51–75%	32 (21.3%)	29 (5.2%)		2 (18.2%)		61 (8.6%)
76–100%	72 (48.0%)	503 (89.7%)		7 (63.6%)		575 (80.9%)
CD98 intensity			$p < 0.001^{\ddagger}$		$p < 0.001^{\ddagger}$	
Absent (0)	76 (50.7%)	32 (5.7%)		0		108 (15.2%)
Weak (1)	41 (27.3%)	128 (22.8%)		0		169 (23.8%)
Moderate (2)	26 (17.3%)	255 (45.5%)		7 (63.6%)		281 (39.5%)
Strong (3)	7 (4.7%)	146 (26.0%)		4 (36.4%)		153 (21.5%)
CD98 expression			$p < 0.001^{\ddagger}$		$p < 0.001^{\ddagger}$	
⩽10%	105 (70.0%)	70 (12.5%)		0		175 (24.6%)
11–25%	22 (14.7%)	151 (26.9%)		0		173 (24.3%)
26–50%	12 (8.0%)	146 (26.0%)		0		158 (22.2%)
51–100%	11 (7.3%)	194 (34.6%)		11 (100%)		205 (28.8%)

Table 1 (*continued*)

	HPV-positive OPSCC Number (percentages)	HPV-negative OPSCC Number (percentages)	*p*-Value*	HPV-positive OPSCC/ CD98 high Number (percentages)	*p*-Value**	All cases Number (percentages)
Treatment modalities			$p < 0.001$‡		$p = 0.44$‡	
SURG + RT	38 (25.3%)	167 (28.5%)		2 (18.2%)		198 (27.8%)
RT	28 (18.7%)	178 (31.7%)		3 (27.3%)		206 (29.0%)
CRT	45 (30.0%)	158 (28.2%)		4 (36.4%)		203 (28.6%)
RT + LND + RT (brachytherapy)	35 (23.3%)	160 (8.6%)		1 (9.1%)		83 (11.7%)
Unknown	4 (2.7%)	17 (3.0%)		1 (9.1%)		21 (3.0%)

NOS means not otherwise specified, SURG means surgery, RT means radiotherapy, CRT means chemoradiation, and LND means lymph node dissection.

† Independent *t*-test.

‡ Chi square.

* Human papillomavirus (HPV)-positive group compared to HPV-negative group.

** HPV-positive patients with CD98 expression >50% compared to HPV-positive patients with CD98 expression <50%.

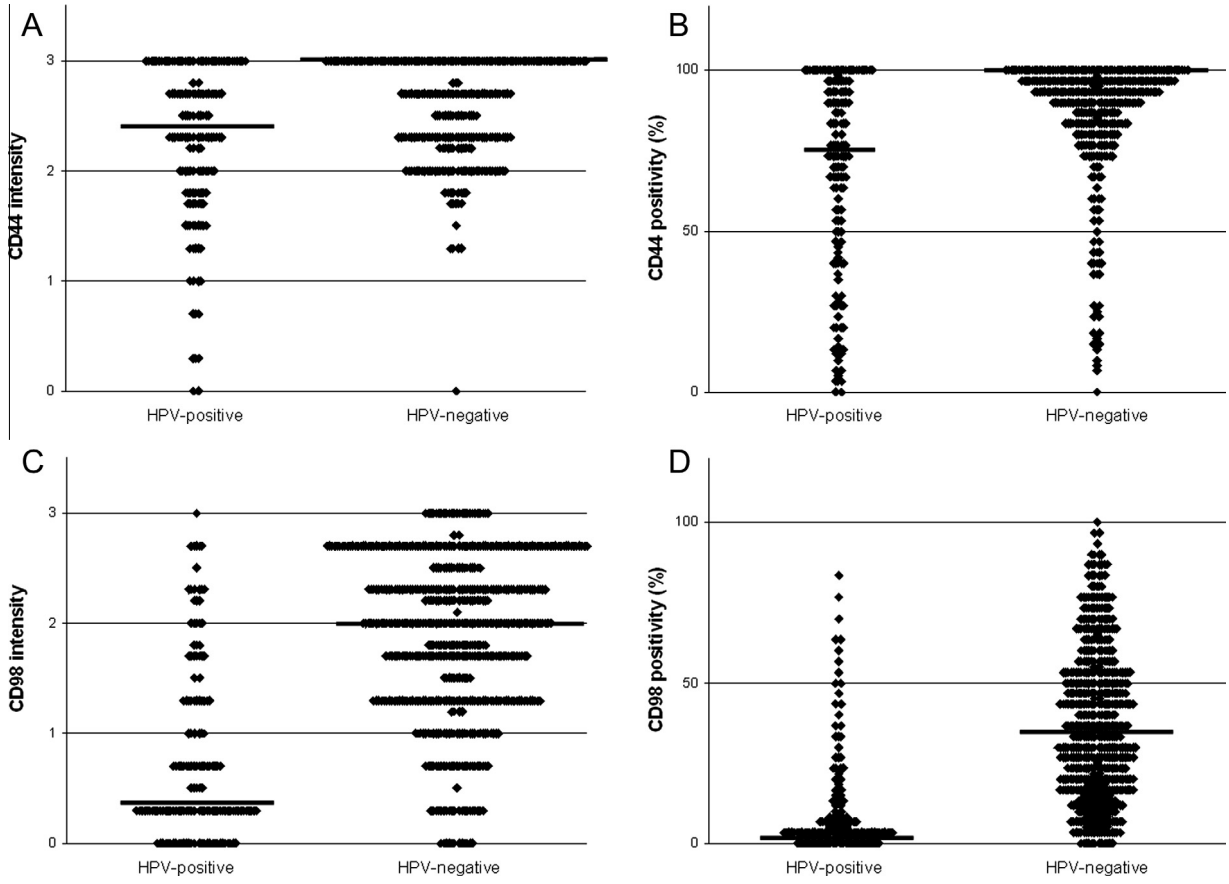

Fig. 1. Results of the scoring percentages of (A) CD44- and (B) CD98-positive tumour cells and the intensities of the immunohistochemical staining (C and D) in the whole patient group ($n = 711$). The data is represented as the mean observation of the three observers. Thick horizontal lines represent the median value observed in the cohort.

3.3. CD44 expression

CD44 intensity as well as percentage of CD44-positive cells was compared between HPV-positive and HPV-negative tumours. In Fig. 2, examples of strong CD44-immunostaining (in an HPV-negative tumour) and weak CD44-immunostaining (in an HPV-positive tumour) are shown. The CD44 intensity, dichotomised as absent (0)/weak (1)/intermediate (2) ($n = 192$) versus strong (3) ($n = 519$), was compared between HPV-positive and HPV-negative tumours. Strong CD44-staining intensity (score = 3) was observed in 445 of 561

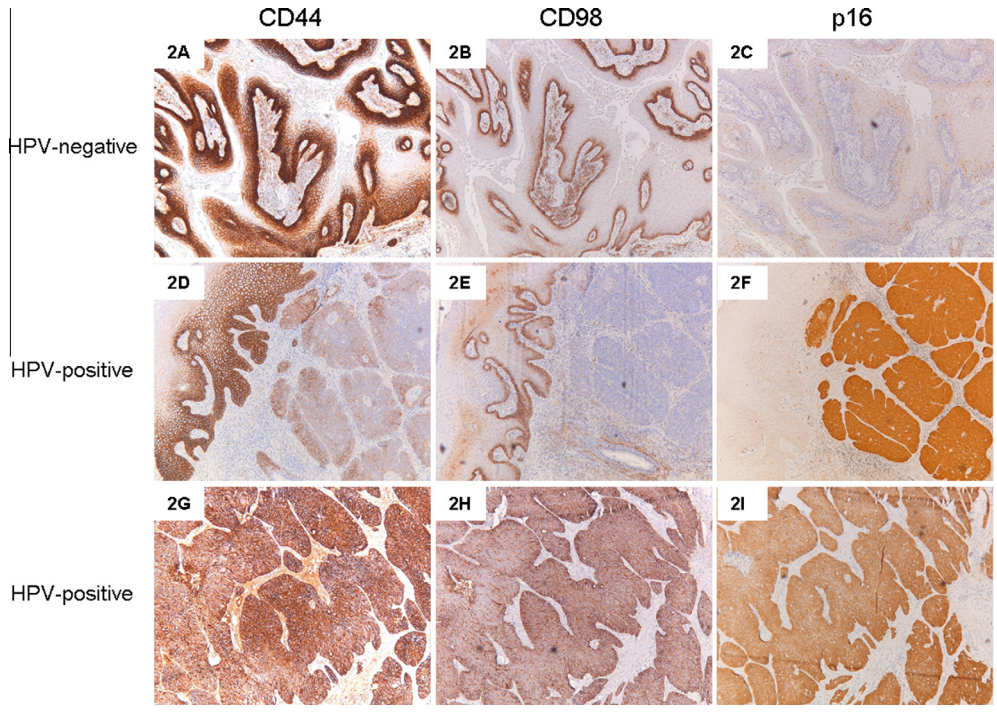

Fig. 2. (A) CD44-immunostaining of an human papillomavirus (HPV)-negative oropharyngeal squamous cell carcinoma (OPSCC). (B) CD98-immunostaining of an HPV-negative OPSCC. (C) Absence of p16^{INK4A}-immunostaining of an HPV-negative OPSCC. (D) CD44-immunostaining of an HPV-positive OPSCC. (E) Absence of CD98-immunostaining of an HPV-positive OPSCC. (F) p16 ^{INK4A}-immunostaining of an HPV-negative OPSCC. (G) CD44-immunostaining of an HPV-positive OPSCC. (H) CD98-immunostaining of an HPV-positive OPSCC. (I) p16 ^{INK4A}-immunostaining of an HPV-positive OPSCC. Pictures were taken through a 10× objective.

(79.3%) HPV-negative tumours compared to 71 of 150 (47.3%) HPV-positive tumours ($p < 0.001$, Pearson χ^2-test). Furthermore, a high percentage of CD44-positive cells (i.e. >75% of malignant cells stained) was found in 72 of 150 HPV-positive tumours (48.0%) versus 503 of 561 HPV-negative tumours (89.7%). This was a significant difference ($p < 0.001$) (Table 1). The interobserver intraclass correlation coefficient (ICC) for fraction of CD44-positive cells was 0.88 (95% CI: 0.87–0.90).

3.4. CD98 expression

CD98-immunostaining was less intense and a lower percentage of tumour cells were stained compared to CD44-immunostaining (Table 1). CD98-intensity was dichotomised as absent (0)/weak (1) ($n = 277$) versus intermediate (2)/strong (3) ($n = 434$). Percentage of CD98-positive cells was classified as 'high' if >50% of tumour cells were stained, in concordance with previously published reports [15,16]. In Fig. 2, examples of weak and strong CD98-immunostaining are depicted. CD98 expression differed significantly between HPV-positive and HPV-negative tumours. An intermediate or strong CD98-staining intensity (score 2 or 3) was observed in 401 of 561 (71.5%) HPV-negative tumours compared to 33 of 150 (22.0%) HPV-positive tumours ($p < 0.001$). Furthermore, HPV-positive tumours showed a significantly smaller percentage of tumour cells with CD98 expression than HPV-negative tumours; a high percentage of CD98-positive cells (i.e. >50% of malignant cells stained) was found in 11 of 150 HPV-positive tumours (7.3%) versus 194 of 561 HPV-negative tumours (34.6%) ($p < 0.001$) (Table 1). The ICC for percentage of CD98-positive cells was 0.83 (95% CI: 0.81–0.84).

3.5. Associations between CD44/CD98 expression and survival

There was no significant difference in survival between the different treatment groups neither for patients with HPV-negative tumours ($p = 0.204$) nor for patients with HPV-positive tumours ($p = 0.277$), after correcting for age and stage of disease.

A univariate survival analysis was performed to evaluate factors potentially associated with OS and PFS. The analyses were subdivided for patients with HPV-positive and HPV-negative tumours. Prognostic factors entered in the univariate analysis were: age, nodal stage, tumour stage, gender, number of pack years, CD44 intensity, percentage of CD44-positive cells, CD98 intensity and percentage of CD98-positive cells. In HPV-negative patients, age, tumour size and nodal stage were all individually associated with OS and PFS outcomes. However, neither CD44 expression nor CD98

Table 2
Univariate models for overall and progression-free survival.

	HPV-positive OPSCC		HPV-negative OPSCC	
	Hazard ratio (95% Confidence Interval)	*p* Value	Hazard ratio (95% Confidence Interval)	*p* Value
Overall survival				
Age (per increase of 1 year)	1.05 (1.02–1.08)	$p < 0.001$	1.02 (1.01–1.04)	$p < 0.001$
Gender (male versus female)	0.79 (0.41–1.52)	$p = 0.47$	0.81 (0.65–1.02)	$p = 0.07$
Tumour size (T3-4 versus T1-2)	1.85 (1.03–3.35)	$p = 0.04$	1.78 (1.42–2.22)	$p < 0.001$
Nodal stage (N2-3 versus N0-1)	0.80 (0.43–1.47)	$p = 0.47$	2.28 (1.84–2.82)	$p < 0.001$
Pack Years		$p = 0.33$		$p = 0.21$
PY: 1–24 PY versus 0 PY	1.56 (0.67–3.61)		1.28 (0.60–2.76)	
PY: >24 PY versus 0 PY	1.75 (0.83–3.68)		1.60 (0.80–3.22)	
CD44 intensity (score 3 versus score 0/1/2)	1.51 (0.79–2.87)	$p = 0.21$	0.92 (0.73–1.15)	$p = 0.46$
CD44 percentage (⩽75% versus >75%)	0.78 (0.44–1.40)	$p = 0.42$	0.87 (0.62–1.22)	$p = 0.42$
CD98 intensity (score 2-3 versus score 0–1)	1.51 (0.79–2.87)	$p = 0.21$	0.92 (0.73–1.15)	$p = 0.46$
CD98 percentage (⩽50% versus >50%)	2.92 (1.30–6.54)	$p = 0.009$	0.86 (0.69–1.07)	$p = 0.18$
Progression-free survival				
Age (per increase of 1 year)	1.05 (1.02–1.08)	$p < 0.001$	1.02 (1.01–1.03)	$p = 0.006$
Tumour size (T3-4 versus T1-2)	1.84 (1.04–3.27)	$p = 0.04$	1.52 (1.21–1.91)	$p < 0.001$
Gender (male versus female)	1.00 (0.56–1.84)	$p = 1.00$	0.81 (0.64–1.02)	$p = 0.08$
Nodal stage (N2-3 versus N0-1)	0.82 (0.45–1.51)	$p = 0.53$	2.16 (1.73–2.69)	$p < 0.001$
Pack Years		$p = 0.38$		$p = 0.63$
PY: 1–24 PY versus 0 PY	1.65 (0.75–3.61)		1.01 (0.49–2.10)	
PY: >24 PY versus 0 PY	1.58 (0.77–3.24)		1.17 (0.60–2.28)	
CD44 intensity (score 3 versus score 0/1/2)	1.62 (0.87–3.02)	$p = 0.13$	0.93 (0.73–1.18)	$p = 0.54$
CD44 percentage (⩽75% versus >75%)	0.86 (0.49–1.51)	$p = 0.60$	0.97 (0.67–1.40)	$p = 0.87$
CD98 intensity (score 2-3 versus score 0–1)	1.62 (0.87–3.02)	$p = 0.13$	0.93 (0.73–1.18)	$p = 0.93$
CD98 percentage (⩽50% versus >50%)	3.57 (1.66–7.68)	$p = 0.001$	0.90 (0.71–1.13)	$p = 0.36$

expression were of significant importance (Table 2). In HPV-positive patients, age, tumour size and percentage of CD98-positive cells were prognostic factors for OS and PFS.

HPV-positive patients with a high percentage of CD98-positive cells (i.e. >50% of malignant cells stained) had significantly worse 5-year OS and PFS rates (36.4% and 27.3%, respectively) compared to patients with a low percentage of CD98-positive cells (71.8% and 69.8%, respectively) (Fig. 3). The percentage of CD44-positive cells did not correlate with OS and PFS survival rates in HPV-positive patients.

Multivariate analysis was performed to estimate the association of all the analysed factors with OS and PFS. Age (⩽55 years), tumour size (T1-2) and nodal stage (N0-1) were independent prognostic factors for OS and PFS in HPV-negative patients. In HPV-positive patients, smoking (⩽24 pack years), tumour size (T1-2) and a low percentage of CD98-positive cells (i.e. <50% of stained tumour cells) were independent prognostic factors for OS and PFS (Table 3).

4. Discussion

Over the past decades, CSCs have been identified in multiple solid tumours and were implicated to play a role in resistance to anticancer treatments [22,23]. As CSCs generally divide slowly and are rich in DNA repair enzymes and detoxification mechanisms, treatment failure likely occurs due to ineffective killing of these CSCs [24]. As many studies have shown that patients with an HPV-positive OPSCC respond better to (chemo) radiation than patients with an HPV-negative OPSCC, we hypothesised that a possible explanation for this could be lower percentages of CSCs in HPV-positive OPSCCs. In this study, we evaluated the immunostaining patterns of two CSC markers, CD44 and CD98, in both HPV-positive and HPV-negative OPSCCs. Expression of both CD44 and CD98 was significantly lower in patients with an HPV-positive OPSCC. This suggests that HPV-positive tumours may have lower numbers of CSCs, which may be reflected by a better therapy response. Therefore, we evaluated whether CD44 and/or CD98 expression could be of potential relevance for predicting treatment in HPV-positive patients. CD44 expression was not associated with survival neither in HPV-positive patients nor in HPV-negative patients. This is likely due to the fact that CD44 was abundantly expressed in almost all tumours.

Several studies have shown that CD44 expression might be used as an outcome predictor in head and neck cancer [7,10], but we cannot confirm this observation.

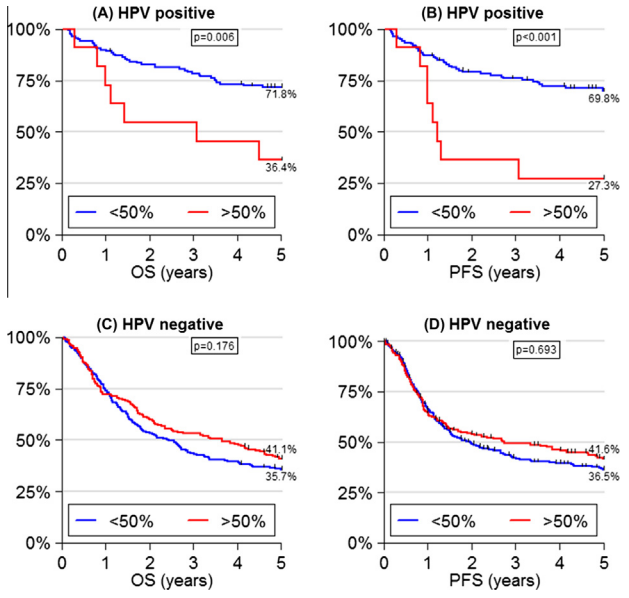

Fig. 3. (A) 5-year overall survival (OS) curves for patients with an human papillomavirus (HPV)-positive oropharyngeal squamous cell carcinoma (OPSCC) and high percentage of CD98-positive cells (i.e. >50% of malignant cells stained; red line) versus patients with a low to intermediate percentage of CD98-positive cells (i.e. ⩽50% of malignant cells stained; blue line) ($p = 0.006$). (B) 5-year progression-free survival (PFS) curves for patients with an HPV-positive OPSCC and high percentage of CD98-positive cells (i.e. >50% of malignant cells stained; red line) versus patients with a low to intermediate percentage of CD98-positive cells (i.e. ⩽50% of malignant cells stained; blue line) ($p < 0.001$). (C) 5-year OS curves for patients with an HPV-negative OPSCC and high percentage of CD98-positive cells (i.e. >50% of malignant cells stained; red line) versus patients with a low to intermediate percentage of CD98-positive cells (i.e. ⩽50% of malignant cells stained; blue line) ($p = 0.176$). (D) 5-year PFS curves for patients with an HPV-negative OPSCC and high percentage of CD98-positive cells (i.e. >50% of malignant cells stained; red line) versus patients with a low to intermediate percentage of CD98-positive cells (i.e. ⩽50% of malignant cells stained; blue line) ($p = 0.693$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Although CD44 seems an interesting marker for CSC enrichment in HNSCC, it is not optimal. In several studies it was shown that CD44 protein expression is not only present on the basal cells (the compartment where the stem cells are assumed to reside) but also on the suprabasal cells, both on normal and malignant squamous tissue [21,25–28]. Subtle differences in CD44 expression might by detected by FACS-sorting, but quantification of CD44-immunostained tissue sections is difficult considering the profuse CD44 expression throughout normal and malignant squamous tissues. In contrast, CD98 expression was restricted to cells in the basal layer and was expressed on a more restricted cell population than CD44, making CD98 more distinctive than CD44. In HPV-negative patients, no association was seen between CD98 expression and survival. However, in patients with HPV-positive OPSCC, OS and PFS were significantly worse for patients with a high percentage of CD98-positive tumour cells.

At this moment, de-escalation trials are being performed for patients with an HPV-positive OPSCC. We suggest it might be useful to evaluate the presence of CSC markers, such as CD98, in addition to HPV-status, as a stratification marker. Consequently, patients with an HPV-positive OPSCC, but a high fraction of CD98-positive tumour cells, might better not be selected for de-escalating oncological treatment. Although a challenging and attractive idea, the use of CD98 as a prognostic marker should first be preceded by a thorough validation phase in prospective clinical trials.

The observation that CD98 expression was not associated with survival in HPV-negative patients, suggests that the molecular characteristics of these cells might be more relevant than the mere numbers. This requires additional investigation and characterisation of these cells in responding and non-responding tumours.

In conclusion, most HPV-positive OPSCCs show low expression levels of the CSC markers CD44 and CD98

Table 3
Multivariate models for overall and progression-free survival.

	HPV-positive OPSCC		HPV-negative OPSCC	
	Hazard ratio (95% Confidence Interval)	p Value	Hazard ratio (95% Confidence Interval)	p Value
Overall survival				
Age (per increase of 1 year)	1.06 (1.03–1.09)	$p < 0.001$	1.03 (1.02–1.05)	$p < 0.001$
Tumour size (T3-4 versus T1-2)	2.22 (1.19–4.16)	$p = 0.01$	1.51 (1.21–1.90)	$p < 0.001$
Nodal stage (N2-3 versus N0-1)			2.33 (1.86–2.91)	$p < 0.001$
CD98 percentage (<50% versus ⩾50%)	0.23 (0.10–0.53)	$p = 0.001$		
Progression-free survival				
Age (per increase of 1 year)	1.05 (1.03–1.08)	$p < 0.001$	1.03 (1.01–1.04)	$p = 0.001$
Tumour size (T3-4 versus T1-2)	2.28 (1.26–4.15)	$p = 0.007$	1.32 (1.05–1.67)	$p = 0.02$
Nodal stage (N2-3 versus N0-1)			2.18 (1.73–2.74)	$p < 0.001$
CD98 percentage (<50% versus ⩾50%)	0.20 (0.09–0.45)	$p < 0.001$		

compared to HPV-negative OPSCCs. Furthermore, OS as well as PFS was markedly better for HPV-positive patients with a low fraction of CD98-positive tumour cells compared to HPV-positive patients with high fraction of CD98-positive tumour cells. In the future, we might use CD98 expression as an additional prognostic marker for selection of HPV-positive patients in clinical trials.

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Conflict of interest statement

None declared.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2013.11.010.

References

[1] Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nat Rev Cancer 2011;11:9–22.
[2] Westra WH. The morphologic profile of HPV-related head and neck squamous carcinoma: implications for diagnosis, prognosis, and clinical management. Head Neck Pathol 2012;6(Suppl. 1):S48–54.
[3] Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 2010;363:24–35.
[4] Fakhry C, Westra WH, Li S, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst 2008;100:261–9.
[5] Kimple RJ, Smith MA, Blitzer GC, et al. Enhanced radiation sensitivity in HPV-positive head and neck cancer. Cancer Res 2013;73:4791–800.
[6] Spanos WC, Nowicki P, Lee DW, et al. Immune response during therapy with cisplatin or radiation for human papillomavirus-related head and neck cancer. Arch Otolaryngol Head Neck Surg 2009;135:1137–46.
[7] de Jong MC, Pramana J, van der Wal JE, et al. CD44 expression predicts local recurrence after radiotherapy in larynx cancer. Clin Cancer Res 2010;16:5329–38.
[8] Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006;444:756–60.
[9] Prince ME, Sivanandan R, Kaczorowski A, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci U S A 2007;104:973–8.
[10] Lindquist D, Ahrlund-Richter A, Tarjan M, Tot T, Dalianis T. Intense CD44 expression is a negative prognostic factor in tonsillar and base of tongue cancer. Anticancer Res 2012;32:153–61.
[11] Martens-de Kemp SR, Brink A, Stigter-van Walsum M, et al. CD98 marks a subpopulation of head and neck squamous cell carcinoma cells with stem cell properties. Stem Cell Res 2013;10:477–88.
[12] Esseghir S, Reis-Filho JS, Kennedy A, et al. Identification of transmembrane proteins as potential prognostic markers and therapeutic targets in breast cancer by a screen for signal sequence encoding transcripts. J Pathol 2006;210:420–30.
[13] Ichinoe M, Mikami T, Yoshida T, et al. High expression of L-type amino-acid transporter 1 (LAT1) in gastric carcinomas: comparison with non-cancerous lesions. Pathol Int 2011;61:281–9.
[14] Imai H, Kaira K, Oriuchi N, et al. Inhibition of L-type amino acid transporter 1 has antitumor activity in non-small cell lung cancer. Anticancer Res 2010;30:4819–28.
[15] Kaira K, Oriuchi N, Imai H, et al. Prognostic significance of L-type amino acid transporter 1 (LAT1) and 4F2 heavy chain (CD98) expression in early stage squamous cell carcinoma of the lung. Cancer Sci 2009;100:248–54.
[16] Kaira K, Takahashi T, Abe M, et al. CD98 expression is associated with the grade of malignancy in thymic epithelial tumors. Oncol Rep 2010;24:861–7.
[17] Sakata T, Ferdous G, Tsuruta T, et al. L-type amino-acid transporter 1 as a novel biomarker for high-grade malignancy in prostate cancer. Pathol Int 2009;59:7–18.
[18] Tang AL, Hauff SJ, Owen JH, et al. UM-SCC-104: a new human papillomavirus-16-positive cancer stem cell-containing head and neck squamous cell carcinoma cell line. Head Neck 2012;34:1480–91.
[19] Rietbergen MM, Leemans CR, Bloemena E, et al. Increasing prevalence rates of HPV attributable oropharyngeal squamous cell carcinomas in The Netherlands as assessed by a validated test algorithm. Int J Cancer 2013;132:1565–71.
[20] Rietbergen MM, Brakenhoff RH, Bloemena E, et al. Human papillomavirus detection and comorbidity: critical issues in selection of patients with oropharyngeal cancer for treatment De-escalation trials. Ann Oncol 2013;24:2740–5.
[21] Van Hal NL, van Dongen GA, Stigter-van Walsum M, Snow GB, Brakenhoff RH. Characterization of CD44v6 isoforms in head-and-neck squamous-cell carcinoma. Int J Cancer 1999;82:837–45.
[22] Hong SP, Wen J, Bang S, Park S, Song SY. CD44-positive cells are responsible for gemcitabine resistance in pancreatic cancer cells. Int J Cancer 2009;125:2323–31.
[23] Rich JN. Cancer stem cells in radiation resistance. Cancer Res 2007;67:8980–4.
[24] Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–11.
[25] Herold-Mende C, Seiter S, Born AI, et al. Expression of CD44 splice variants in squamous epithelia and squamous cell carcinomas of the head and neck. J Pathol 1996;179:66–73.
[26] Mack B, Gires O. CD44s and CD44v6 expression in head and neck epithelia. PLoS One 2008;3:e3360–7.
[27] Soukka T, Salmi M, Joensuu H, et al. Regulation of CD44v6-containing isoforms during proliferation of normal and malignant epithelial cells. Cancer Res 1997;57:2281–9.
[28] van Zeeburg HJ, van Beusechem V, Huizenga A, et al. Adenovirus retargeting to surface expressed antigens on oral mucosa. J Gene Med 2010;12:365–76.