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Martens-de Kemp, S.R.

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## Chapter 7

### Cancer stem cell enrichment marker CD98: a prognostic factor for survival in patients with HPV-positive oropharyngeal cancer.

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European Journal of Cancer, in press.

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Michelle M. Rietbergen\*  
Sanne R. Martens-de Kemp\*  
Elisabeth Bloemena  
Birgit I. Witte  
Arjen Brink  
Robert J. Baatenburg de Jong  
C. René Leemans  
Boudewijn J.M. Braakhuis  
Ruud H. Brakenhoff

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\*These authors contributed equally to this work.

**ABSTRACT**

**Objective:** Several hypotheses have been proposed to explain the relatively good prognosis of patients with an 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 percentage of CSCs and prognosis in patients with an HPV-positive OPSCC.

**Methods:** All OPSCC patients (n=711) treated between 2000 and 2006 in two Dutch university hospitals were included. Presence of HPV in a tumor tissue specimen was tested by p16-immunostaining followed by an HPV DNA GP5+/6+-PCR. The presence and intensity of tumor 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 tumors expressed less CD44 and CD98 than HPV-negative tumors ( $p < 0.001$ ,  $\chi^2$ -test). Within the group of patients with an HPV-positive OPSCC, a high percentage of CD98-positive tumor cells was associated with a significantly worse 5-years 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) ( $p < 0.001$ ).

**Conclusion:** HPV-positive OPSCCs harbor fewer cells expressing the CSC enrichment markers CD44 and CD98. Furthermore, OS and PFS were significantly worse for patients with an HPV-positive OPSCC with a high CD98 expression.

## **INTRODUCTION**

Infection with high-risk human papillomavirus (HPV) is etiologically 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 characterized by an epidemiologic, demographical and clinical profile that deviates from that of HPV-negative OPSCCs<sup>(1,2)</sup>. The most important difference is related to prognosis, which is markedly better for patients with an HPV-positive tumor compared to those with an HPV-negative tumor. Several hypotheses have been proposed to explain the improved outcome for patients with an 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 cancerization<sup>(3-6)</sup>. 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)<sup>(7)</sup>. CSCs represent a small subpopulation of tumor cells that maintain tumor growth by fuelling the expansion of the malignant cell population infinitely<sup>(8)</sup>. CSCs can be distinguished from the bulk of the tumor 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<sup>high</sup> HNSCC cells have been shown to initiate tumor growth in mice much more efficiently than CD44<sup>low</sup> cells, indicating that CSCs are enriched in the CD44<sup>high</sup> subpopulation of HNSCC<sup>(9)</sup>. Moreover, a high expression of CD44 seems associated with a poor prognosis in patients with HNSCC<sup>(10)</sup>.

Recently, we examined CD98 as a novel, putative CSC enrichment marker in HNSCC and showed that CD98<sup>high</sup> cells, in contrast to CD98<sup>low</sup> cells, are able to generate tumors in immune-deficient mice<sup>(11)</sup>. Studies in a multitude of cancer types showed a higher CD98 expression in progressive and metastatic tumors, which relates to a poor prognosis<sup>(12-17)</sup>.

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<sup>(18)</sup>. As patients with an HPV-positive OPSCC respond better to treatment and have a more favorable prognosis compared to patients with an HPV-negative OPSCC, we hypothesized 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<sup>(19)</sup>. Furthermore, we evaluated whether CD44 and CD98 expression could be of potential relevance for predicting treatment outcome in patients with an HPV-positive OPSCC.

## **MATERIAL AND METHODS**

### **Patients and tumor samples**

To evaluate CD44/CD98 immunostaining and the relation to HPV, a test cohort was composed, which included 85 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 RT-PCR<sup>(19)</sup>.

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<sup>(20)</sup>. A sample was scored HPV-positive based on a positive p16<sup>INK4A</sup>-immunohistochemistry (p16-IHC) and a subsequent positive HPV DNA GP5+/6+-PCR, according to a previously validated algorithm<sup>(19)</sup>. 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](http://www.federa.org)).

### **Immunohistochemical staining of CD44 and CD98**

Formalin-fixed paraffin-embedded sections of HNSCC tumor biopsies were deparaffinized and subjected to Tris/EDTA (10 mM/1 mM, pH 9.0) antigen retrieval. Primary antibodies U36 (anti-CD44v6, developed at our laboratory<sup>(21)</sup>) 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) was employed according to the description of the manufacturer. The staining was developed with diaminobenzidine and H<sub>2</sub>O<sub>2</sub> 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 over 70% of viable HNSCC tissue.

### **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 percentages of stained malignant cells: 1 ( $\leq 10\%$  of tumor cells stained), 2 (11-50% stained), 3 (51-75% stained) or 4 ( $>75\%$  stained)<sup>(10)</sup>. Percentage of CD98-positive cells was classified differently, in accordance to previous reports, as overall staining was lower: 1 ( $\leq 10\%$  of tumor cells stained), 2 (11-25% stained), 3 (26-50% stained) and 4 ( $>50\%$  stained)<sup>(15,16)</sup>. The intensity of CD44 and CD98 staining was scored separately and evaluated as absent (0), weak (1), moderate (2) and strong (3)<sup>(10)</sup>.

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.

### **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 OPSCCs were assessed using the Pearson  $\chi^2$ -test or Student's t-test. Bonferroni correction was used to compare subgroups for specific variables. The endpoints 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 categorized as local-regional recurrence or distant metastases. Patients who developed a second primary tumor were censored at the incidence date of that tumor. Survival rates were estimated by means of the Kaplan-Meier method and associations were analyzed 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 (ICC, with 95% confidence intervals [CI]) were computed to determine interobserver variability of the three investigators for percentages of CD44 and CD98 positive cells.

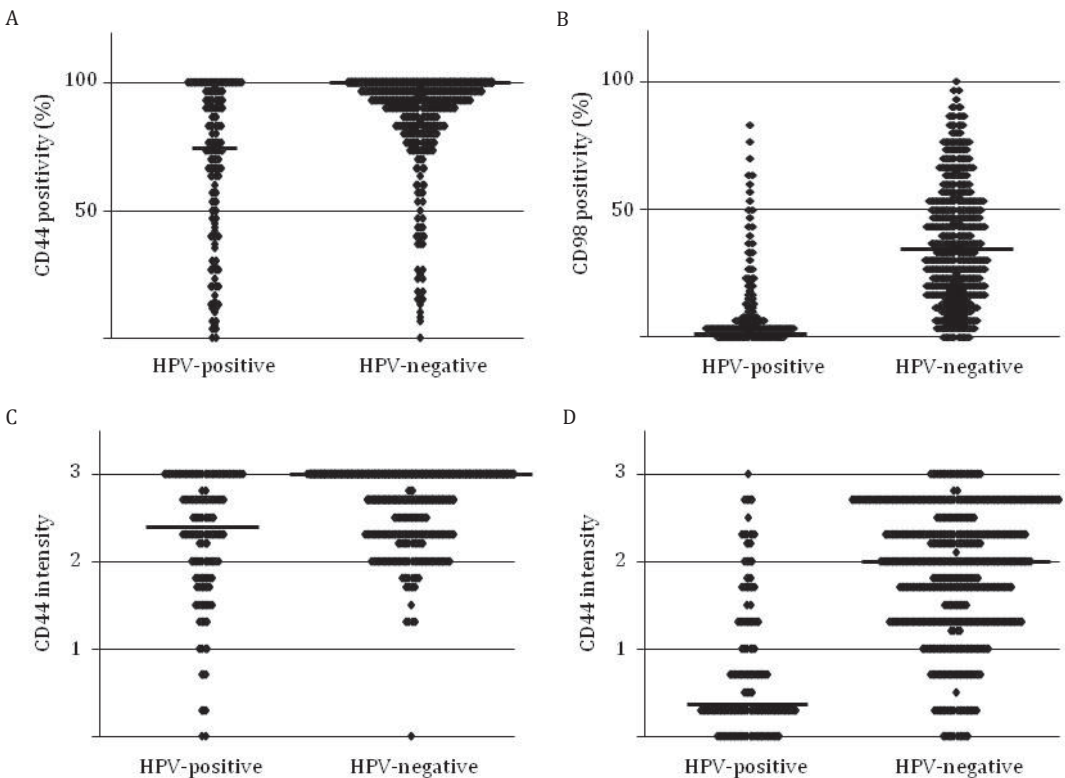
## RESULTS

### 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 OPSCCs and to evaluate the chosen cut-off values. CD44 staining intensity and percentage of CD44-positive cells were significantly higher in HPV-negative tumors (n=63) compared to HPV-positive tumors (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 tumors compared to HPV-positive tumors. Distribution curves for percentage of CD44/CD98-positive cells and CD44/CD98 intensity for this test cohort are depicted in Supplementary Fig. S1.

### Patient and tumor characteristics

Next, we investigated the relation between CD44/CD98 expression and survival in tumor biopsies obtained from a large, consecutive cohort of 711 OPSCC patients. Patient and tumor characteristics are depicted in Table 1. As published before, patients with HPV-positive tumors had less advanced tumor stages than patients with HPV-negative tumors, 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)<sup>(20)</sup>.



**Figure 1. Distribution curves for percentage of CD44/CD98-positive cells and CD44/CD98 intensity.**

Results of the scoring percentages of (A) CD44- and (B) CD98-positive tumor 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.

**Table 1. Patient and tumor characteristics.**

	HPV-positive OPSCCs	HPV-negative OPSCCs	p-value*	HPV-positive OPSCCs/ CD98 high	p-value**	All OPSCCs
	number (percentages)	number (percentages)		number (percentages)		number (percentages)
<i>No. of cases</i>	150 (21.1%)	561 (78.9%)		11		711
<i>Age at diagnosis</i>						
mean	61.0	60.6	$p=0.722†$	57.3	$p=0.31†$	60.7
median	58.8	59.4		55.6		59.3
<i>Gender</i>			$p=0.374‡$		$p=0.93‡$	
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%)
<i>Oropharyngeal subsite</i>			$p<0.001‡$		$p=0.23‡$	
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%)
<i>Smoking</i>			$p<0.001‡$		$p=0.37‡$	
never	44 (29.3%)	17 (3.0%)		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%)
<i>T-stage</i>			$p=0.001‡$		$p=0.93‡$	
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%)
<i>N-stage</i>			$p<0.001‡$		$p=0.08‡$	
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%)
<i>Tumor differentiation</i>			$p<0.001‡$		$p=0.26‡$	
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%)

Continued: Table 1. Patient and tumor characteristics.

	HPV-positive OPSCCs	HPV-negative OPSCCs	p-value*	HPV-positive OPSCCs/ CD98 high	p-value**	All OPSCCs
	number (percentages)	number (percentages)		number (percentages)		number (percentages)
<i>CD44 intensity</i>			$p < 0.001 \ddagger$		$p = 0.07 \ddagger$	
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%)
<i>CD44 expression</i>			$p < 0.001 \ddagger$		$p = 0.66 \ddagger$	
≤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%)
<i>CD98 intensity</i>			$p < 0.001 \ddagger$		$p < 0.001 \ddagger$	
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%)
<i>CD98 expression</i>			$p < 0.001 \ddagger$		$p < 0.001 \ddagger$	
≤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%)
<i>Treatment modalities</i>			$p < 0.001 \ddagger$		$p = 0.44 \ddagger$	
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, not otherwise specified; SURG, surgery; RT, radiotherapy; LND, lymph node dissection; CRT, chemoradiotherapy.

‡ Independent *t* test, † Chi square.

\*, HPV-positive group compared to HPV-negative group.

\*\*, HPV-positive OPSCCs with CD98 expression > 50% compared to HPV-positive OPSCCs with CD98 expression < 50%.



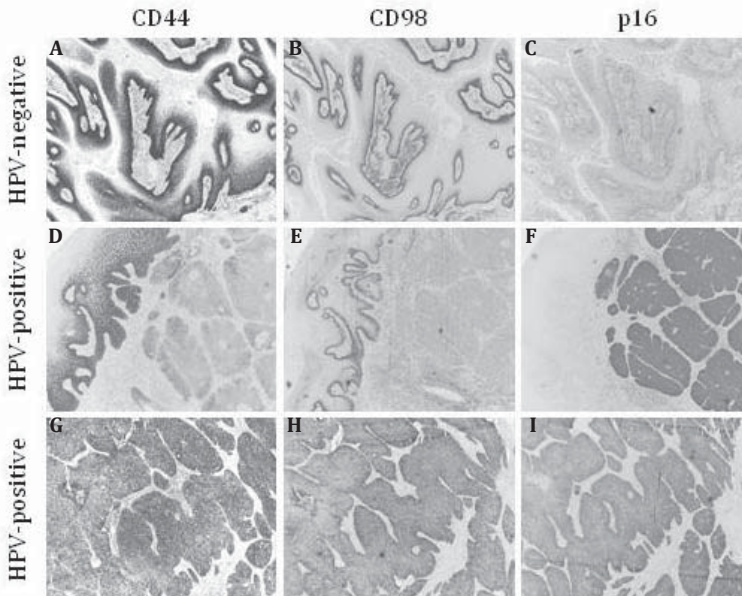
With regard to differentiation, the majority of all HPV-positive tumors (71.3%) were poorly differentiated, while the majority of the HPV-negative tumors (72.5%) were moderately differentiated. The intensity and percentage of CD44 and CD98 positive cells were not statistically different in poorly differentiated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

### CD44 expression

CD44 intensity as well as percentage of CD44-positive cells was compared between HPV-positive and HPV-negative tumors. In Fig. 2, examples of strong CD44-immunostaining (in an HPV-negative tumor) and weak CD44-immunostaining (in an HPV-positive tumor) are shown. The CD44 intensity, dichotomized as absent (0)/weak (1)/intermediate (2) (n=192) versus strong (3) (n=519), was compared between HPV-positive and HPV-negative tumors. Strong CD44-staining intensity (score=3) was observed in 445 of 561 (79.3%) HPV-negative tumors compared to 71 of 150 (47.3%) in HPV-positive tumors ( $p < 0.001$ , Pearson  $\chi^2$ -test). Furthermore, a high percentage of CD44-positive cells (i.e. >75% of malignant cells stained) was found in 72 of 150 HPV-positive tumors (48.0%) versus 503 of 561 in HPV-negative tumors (89.7%). This was a significant difference ( $p < 0.001$ ) (Table 1). The interobserver intraclass correlation coefficient (ICC) for percentage of CD44-positive cells was 0.88 (95% CI: 0.87-0.90).

### CD98 expression

CD98-immunostaining was less intense and a lower percentage of tumor cells was stained compared to CD44-immunostaining (Table 1). CD98-intensity was dichotomized 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 tumor cells were stained, in concordance with



**Figure 2.** CD44, CD98 and p16 staining on HPV-positive and HPV-negative OPSCC tissue specimens.

(A) CD44-immunostaining of an HPV-negative OPSCC. (B) CD98-immunostaining of an HPV-negative OPSCC. (C) Absence of p16<sup>INK4A</sup>-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<sup>INK4A</sup>-immunostaining of an HPV-negative OPSCC. (G) CD44-immunostaining of an HPV-positive OPSCC. (H) CD98-immunostaining of an HPV-positive OPSCC. (I) p16<sup>INK4A</sup>-immunostaining of an HPV-positive OPSCC. Pictures were taken through a 10x objective.

previously published reports<sup>(15,16)</sup>. In Fig. 2, examples of weak and strong CD98-immunostaining are depicted. CD98 expression differed significantly between HPV-positive and HPV-negative tumors. An intermediate or strong CD98-staining intensity (score 2/3) was observed in 401 of 561 (71.5%) HPV-negative tumors compared to 33 of 150 (22.0%) in HPV-positive tumors ( $p<0.001$ ). Furthermore, HPV-positive tumors showed a significantly smaller percentage of tumor cells with CD98 expression than HPV-negative tumors; a high percentage of CD98-positive cells (i.e.  $>50\%$  of malignant cells stained) was found in 11 of 150 HPV-positive tumors (7.3%) versus 194 of 561 in HPV-negative tumors (34.6%) ( $p<0.001$ ) (Table 1). The ICC for percentage of CD98-positive cells was 0.83 (95% CI: 0.81-0.84).

**Table 2. Univariate models for OS and PFS.**

	HPV-positive OPSCCs		HPV-negative OPSCCs	
	Hazard ratio (95% Confidence Interval)	p-value	Hazard ratio (95% Confidence Interval)	p-value
<b>Overall Survival</b>				
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 vs female)	0.79 (0.41-1.52)	$p=0.47$	0.81 (0.65-1.02)	$p=0.07$
Tumor size (T3-4 vs T1-2)	1.85 (1.03-3.35)	$p=0.04$	1.78 (1.42-2.22)	$p<0.001$
Nodal stage (N2-3 vs N0-N1)	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 vs 0 PY	1.56 (0.67-3.61)		1.28 (0.60-2.76)	
PY: $>24$ PY vs 0 PY	1.75 (0.83-3.68)		1.60 (0.80-3.22)	
CD44 intensity (score 3 vs score 0/1/2)	1.51 (0.79-2.87)	$p=0.21$	0.92 (0.73-1.15)	$p=0.46$
CD44 expression ( $\leq 75\%$ vs $>75\%$ )	0.78 (0.44-1.40)	$p=0.42$	0.87 (0.62-1.22)	$p=0.42$
CD98 intensity (score 2-3 vs score 0-1)	1.51 (0.79-2.87)	$p=0.21$	0.92 (0.73-1.15)	$p=0.46$
CD98 expression ( $\leq 50\%$ vs $>50\%$ )	2.92 (1.30-6.54)	$p=0.009$	0.86 (0.69-1.07)	$p=0.18$
<b>Progression-Free Survival</b>				
Age (per increase of 1 year)	1.05 (1.02-1.08)	$p<0.001$	1.02 (1.01-1.03)	$p=0.006$
Tumor size (T3-4 vs T1-2)	1.84 (1.04-3.27)	$p=0.04$	1.52 (1.21-1.91)	$p<0.001$
Gender (male vs female)	1.00 (0.56-1.84)	$p=1.00$	0.81 (0.64-1.02)	$p=0.08$
Nodal stage (N2-3 vs 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 vs 0 PY	1.65 (0.75-3.61)		1.01 (0.49-2.10)	
PY: $>24$ PY vs 0 PY	1.58 (0.77-3.24)		1.17 (0.60-2.28)	
CD44 intensity (score 3 vs score 0/1/2)	1.62 (0.87-3.02)	$p=0.13$	0.93 (0.73-1.18)	$p=0.54$
CD44 expression ( $\leq 75\%$ vs $>75\%$ )	0.86 (0.49-1.51)	$p=0.60$	0.97 (0.67-1.40)	$p=0.87$
CD98 intensity (score 2-3 vs score 0-1)	1.62 (0.87-3.02)	$p=0.13$	0.93 (0.73-1.18)	$p=0.93$
CD98 expression ( $\leq 50\%$ vs $>50\%$ )	3.57 (1.66-7.68)	$p=0.001$	0.90 (0.71-1.13)	$p=0.36$

### **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 tumors ( $p=0.204$ ) nor for patients with HPV-positive tumors ( $p=0.277$ ), 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 tumors. Prognostic factors entered in the univariate analysis were: age, nodal stage, tumor stage, gender, number of pack years, CD44 intensity, percentage of CD44-positive cells, CD98 intensity, and percentage of CD98-positive cells. In patients with an HPV-negative OPSCC, age, tumor size and nodal stage were all individually associated with OS and PFS outcomes. However, neither CD44 expression nor CD98 expression were of significant importance (Table 2). In patients with an HPV-positive OPSCC, age, tumor size and percentage of CD98-positive cells were prognostic factors for OS and PFS.

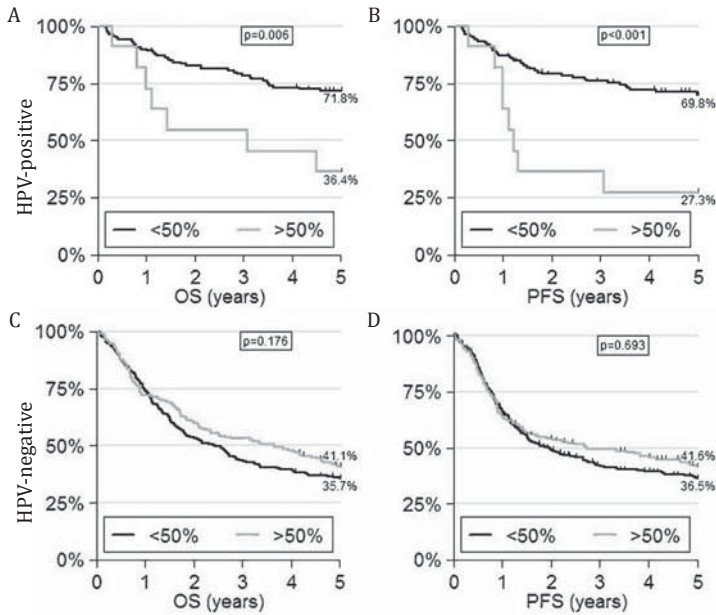
Patients with an HPV-positive OPSCC with a high percentage of CD98-positive cells (i.e.  $>50\%$  of malignant cells stained) had significantly worse 5-years OS and PFS rates (36.4% and 27.3%, respectively) compared to patients with a low percentage of CD98-positive cells (71.8% and 69.8%, respectively)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

Multivariate analysis was performed to estimate the association of all the analyzed factors with OS and PFS. Age ( $\leq 55$  years), tumor size (T1-2), and nodal stage (N0-N1) were independent prognostic factors for OS and PFS in patients with an HPV-negative OPSCC. In the group of patients with an HPV-positive OPSCC, smoking ( $\leq 24$  pack years), tumor size (T1-2), and a low percentage of CD98-positive cells (i.e.  $<50\%$  of stained tumor cells) were independent prognostic factors for OS and PFS (Table 3).

### **DISCUSSION**

Over the past decades, CSCs have been identified in multiple solid tumors and were implicated to play a role in resistance to anticancer treatments<sup>(22,23)</sup>. 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<sup>(24)</sup>. 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 hypothesized 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 tumors may have lower percentages 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 outcome in patients with an HPV-positive OPSCC. CD44 expression was not associated with survival neither in patients with an HPV-positive tumor nor in those with an HPV-negative OPSCC. This is likely due to the fact that CD44 was abundantly expressed in almost all tumors.

Several studies have shown that CD44 expression might be used as an outcome predictor in head and neck cancer<sup>(7,10)</sup>, but we cannot confirm this observation. 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 CSCs are assumed to reside) but also on the suprabasal cells, both on normal and malignant squamous tissue<sup>(21,25-28)</sup>. Subtle differences in CD44 expression might be 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



**Figure 3. Low percentage of CD98 is an independent prognostic factor of OS and PFS in HPV-positive OPSCCs.**

(A) 5-year OS curves for patients with an HPV-positive OPSCC and high percentage of CD98-positive cells (i.e. >50% of malignant cells stained; grey line) versus patients with a low to intermediate percentage of CD98-positive cells (i.e. ≤50% of malignant cells stained; black line) ( $p=0.006$ ). (B) 5-year PFS curves for patients with an HPV-positive OPSCC and high percentage of CD98-positive cells (i.e. >50% of malignant cells stained; grey line) versus patients with a low to intermediate percentage of CD98-positive cells (i.e. ≤50% of malignant cells stained; black 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; grey line) versus patients with a low to intermediate percentage of CD98-positive cells (i.e. ≤50% of malignant cells stained; black 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; grey line) versus patients with a low to intermediate percentage of CD98-positive cells (i.e. ≤50% of malignant cells stained; black line) ( $p=0.693$ ).

**Table 3. Multivariate models for OS and PFS.**

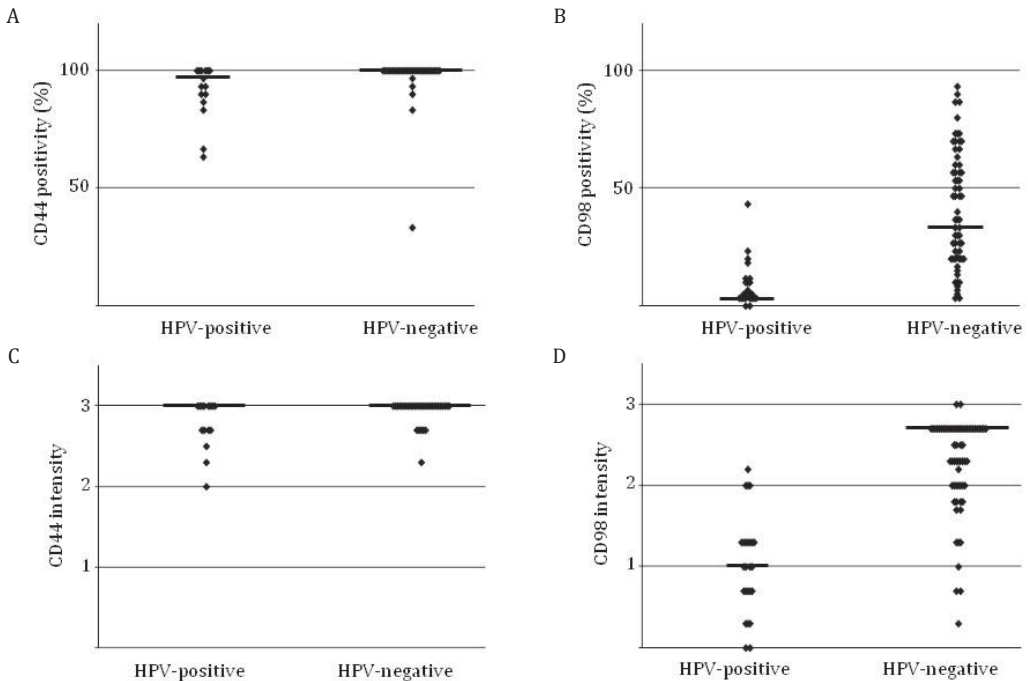
	HPV-positive OPSCCs		HPV-negative OPSCCs	
	Hazard ratio (95% Confidence Interval)	p-value	Hazard ratio (95% Confidence Interval)	p-value
<b>Overall Survival</b>				
Age (per increase of 1 year)	1.06 (1.03-1.09)	$p<0.001$	1.03 (1.02-1.05)	$p<0.001$
Tumor size (T3-4 vs T1-2)	2.22 (1.19-4.16)	$p=0.01$	1.51 (1.21-1.90)	$p<0.001$
Nodal stage (N2-3 vs N0-N1)			2.33 (1.86-2.91)	$p<0.001$
CD98 expression (<50% vs ≥ 50%)	0.23 (0.10-0.53)	$p=0.001$		
<b>Progression-Free Survival</b>				
Age (per increase of 1 year)	1.05 (1.03-1.08)	$p<0.001$	1.03 (1.01-1.04)	$p=0.001$
Tumor size (T3-4 vs T1-2)	2.28 (1.26-4.15)	$p=0.007$	1.32 (1.05-1.67)	$p=0.02$
Nodal stage (N2-3 vs N0-1)			2.18 (1.73-2.74)	$p<0.001$
CD98 expression (<50% vs ≥ 50%)	0.20 (0.09-0.45)	$p<0.001$		

cell population than CD44, making CD98 more distinctive than CD44. In patients with an HPV-negative OPSCC, no association was seen between CD98 expression and survival. However, in patients with an HPV-positive OPSCC, OS and PFS were significantly worse for patients with a high percentage of CD98-positive tumor 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 percentage of CD98-positive tumor 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 patients with an HPV-negative OPSCC, suggests that the molecular characteristics of these cells might be more relevant than the mere numbers. This requires additional investigation and characterization of these cells in responding and non-responding tumors.

In conclusion, most HPV-positive OPSCCs show low expression levels of the CSC markers CD44 and CD98 compared to HPV-negative OPSCCs. Furthermore, OS as well as PFS was markedly better for patients with an HPV-positive OPSCC with a low percentage of CD98-positive tumor cells compared to patients with an HPV-positive OPSCC with high percentage of CD98-positive tumor cells. In the future, we might use CD98 expression as an additional prognostic marker for selection of patients with HPV-positive OPSCCs in clinical trials.



**Supplementary Fig. S1. Distribution curves for percentage of CD44/CD98-positive cells and CD44/CD98 intensity in our test cohort.** Results of the scoring percentages of (A) CD44- and (B) CD98-positive tumor cells and the intensities of the immunohistochemical staining (C and D) in the test cohort, which contained 63 HPV-negative tumors and 25 HPV-positive tumors. The data is represented as the mean observation of the three observers. Thick horizontal lines represent the median value observed in the cohort.

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