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ONCOLOGY OF STEM CELL MARKERS' PROGNOSTIC FUNCTIONS IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS RECEIVING CHEMOTHERAPY

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Abstract

Oral squamous cell carcinoma (OSCC) is a top-ranked cancer in the global population, and patient survival has remained unchanged at ~50% for several decades. Recent advances have claimed that a subset of tumour cells, called cancer stem cells (CSCs), are responsible for tumour progression, treatment resistance, and metastasis, which leads to a poor prognosis. Anti-EGFR-based therapies have limited success in OSCC patients. Predictive biomarkers are needed to identify the patients most likely to benefit from these therapies. Here, we studied prognostic associations of different cancer stem cell markers in HPV-negative locally advanced (LA) OSCC patients. Pretreatment tumour tissues of 404 HPV-negative LA-OSCCs patients and a subset of study were comparing cisplatin-radiation (CRT) and nimotuzumab plus cisplatin-radiation(NCRT). The expression levels of CD44, CD44v6, CD98hc, ALDH1A1, SOX2 and OCT4A were evaluated using immunohistochemistry. Progression-free survival (PFS), loco-regional control(LRC),- and overall survival(OS) were estimated by Kaplan–Meier method. Hazard ratios were estimated by Cox proportional hazard models. NCRT showed significantly improved OS with low membrane expression of CD44 compared to CRT [HR (95% CI) = 0.69 (0.44–0.98)]. Patients with low CD44v6 also showed better outcomes with NCRT [LRC: HR (95% CI) = 0.25 (0.09–0.64); OS: HR (95% CI) = 0.39 (0.17–0.68)]. No similar benefit with NCRT observed in patients with high CD44 or CD44v6 expression. It was concluded that CD44 and CD44v6 are potential predictive biomarkers for NCRT response. CD98hc emerged as an independent negative prognostic biomarker.

Keywords: oncology, stem cell markers, prognostic functions, oral squamous cell carcinoma, chemotherapy

INTRODUCTION

The mainstay of care for patients with head and neck squamous cell carcinoma (HNSCC) is often concomitant chemoradiation for patients who have locally advanced (LA) primary disease [1]. One characteristic of HNSCCs is the presence of the epidermal growth factor receptor (EGFR), which is present in >80% of HNSCC tumours [2]. The Food and Drug Administration has only authorised EGFR-targeting treatment for LA-HNSCC patients [3]. Nevertheless, there hasn't been much success adding an EGFR-targeting monoclonal antibody (mAb) to radiation or chemoradiation therapy [4]. Furthermore, new information indicates that in patients with human papillomavirus (HPV)-positive HNSCC, EGFR targeting in conjunction with radiation is not a suitable replacement for cisplatin-radiation [5, 6]. High expression of EGFR ligands, HER3, Src family kinases, and the HGF/MET axis are among the resistance mechanisms for anti-EGFR therapy in HNSCCs that have been documented in the literature [7]. They have not yet been proven to be clinically useful in treatment decision-making, in contrast to colorectal and non-small cell lung cancer. Currently, these medications are given to patients randomly since prognostic biomarkers for HNSCC are lacking, which results in a low benefit to risk ratio [8]. EGFR-targeted treatments are costly, frequently hazardous, and only somewhat effective. Finding the patients who will benefit from these medicines is therefore essential. As of right now, these medicines lack validated indicators to predict treatment response. As demonstrated by our earlier study and others [9, 10], EGFR protein expression and gene amplification are not helpful in predicting responsiveness to EGFR mAbs in HNSCC patients. While its prognostic value in HNSCC patients is unknown, the severity of EGFR mAbs-induced skin rashes to cetuximab and panitumumab is linked to a greater response to these treatments [11]. Furthermore, skin rashes have a significant negative impact on patients' quality of life [12]. When compared to other anti-EGFR mAbs, nimotuzumab (h-R3), a humanised IgG1 mAb against EGFR, has been demonstrated to have low toxicity [13, 14]. Patil et al. reported enhanced loco-regional control (LRC) and progression-free survival (PFS) in unselected LA-HNSCC (>94% HPV-negative) patients treated with nimotuzumab plus cisplatin-radiation compared to patients treated with only cisplatin-radiation (CRT) in a Phase 3-randomised trial carried out in India [15]. High HIF1α was formerly thought to indicate a poor response to CRT. Furthermore, when compared to CRT, patients with low HIF1α did not exhibit the same improvement in response to NCRT as did those with high HIF1α. However, HIF1α expression did not show any signs of being predictive of a distinct response to the NCRT response [9]. Thus, in the current investigation, we assessed prognostic and predictive functions of various putative cancer stem cell (CSC) markers in the same patient cohort in order to identify predictive biomarkers for NCRT. One possible marker of the CSC in HNSCC is the cluster of differentiation (CD)44 [16]. It is a membrane glycoprotein that functions as a key hyaluronic acid receptor and mediates interactions between cells and between cells and matrices. Glycosylation and alternative splicing of ten variant exons, resulting in the production of distinct isoforms, control the function of CD44 [17]. The majority of vertebrate cells, including epithelial, immunological, and mesenchymal cells, express the smallest isoform, CD44s or CD44H. Other splice variants (CD44v1–v10), however, exhibit tissue-specific expression [18]. A role in tumour growth and metastasis is played by CD44s and their variant isoforms, which are overexpressed in a variety of malignancies, including HNSCC [19–21]. Another potential CSC marker for HNSCC is CD98 [22]. One type II single-pass transmembrane glycoprotein is the CD98 heavy chain (also known as CD98hc, 4F2hc, and SLC3A2). A component of β-integrin signalling, which is linked to carcinogenesis and cell dissemination, is CD98hc [23]. Along with its interactions, CD98hc functions as a chaperone to facilitate LAT1 trafficking, functional insertion, and stabilisation into the plasma membrane [24]. LAT1 is a multi-pass light chain of large neutral amino acid transporters. High expression levels of CD98hc are associated with radiation resistance and a poor prognosis in HNSCC, as demonstrated by Digomann et al. [25]. Moreover, octamer-binding

transcription factor 4 (OCT4, also known as POU5F1) and sex-determining region-Y homeobox-2 (SOX2) are significant pluripotency-associated transcription factors implicated in the preservation of embryonic stem cells' ability to self-renew [26–34]. A deeper understanding of the molecular profiles and how they affect treatment outcomes is necessary to increase a treatment's efficacy. Here, we evaluated the prognostic and predictive roles of different recognised CSC markers in OSCC patients treated with concurrent cisplatin-radiation with or without nimotuzumab. Main objective of study was to find out the oncology of stem cell markers' prognostic functions in oral squamous cell carcinoma patients receiving chemotherapy.

METHODOLOGY

Those who compared CRT with NCRT in 150 ONSCC patients were included in this study [15]. The inclusion and exclusion criteria were derived from a prior published study [15]. Formalin-fixed paraffin-embedded biopsy tumour samples for 122 patients who underwent HPV screening were available, as previously reported [35]. The cancer tissues had sufficient size. The remaining 104 HPV-negative tumour samples underwent biomarker expression analysis while being blinded to the patient's clinical results and treatment assignment. Every experimental procedure was carried out in compliance with the Helsinki Declaration. The Sher-i-Kashmir Institute of Medical Sciences in Soura, Srinagar, Jammu and Kashmir's Institutional Ethics Committee approved the project. The levels of protein expression were examined using the universal kit for immunohistochemistry (IHC). For CD44 (pH 9 for 12 min at 700 W), CD44v6 (pH 8 for 12 min at 700 W), CD98hc (pH 6 for 16 min at 560 W), ALDH1A1 (pH 8 for 16 min at 700 W), SOX2 (pH 6 for 5 min at 700 W following 10 min at 560 W), and OCT4A (pH 9 for 16 min at 560 W), antigen retrieval was done in a microwave oven using the appropriate buffer. After that, the sections were exposed for 14 hours with the main antibody. The primary antibodies were directed against ALDH1A1, SOX2, CD44, CD44v6, CD98hc, and OCT4A. Each antigen's IHC staining procedure was uniformed on the corresponding positive-control tissues. Two board-certified pathologists examined and authorised the staining. Two clinicians blind to the individual's outcomes and therapy administration assessed IHC staining in a semi-quantitative and impartial manner. While discontinuous staining was regarded as a "insufficient membrane staining pattern," complete membrane indicates continuous staining of the cancer cell membrane. By calculating the HScore, the expression levels of CD44, CD44v6, CD98hc, ALDH1A1, SOX2, and OCT4A were assessed. While continuous data are displayed as the median and range or interquartile range (IQR), categorical data are shown as frequencies and percentages. Correlations between continuous variables were found using Spearman's rank test. Utilising Pearson's chi-square test, the relationship between categorical variables was ascertained. The primary outcome was progression-free survival (PFS), with secondary endpoints being overall survival (OS) and locoregional control (LRC). The Kaplan-Meier approach was used to estimate PFS, LRC, and OS, and log-rank tests were used to compare the results. Using a univariate Cox regression model, the prognostic influence of each biomarker on clinical outcomes was examined, and hazard ratios (HRs) and 95% confidence intervals (CIs) were obtained. Once possible confounders were taken into account, multivariate analysis was performed using the backward likelihood ratio approach to evaluate the independent prognostic significance (clinical parameters, such as age, sex, clinical stage, and cancer site, associated with PFS, LRC, or OS with $P<0.20$). Cox models including treatments (NCRT versus CRT), biomarker status (low versus high), and the interaction between treatment impact and biomarker status were fitted in order to evaluate the predictive relevance of biomarkers [36]. The bootstrap resampling approach was used to perform internal validation of the prognostic and predictive models, and concordance indices (c-indices) were computed. Version 23.0 of the SPSS programme was used for all statistical analyses. P-values were all two-sided and considered statistically significant if they were less than 0.05.

RESULTS AND DISCUSSION

As previously reported [9], the baseline characteristics of the 104 study patients were representative of the study and balanced between the two therapy groups. Table 1 provides a list of the patients' demographic information that was part of this investigation. In 104 HPV-negative cases—of which 06 underwent CRT and 98 underwent NCRT treatment—we performed biomarker analysis. Between the two treatment groups, the overall frequency distribution of all biomarkers was similar (Figure 1a-e). Around 74%, 92%, and 77% of the HNSCC tumours showed full membrane expression of CD44, CD44v6, and CD98hc, respectively. The immune cells were also found to express CD44 and CD98hc [38]. These cells did not, however, express CD44v6 [39]. Figure 2a-c displays representative IHC staining images showing full membrane expression of CD44, CD44v6, and CD98hc. The majority of ALDH1A1 expression was seen in the cancer cells' cytoplasm. In about 51.4% of cases, ALDH1A1 expression was detected. Of the patients, nuclear SOX2 staining was visible in about 73.6%. Supplementary Fig. 2A, B displays representative IHC staining images demonstrating nuclear SOX2 expression and cytoplasmic ALDH1A1 expression. Nuclear staining of OCT4A was not observed in any of the tumour tissues, although testicular seminoma tissue used as positive control showed strong nuclear OCT4A staining (Figure 3). Univariate Cox analysis revealed that low CD98hc expression defined using the cut-off of 40 was significantly associated with longer OS (HR = 0.63, 95% CI = 0.41–0.96; 53.9 vs 33.4 months) when compared to high CD98hc expression (n = 111). No significant association was found for PFS (HR = 0.75, 95% CI = 0.50–1.13; 49.7 vs 36.3 months) and LRC (HR = 0.66, 95% CI = 0.41–1.04; 59.6 vs 43.8 months) (Figure 3a-c). Correlation among biomarkers and between biomarker status and clinicopathological parameters Correlations between different biomarkers (continuous and categorical) are given in Supplementary Table 2A, B respectively. A weak but significant positive correlation was detected between CD44-CD44v6 (rho = 0.45). ALDH1A1 and SOX2 showed a moderate positive correlation (rho = 0.69) [36].

Table1: Demographic characteristics of patients

Characteristics	CRT (n=54)	NCRT (n= 50)
Age (Years)		
40 or below	7.8%	9.6%
>40 and <60	64.1%	55.6%
60 and above	28.1%	34.8%
Gender		
Male	88.3%	86.4%
Female	11.7%	13.6%
ECOG PS		
0	22.8%	22.2%
1-2	77.2%	77.8%
Site of tumor		
Hypopharynx	20.4%	24.7%
Larynx	32%	24.7%
Oral Cavity	1%	0%
Oropharynx	46.6%	50.5%
Clinical stage[a]		
II	0%	0%
III	28.2%	20.2%
IVA	27.7%	32.8%
IVB	44.2%	47.0%
T stage[a]		
T1-T2	19.9%	17.2
T3-T4	80.1%	82.8%
N stage[a]		
N0-N1	38.8%	32.3%
N2-N3	61.2%	67.7%
No habits	6.8%	8.1%
No information	1.9%	3.5%

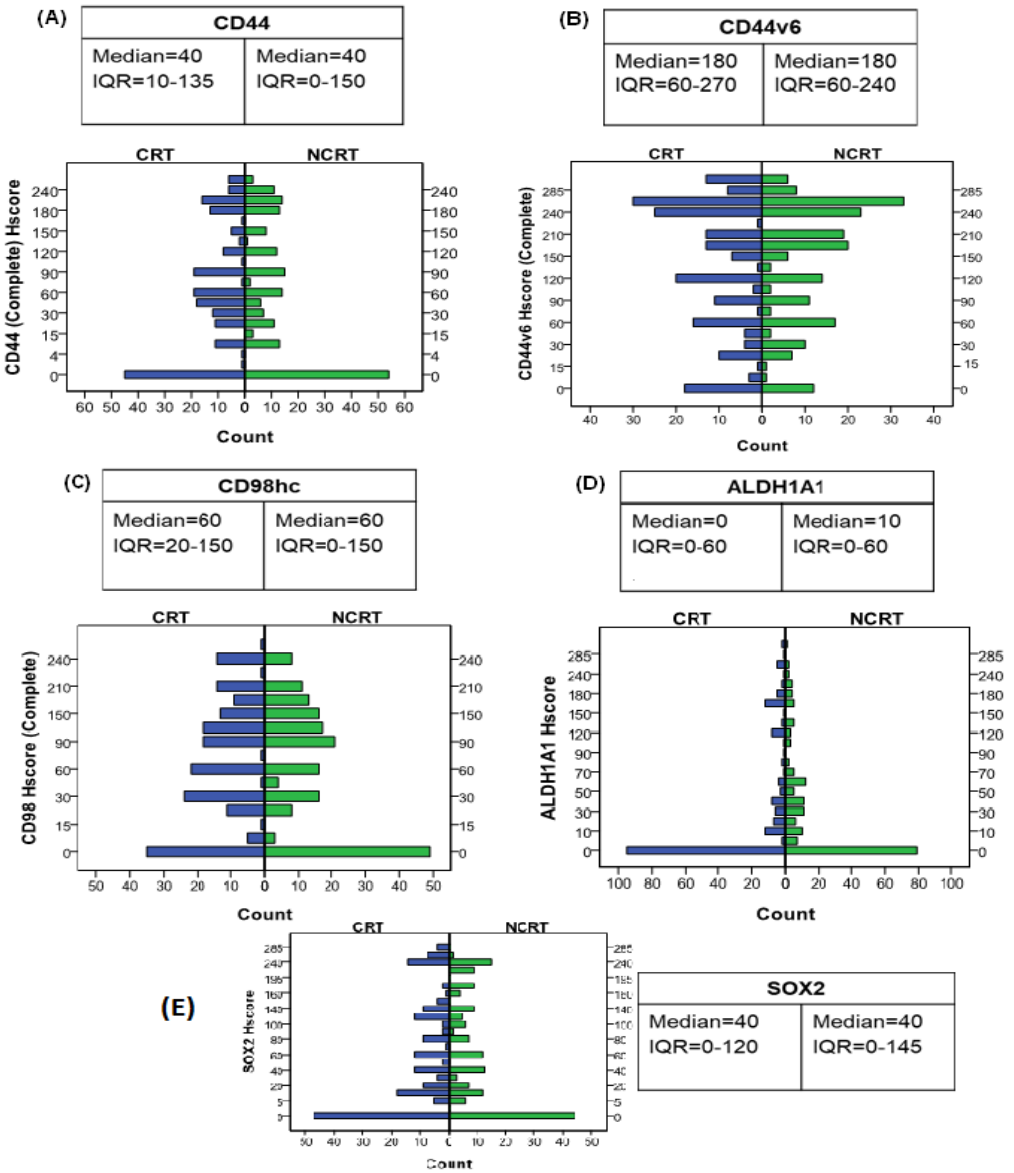

Figure 1: Histograms of frequency distributions of different biomarker HScore. (A) Membrane CD44, (B) membrane CD44v6, (C) membrane CD98hc, (D) cytoplasmic ALDH1A1, and (E) nuclear SOX2. The abbreviations are: IQR=inter quartile range; CRT=cisplatin radiation therapy; and NCRT=nimotuzumab plus cisplatin radiation therapy.

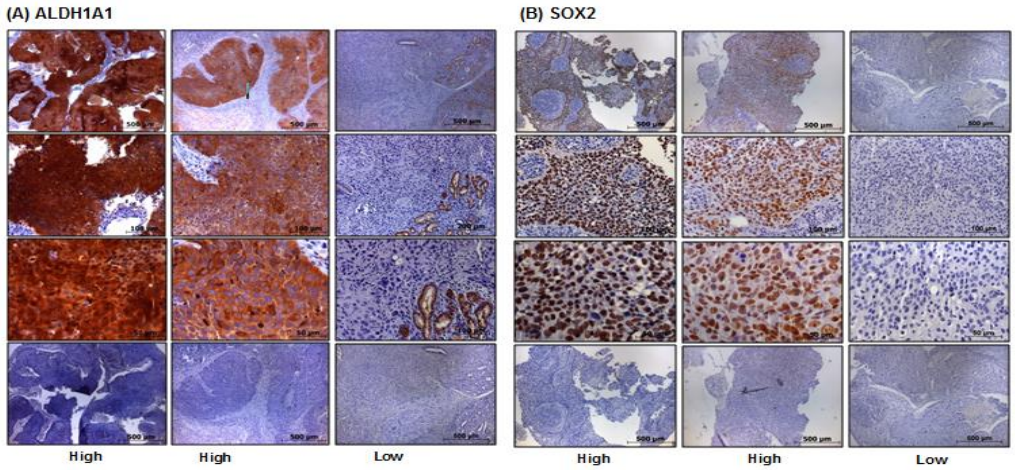

Figure 2: Representative images of immunohistochemistry (IHC) staining.

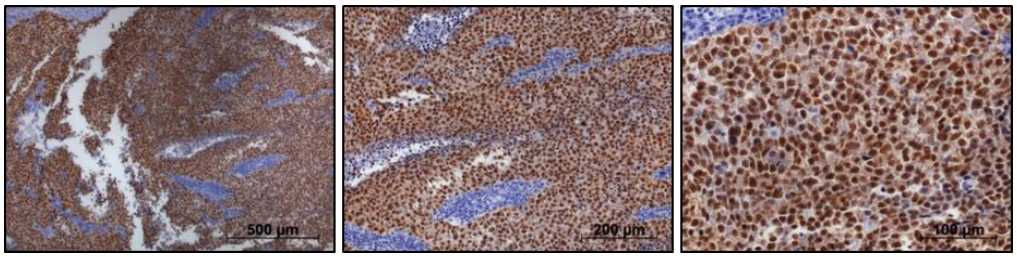

Figure 3: Representative Immunohistochemistry (IHC) staining results. Strong nuclear staining of OCT4A in positive control tissue (testicular seminoma)

Table 2: Correlation among different biomarkers (continuous)

		CD44v6	CD98hc	ALDH1A1	SOX2	HIF1α	EGFR
CD44	R	.45[a]	.24[a]	-.12[b]	-.12[b]	0.21[a]	.20[a]
	P	<0.0001	<0.0001	0.027	0.032	<0.0001	<0.0001
CD44v6	R	1	.24[a]	-0.02	0	0.15[a]	.15[a]
	P	.	<0.0001	0.663	0.993	0.003	0.002
CD98hc	R		1	-.13[b]	-0.10	0.22[a]	.18[a]
	P		.	0.013	0.058	<0.0001	0.001
ALDH1A1	R			1	.69[a]	-0.017	-.12[b]
	P			.	<0.0001	0.757	0.019
SOX2	R				1	-0.048	-.12[b]
	P				.	0.376	0.03

rho= Spearman's correlation coefficient; ([a]) Correlation is significant at the 0.01 level (2-tailed); ([b]) Correlation is significant at the 0.05 level (2-tailed).

Table 2B: Correlation among different biomarkers (categorical)

		CD44v6		CD98hc		ALDH1A1		SOX2		HIF1α		EGFR	
		Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
CD44	Low, (%)	23.5	76.5	45.2	54.8	77.9	22.1	48.7	51.3	54.2	45.8	54.7	45.3
	High, (%)	1.2	98.8	30.8	69.2	84.7	15.3	65.7	34.3	43.0	57.0	36.1	63.9
	P	<0.0001		0.027		0.253		0.015		0.099		0.003	
	R	0.24		0.12		-0.07		-0.14		0.09		0.15	
CD44v6	Low, (%)			53.1	46.9	86.7	13.3	58.3	41.7	62.5	37.5	52.1	47.9
	High, (%)			40.5	59.5	77.6	22.4	52.5	48.8	48.3	51.7	50	50
	P			0.041		0.112		0.324		0.036		0.751	
	R			0.10		0.08		0.05		0.11		0.016	
CD98hc	Low, (%)					76.7	23.3	45.9	54.1	61.0	39.0	57.3	42.7
	High, (%)					81.0	19.0	57.4	42.6	44.9	55.1	44.6	55.4
	P					0.356		0.038		0.003		0.016	
	R					-0.05		-0.11		0.16		0.13	
ALDH1A1	Low, n (%)							64.0	36.0	52.7	47.3	49.5	50.4
	High, n (%)							8.3	91.7	46.6	53.4	54.1	45.9
	P							<0.0001		0.360		0.5	
	R							0.46		0.05		-0.036	
SOX2	Low, n (%)									50.8	49.2	46.4	53.6
	High, n (%)									53.1	46.9	53.7	46.3
	P									0.745		0.179	
	R									-0.022		-0.072	

R=Pearson's correlation coefficient; P values <0.05 were considered statistically significant. For categorizing biomarkers HScore cutpoint used were 150 (CD44), 40 (CD44v), 40 (CD98hc), 70 (ALDH1A1), 40 (SOX2), 90 (HIF1α) and 100 (membrane EGFR)

Prognostic association of biomarkers Univariate Cox regression analysis was performed in the CRT group to determine the prognostic significance of the biomarkers. At the median HScore cut-off,

CD44 or CD44v6 did not show any association with PFS, LRC or OS. Additionally, CD44 or CD44v6 did not show any significant association with PFS, LRC or OS when dichotomised at different possible HScore cut-offs, suggesting no prognostic role of these biomarkers in these patients (Table 3A, B). HRs for disease progression, loco-regional failure and death were lower for patients with low CD98hc when dichotomised at lower cut-off points (H score = 0 or ≤20 or ≤40), suggesting better clinical outcomes in these patients than in patients expressing high CD98hc (Table 3C). We did not observe any prognostic association of ALDH1A1 expression at any of the studied cut-offs (Table 3D). HRs for disease progression, loco-regional failure and death were >1.0 for the patients with low SOX2 defined using most of the cut-offs. However, no statistically significant association was observed between SOX2 status and any of the studied clinical endpoints, suggesting no prognostic role of SOX2 in these patients (Table 3E). Multivariable Cox analyses included clinical characteristics (age, clinical stage and tumour site) associated with PFS, LRC or OS ($P < 0.20$) reported previously [9]. In multivariable analysis, CD98hc expression maintained an independent prognostic significance for LRC (HR = 0.63, 95% CI = 0.39–1.0, P = 0.049) and OS (HR = 0.62, 95% CI = 0.40–0.95, P = 0.028) (Table 4). Previously, we have reported prognostic association of HIF1α expression. Low-HIF1α expression defined at median cut-off of 90 (n = 108) was significantly associated with better LRC (HR = 0.58, 95% CI = 0.38–0.89) and OS (HR = 0.62, 95%CI = 0.42–0.91) but not with PFS (HR = 0.69, 95% CI = 0.47–1.01) when compared high HIF1α expression (n = 91) in univariate Cox analysis. Therefore, we constructed a second multivariable model with previously analysed biomarkers (pEGFRY1068, pEGFRY1173 and HIF1α) associated with PFS, LRC or OS (at $P < 0.20$ in univariate Cox analysis) [9]. The results implicated both HIF1α and CD98hc as negative prognostic biomarkers, although the prognostic impact of HIF1α expression was stronger than that of CD98hc expression. We did not find a statistically significant interaction between CD44 status and treatment effect for PFS and LRC at any of the studied cut-offs.

Table 3 (A): Analysis to assess the prognostic role of complete membrane CD44 HScore

CRT (n=196)			**PFS**		**LRC**		**OS**	
Cutpoint	**Low**	**High**	**HR (95% CI)**	***P*** [a]	**HR (95% CI)**	***P*** [a]	**HR (95% CI)**	***P*** [a]
0 &>0	45	51	0.89 (0.57-1.41)	0.620	0.98 (0.60-1.59)	0.923	1.08 (0.69-1.67)	0.740
≤15 &>15	58	38	0.88 (0.58-1.34)	0.550	0.96 (0.61-1.51)	0.863	1.09 (0.73-1.65)	0.666
≤20 &>20	69	27	1.07 (0.72-1.58)	0.757	1.02 (0.66-1.57)	0.945	1.24 (0.84-1.84)	0.281
≤40 &>40	**99**	**97**	**1.05 (0.72-1.54)**	**0.791**	**1.11 (0.73-1.69)**	**0.621**	**1.15 (0.78-1.69)**	**0.496**
≤60 &>60	98	78	1.01 (0.68-1.49)	0.979	1.11 (0.72-1.71)	0.650	1.16 (0.77-1.74)	0.479
≤90 &>90	38	58	1.15 (0.75-1.76)	0.534	1.13 (0.71-1.81)	0.601	1.22 (0.78-1.90)	0.391
≤120 &>120	47	49	1.40 (0.87-2.24)	0.163	1.28 (0.77-2.13)	0.338	1.46 (0.89-2.40)	0.139
≤150 &>150	54	42	1.27 (0.78-2.06)	0.343	1.19 (0.70-2.02)	0.525	1.43 (0.84-2.44)	0.187
≤180 &>180	68	28	0.89 (0.52-1.52)	0.675	0.81 (0.46-1.44)	0.470	1.01 (0.56-1.81)	0.971

Table 3 (B): Analysis to assess the prognostic role of complete membrane CD44v6 HScore

CRT (n=201)			**PFS**		**LRC**		**OS**	
Cutpoint	**Low**	**High**	**HR (95% CI)**	***P*** [a]	**HR (95% CI)**	***P*** [a]	**HR (95% CI)**	***P*** [a]
≤15 &>15	22	79	0.88 (0.47-1.65)	0.700	0.77 (0.37-1.59)	0.474	1.14 (0.64-2.05)	0.652
≤30 &>30	36	76	1.09 (0.68-1.76)	0.722	1.06 (0.63-1.81)	0.820	1.24 (0.78-1.98)	0.355
≤40 &>40	40	66	1.14 (0.72-1.79)	0.583	1.15 (0.70-1.90)	0.574	1.28 (0.82-1.99)	0.278
≤60 &>60	56	99	0.92 (0.60-1.41)	0.693	0.92 (0.58-1.47)	0.732	1.08 (0.71-1.63)	0.730
≤90 &>90	68	103	0.82 (0.55-1.24)	0.353	0.82 (0.52-1.28)	0.384	0.86 (0.57-1.29)	0.463
≤120 &>120	90	101	1.13 (0.77-1.66)	0.538	1.02 (0.67-1.56)	0.919	1.13 (0.77-1.66)	0.538
≤150 &>150	98	103	1.04 (0.71-1.52)	0.840	1.10 (0.73-1.68)	0.646	1.21 (0.83-1.79)	0.323
≤180 &>180	**90**	**101**	**1.16 (0.79-1.71)**	**0.447**	**1.19 (0.77-1.81)**	**0.435**	**1.28 (0.87-1.90)**	**0.215**
≤210 &>210	77	94	1.05 (0.71-1.57)	0.795	1.06 (0.69-1.64)	0.787	1.22 (0.82-1.84)	0.330
≤240 &>240	51	95	0.93 (0.60-1.44)	0.748	1.01 (0.62-1.65)	0.969	1.01 (0.64-1.58)	0.979
≤270 &>270	21	98	0.78 (0.43-1.43)	0.430	0.77 (0.40-1.49)	0.442	0.76 (0.40-1.42)	0.383

Table 3 (C): Analysis to assess the prognostic role of complete membrane CD98hc HScore

CRT (n=188)			PFS		LRC		OS	
Cutpoint	**Low**	**High**	**HR (95% CI)**	***P*** [a]	**HR (95% CI)**	***P*** [a]	**HR (95% CI)**	***P*** [a]
0 &>0	35	153	0.55 (0.30-1.0)	0.050	0.36 (0.17-0.79)	0.011	0.59 (0.32-1.08)	0.086
≤20 &>20	52	136	0.69 (0.43-1.11)	0.122	0.58 (0.34-1.01)	0.054	0.67 (0.41-1.09)	0.103
≤40 &>40	77	111	0.75 (0.50-1.13)	0.171	0.66 (0.41-1.04)	0.071	0.63 (0.41-0.96)	0.032
≤60 &>60	**99**	**89**	**0.87 (0.58-1.29)**	**0.476**	**0.77 (0.50-1.20)**	**0.250**	**0.67 (0.45-1.01)**	**0.054**
≤90 &>90	118	70	0.96 (0.64-1.44)	0.837	0.86 (0.55-1.35)	0.510	0.67 (0.45-1.0)	0.051
≤120 &>120	136	52	0.94 (0.61-1.46)	0.788	0.86 (0.54-1.40)	0.550	0.69 (0.45-1.06)	0.088
≤180 &>180	158	30	0.92 (0.54-1.57)	0.749	0.91 (0.50-1.65)	0.758	0.67 (0.41-1.11)	0.119

Table 3 (D): Analysis to assess the prognostic role of ALDH1A1 HScore

CRT (n=181)			PFS		LRC		OS	
Cutpoint	**Low**	**High**	**HR (95% CI)**	***P*** [a]	**HR (95% CI)**	***P*** [a]	**HR (95% CI)**	***P*** [a]
0 & >0	95	86	0.84 (0.56-1.27)	0.842	0.79 (0.50-1.24)	0.306	0.88 (0.58-1.33)	0.536
≤5 &>5	**97**	**84**	**0.85 (0.56-1.27)**	**0.426**	**0.80 (0.51-1.26)**	**0.342**	**0.84 (0.55-1.26)**	**0.393**
≤10 &>10	109	72	1.02 (0.67-1.55)	0.927	1.0 (0.63-1.58)	0.997	0.93 (0.61-1.42)	0.744
≤30 &>30	122	59	0.96 (0.62-1.48)	0.841	0.89 (0.55-1.43)	0.631	1.04 (0.66-1.63)	0.878
≤50 &>50	133	48	1.21 (0.74-2.0)	0.450	0.94 (0.57-1.56)	0.824	1.21 (0.74-2.0)	0.450
≤60 &>60	137	44	1.14 (0.70-1.86)	0.593	1.20 (0.70-2.05)	0.518	1.40 (0.82-2.37)	0.217
≤70 &>70	138	43	1.14 (0.70-1.86)	0.597	1.19 (0.70-2.05)	0.521	1.50 (0.87-2.58)	0.142
≤120 &>120	150	31	1.0 (0.58-1.71)	0.494	1.02 (0.56-1.85)	0.951	1.24 (0.67-2.27)	0.494

Table 3 (E): Analysis to assess the prognostic role of SOX2 HScore

CRT (n=176)			PFS		LRC		OS	
Cut point	**Low**	**High**	**HR (95% CI)**	***P*** [a]	**HR (95% CI)**	***P*** [a]	**HR (95% CI)**	***P*** [a]
0 & >0	47	79	1.13 (0.71-1.80)	0.597	1.04 (0.62-1.75)	0.885	1.31 (0.84-2.06)	0.231
≤10 &>10	70	102	1.01 (0.66-1.53)	0.970	0.96 (0.60-1.54)	0.867	1.19 (0.78-1.80)	0.424
≤30 &>30	83	93	0.99 (0.66-1.50)	0.977	0.81 (0.51-1.29)	0.381	1.09 (0.72-1.64)	0.695
≤40 &>40	**95**	**81**	**1.08 (0.72-1.64)**	**0.703**	**0.87 (0.55-1.37)**	**0.538**	**1.12 (0.74-1.70)**	**0.599**
≤60 &>60	109	67	1.05 (0.69-1.62)	0.809	0.92 (0.58-1.47)	0.732	1.01 (0.66-1.56)	0.952
≤90 &>90	121	55	1.21 (0.77-1.92)	0.415	1.08 (0.66-1.78)	0.767	1.05 (0.66-1.65)	0.841
≤120 &>120	135	41	1.25 (0.74-2.12)	0.402	1.12 (0.64-1.98)	0.694	1.04 (0.62-1.75)	0.875
≤150 &>150	148	28	1.39 (0.74-2.61)	0.308	1.16 (0.60-2.26)	0.662	1.41 (0.73-2.71)	0.311

([a]) Univariate Cox regression analysis. Results at median cutpoint are highlighted in bold. CRT=cisplatin-radiation alone; HR=hazard ratio; CI=confidence interval; PFS=progression free survival; LRC=loco-regional control; OS=overall survival.

Table 4: Prognostic significance of clinical parameters and biomarkers in the CRT group

Variables	**Univariate Cox analysis**		**Multivariable Cox analysis**[a]	
	HR (95% CI)	***P* value**	**HR (95% CI)**	***P* value**
Progression free survival (PFS)				
Age (< 60 vs ≥ 60 years)	1.46 (0.94-2.28)	0.002	1.51 (0.94-2.43)	0.001
[c]linical stage (III vs. IV)	0.48 (0.30-0.78)	0.000	0.46 (0.27-0.77)	0.000
Site of tumor (oropharynx vs others)	1.74 (1.19-2.56)	0.001	-	-
CD98hc (low vs high)	0.75 (0.50-1.13)	0.131	-	-
Loco-regional control (LRC)				
Age (< 60 vs ≥ 60 years)	1.49 (0.91-2.43)	0.001	1.56 (0.91-2.68)	0.005
[c]linical stage (III vs. IV)	0.43 (0.25-0.75)	0.003	0.39 (0.21-0.70)	0.002
Site of tumor (oropharynx vs others)	1.58 (1.05-2.40)	0.030	-	-
CD98hc (low vs high)	0.66 (0.41-1.04)	0.071	**0.63 (0.39-1.0)**	**0.040**
Overall survival (OS)				
Age (< 60 vs ≥ 60 years)	1.59 (1.0-2.53)	0.042	1.55 (0.95-2.55)	0.082
[c]linical stage (III vs. IV)	0.64 (0.40-1.00)	0.041	-	-
Site of tumor (oropharynx vs others)	1.62 (1.10-2.37)	0.010	1.60 (1.06-2.40)	0.022
CD98hc (low vs high)	0.63 (0.41-0.96)	0.002	**0.62 (0.40-0.85)**	**0.021**

([a]) A multivariate Cox model using backward likelihood ratio method was applied to adjust for potential confounders (clinical characteristics associated with PFS, LRC or OS at *P*<0.20 in univariate analysis). HR=hazard ratio; CI=confidence interval. (-) data not available; ([b]) According to AJCC-UICC system (8th edition).

Our earlier research revealed the dual prognostic functions of HIF1α expression status in HPV-negative OSCC patients receiving CRT or NCRT [9]. Using the same patient group, we assessed the

prognostic significance of potential CSC markers in OSCCs in the current investigation. When compared to CRT alone, the results showed that full membrane expression of CD44 and CD44v6 could predict clinical improvement from nimotuzumab added to CRT treatment. These biomarkers have the potential to reduce overtreatment by identifying individuals (low CD44 or CD44v6) who need NCRT treatment for an improved clinical result and designating those (high CD44 or CD44v6) who might not benefit from NCRT. Furthermore, we demonstrated the significance of full CD98hc membrane expression in prognosis. The research has reported on the predictive importance of CD98hc in HNSCC [25, 37]. Many studies have been conducted on the prognostic significance of CD44 and its variant isoforms in HNSCC. The results, meanwhile, have been contradictory [21]. No predictive correlation between CD44 or CD44v6 was observed at any of the HScore cut-offs that we looked at. Patients with HPV-DNA-negative HNSCC have been found to have no prognostic correlation with CD44 [38]. Our findings further suggest that resistance to EGFR-based therapy may be linked to increased expression of CD44 or CD44v6. Comparable in vitro investigations following nimotuzumab or cetuximab treatment do not demonstrate this connection. Nonetheless, a variety of in vitro investigations have documented interactions between CD44 and EGFR and erbB2 in several malignancies, including HNSCC, and these interactions may be a factor in the development of resistance to EGFR-targeted treatments [39–41]. Additionally, it has been noted that in HNSCC, the hyaluronan-CD44 association stimulates EGFR activation independently of EGF [42, 43]. The current study offers the first proof of the significance of CD44 and CD44v6 in EGFR-based therapy response prediction in patients with HPV-negative OSCC. Recent research has revealed a negative prognostic correlation between the expression of the CD98hc (SLC3A2) gene and HPV-negative LA-HNSCC [42-44].

CONCLUSION

In summary, for HPV-negative ONSCC patients, this is the first thorough study demonstrating the potential of CD44 and CD44v6 alone or in combination for NCRT therapy response. Furthermore, HIF1α has been shown to be a more reliable predictive biomarker than CD98hc. Making an investigation into the relationship between HIF1α and potential CSC indicators and clinical outcomes in patients with HNSCC will be very helpful in guiding treatment choices. These biomarkers may aid in the classification of HNSCC patients for either conventional or EGFR-based targeted therapy following validation of the findings in a larger cohort.

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