British Journal of Cancer www.nature.com/bjc

ARTICLE

Prognostic and predictive roles of cancer stem cell markers in head and neck squamous cell carcinoma patients receiving chemoradiotherapy with or without nimotuzumab

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BACKGROUND: Anti-EGFR-based therapies have limited success in HNSCC patients. Predictive biomarkers are needed to identify the patients most likely to benefit from these therapies. Here, we present predictive and prognostic associations of different cancer stem cell markers in HPV-negative locally advanced (LA) HNSCC patients.

METHODS: Pretreatment tumour tissues of 404 HPV-negative LA-HNSCCs patients, a subset of—phase 3-randomised study comparing cisplatin-radiation(CRT) and nimotuzumab plus cisplatin-radiation(NCRT) were examined. 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.

RESULTS: NCRT showed significantly improved OS with low membrane expression of CD44 compared to CRT [HR (95% CI) = 0.63 (0.46–0.88)]. Patients with low CD44v6 also showed better outcomes with NCRT [LRC: HR (95% CI) = 0.25 (0.10–0.62); OS: HR (95% CI) = 0.38 (0.19–0.74)]. No similar benefit with NCRT observed in patients with high CD44 or CD44v6 expression. Bootstrap resampling confirmed the predictive effect of CD44 (Interaction $P = 0.015$) and CD44v6 (Interaction $P = 0.041$) for OS. Multivariable Cox analysis revealed an independent negative prognostic role of CD98hc membrane expression for LRC [HR (95% CI) = 0.63 (0.39–1.0)] and OS[HR (95% CI) = 0.62 (0.40–0.95)].

CONCLUSIONS: CD44 and CD44v6 are potential predictive biomarkers for NCRT response. CD98hc emerged as an independent negative prognostic biomarker.

CLINICAL TRIAL REGISTRATION: Registered with the Clinical Trial Registry of India (Trial registration identifier—CTRI/2014/09/004980).

British Journal of Cancer (2022) 126:1439–1449; https://doi.org/10.1038/s41416-022-01730-9

INTRODUCTION

Patients with head and neck squamous cell carcinoma (HNSCC) frequently present with locally advanced (LA) primary disease with concurrent chemoradiation as the standard treatment of care [1]. Epidermal growth factor receptor (EGFR) expression occurs in >80% of HNSCC tumours and is a hallmark of HNSCCs [2]. The only targeted therapy approved for LA-HNSCC patients by the Food and Drug Administration is that against EGFR [3]. However, the addition of an EGFR-targeting monoclonal antibody (mAb) to radiation or chemoradiation therapy has shown limited success [4]. In addition, recent data suggest that EGFR targeting in combination with radiation is not an acceptable substitute for cisplatin-radiation in human papillomavirus (HPV)-positive HNSCC patients [5, 6]. Resistance mechanisms for anti-EGFR therapies in HNSCCs are reported in the literature including high expression of EGFR ligands, HER3, Src family kinases and HGF/MET axis [7]. However, unlike non-small cell lung cancer and colorectal cancer, their clinical use in treatment decision making is yet to be established. At present, due to the lack of predictive biomarkers in HNSCC, these therapies are offered indiscriminately to patients leading to a poor benefit to risk ratio [8]. EGFR-targeted therapies are only marginally effective, expensive and often associated with toxicity. Thus, it is necessary to identify patients most likely to benefit from these treatments. Currently, there are no established biomarkers for predicting treatment response to these therapies. EGFR protein expression and gene amplification are not useful to predict response to EGFR mAbs in HNSCC patients, as shown by our previous study and others [9, 10]. The severity of EGFR mAbs-cetuximab and panitumumab induced

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Received: 17 August 2021 Revised: 7 January 2022 Accepted: 28 January 2022
Published online: 9 February 2022

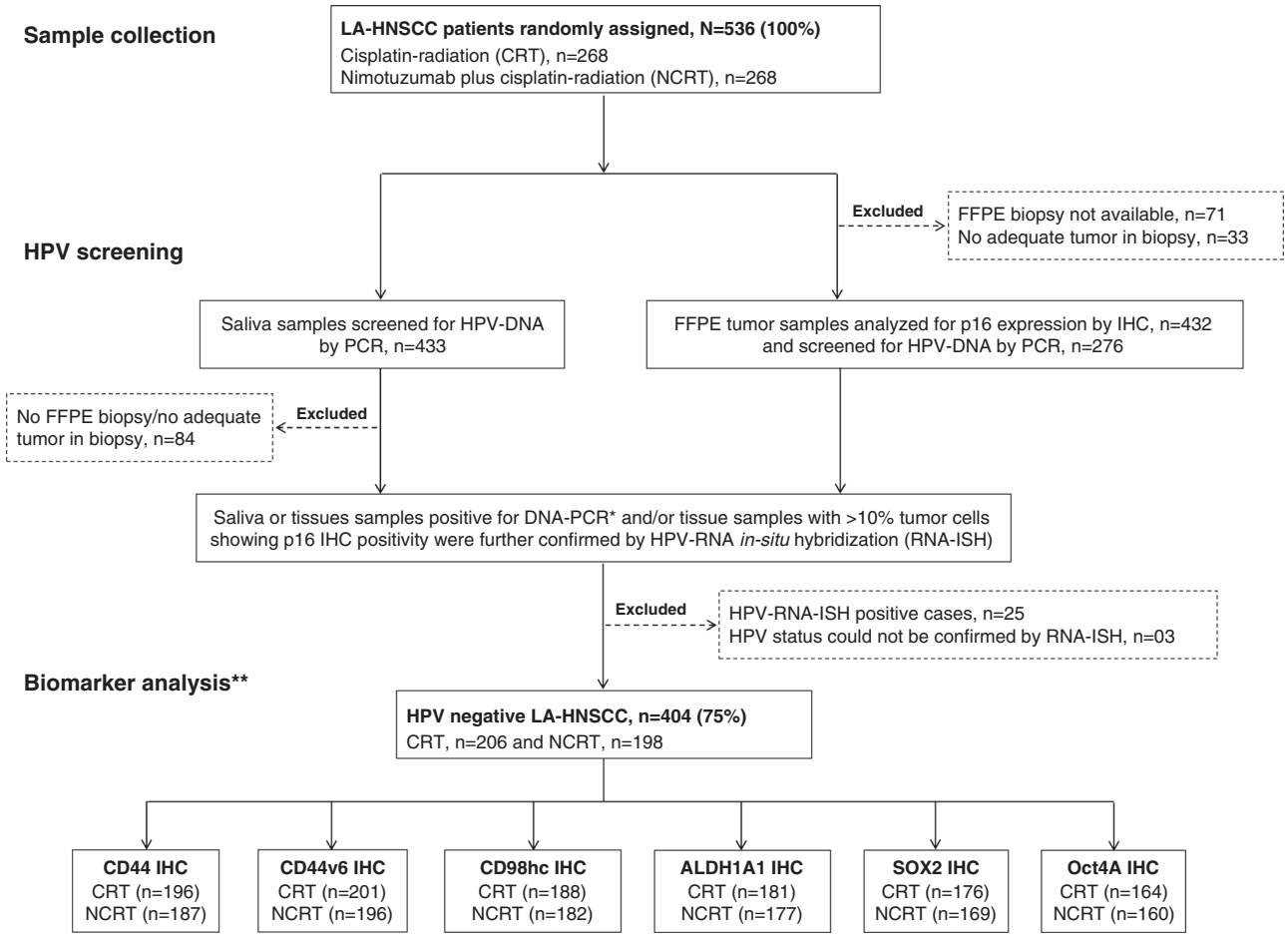

Fig. 1 Flow diagram of the study. (*) Both saliva and tumour tissue were screened in 222 cases for HPV–DNA by PCR; for 128 cases, only saliva samples, and for 54 cases only tumour tissue were analysed for HPV–DNA by PCR. (**) Biomarker groups differed in sample size due to limited availability of biopsy tumour tissue; LA-HNSCC locally advanced HNSCC, HPV human papillomavirus.

skin rashes is associated with a better response to these treatments, although, its predictive role is not established in HNSCC patients [11]. In addition, skin rashes highly affect the quality of life of the patients [12].

Nimotuzumab (h-R3), is a humanised IgG1 mAb against EGFR shown to have low toxicity as compared to other anti-EGFR mAbs [13, 14]. In a Phase 3-randomised trial conducted in India, Patil et al. reported improved progression-free survival (PFS) and loco-regional control (LRC) in unselected LA-HNSCC (> 94% HPV-negative) patients treated with nimotuzumab plus cisplatin-radiation compared (NCRT) to the patients treated with only cisplatin-radiation (CRT) [15]. We have analysed tumour samples of HPV-negative LA-HNSCC patients, participants of the above-mentioned clinical trial. Previously, we showed that high HIF1α is a marker for poor response to CRT. In addition, patients with high HIF1α markedly showed improved response to NCRT; no such improvement observed in HIF1α low patients with NCRT when compared to CRT. HIF1α expression, however, did not emerge predictive of differential response to NCRT response [9]. Therefore, to find predictive biomarkers for NCRT, in the present study, we evaluated prognostic and predictive roles of different putative cancer stem cell (CSC) markers in the same patient cohort.

The cluster of differentiation (CD)44 is a putative CSC marker of HNSCC [16]. It is a membrane glycoprotein that mediates cell-cell and cell-matrix interactions and is a major receptor for hyaluronic acid [17]. CD44 function is regulated by glycosylation and alternative splicing of 10 variant exons, giving rise to different isoforms [17]. The smallest isoform (CD44s or CD44H) is expressed on most vertebrate cells, including epithelial, immune and mesenchymal cells. However, the expression of other splice variants (CD44v1-v10) is tissue-specific [18]. CD44s and their variant isoforms are overexpressed in different cancers, including HNSCC and play a role in tumour progression and metastasis [19–21]. CD98 is another putative CSC marker of HNSCC [22]. The CD98 heavy chain (CD98hc, 4F2hc, SLC3A2) is a type II single-pass transmembrane glycoprotein. CD98hc participates in β-integrin signaling, which is involved in cell spreading and tumorigenesis [23]. CD98hc also interacts with LAT1, a multi-pass light chain of large neutral amino acid transporters, and acts as a chaperone that promotes LAT1 trafficking, functional insertion and stabilisation into the plasma membrane [24]. Digomann et al. recently showed that high expression levels of CD98hc lead to radiation resistance and poor prognosis in HNSCC [25]. Aldehyde dehydrogenase 1 (ALDH1) is yet another putative CSC marker of HNSCC [26]. ALDH1A1 oxidises retinal to retinoic acid, which binds to its nuclear receptor and drives transcription of genes involved in growth and differentiation. High expression of ALDH1A1 is reportedly a negative prognostic factor in many cancers, including HNSCC [27]. Further, sex-determining region-Y homeobox-2 (SOX2) and octamer-binding transcription factor 4 (OCT4, also termed POU5F1) are important pluripotency-associated transcription factors involved in the maintenance of self-renewal capacity of embryonic stem cells [28, 29]. The prognostic roles of SOX2 and OCT4 overexpression in HNSCC

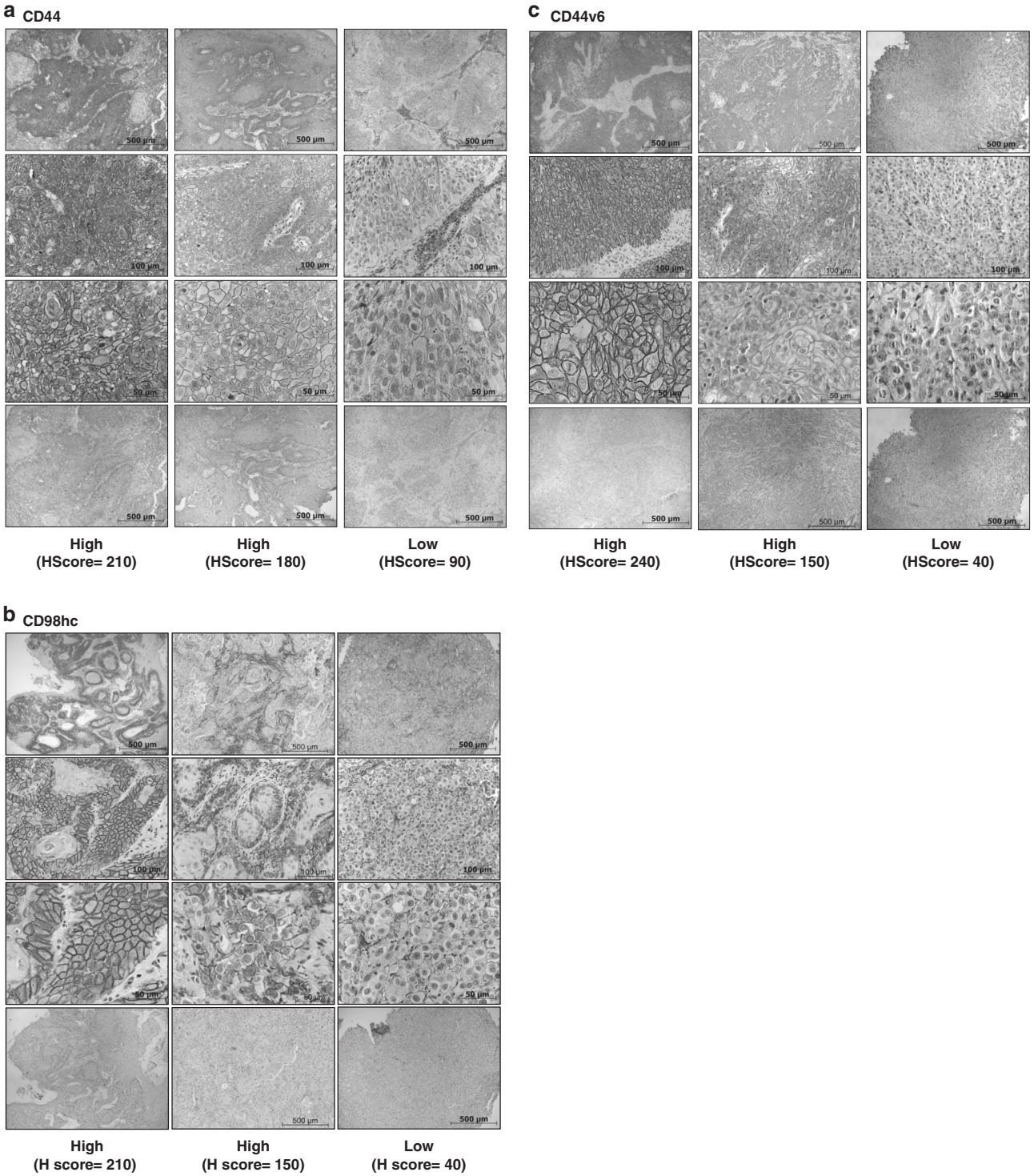

Fig. 2 Representative images of immunohistochemistry (IHC) staining in HPV-negative LA-HNSCC patients. Images showing high and low complete membrane IHC staining of **a** CD44, **b** CD44v6 and **c** CD98hc. The bottom panel shows staining in the respective negative control.

patients are debatable [30–32]. HNSCC cells positive for SOX2 and OCT4 exhibit CSC-like properties [33, 34].

To improve the efficacy of a given treatment, better knowledge of the molecular profiles and their impact on treatment outcomes is required. Here, we evaluated the prognostic and predictive roles of different recognised CSC markers (CD44, CD44v6, CD98hc, ALDH1A1, SOX2 and OCT4A) in HPV-negative LA-HNSCC patients treated with concurrent cisplatin-radiation with or without nimotuzumab. Additionally, a combined predictive analysis of HIF1α and CSC markers was performed to explore potential predictive associations.

MATERIALS AND METHODS

Study design and samples

The participants in this study were those included in a previously reported randomised phase 3 clinical trial (Clinical Trial Registry of India, trial registration identifier: CTRI/2014/09/004980) that compared CRT with NCRT

in 536 LA-HNSCC patients [15]. Inclusion and exclusion criteria for the trial were published earlier [15]. Formalin-fixed paraffin-embedded biopsy tumour tissues with adequate tumour were available for 432 patients which were screened for HPV as previously reported [35]. Biomarker expression was analysed in the remaining 404 HPV-negative tumour tissues blinded to treatment allocation and the patient's clinical outcomes. Figure 1 outlines the workflow of the study. All experimental procedures were conducted in accordance with the Declaration of Helsinki. The study was approved by the Institutional Ethics Committee of Tata Memorial Center (IEC approval 50 of 2011).

Immunohistochemistry

The protein expression levels were analysed using immunohistochemistry (IHC) with the VECTASTATIN® Elite ABC-HRP universal kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Antigen retrieval was carried out in a microwave oven in an appropriate buffer, for CD44 (10 mM Tris-1 mM EDTA buffer, pH 9 for 12 min at 700 W), CD44v6 (1 mM EDTA, pH 8 for 12 min at 700 W), CD98hc (10 mM sodium citrate, pH 6 for 16 min at 560 W), ALDH1A1 (1 mM EDTA, pH 8 for 16 min at 700 W), SOX2 (10 mM sodium citrate, pH 6 for 5 min at 700 W followed by 10 min at 560 W) and OCT4A (10 mM Tris-1 mM EDTA, pH 9 for 16 min at 560 W). The sections were then incubated with the primary antibody for 14 h. The primary antibodies were against CD44 (1:500 dilution; RRID: AB_10003817; Novus Biologicals, Centennial, CO, USA: NBP1-31488), CD44v6 (1:600 dilution; Novus Biologicals: NBP2-29853), CD98hc (1:800 dilution; RRID: AB_11124521; MBL Life Science, Tokyo, Japan: BMP090), ALDH1A1 (1:200 dilution; RRID: AB_867566; Abcam, Cambridge, UK: ab52492), SOX2 (1:100 dilution; RRID: AB_2798664; Cell Signaling Technology, Beverly, MA, USA: #14962) and OCT4A (1:100 dilution; RRID: AB_2167725; Cell Signaling Technology: #2890). The IHC staining protocol for each antigen was standardised on respective positive-control tissues. The staining was verified and approved by two experienced pathologists.

Assessment of immunostaining

IHC staining was evaluated semi-quantitatively and independently by two pathologists who were blinded to the treatment allocation and patient outcomes. "Complete membrane" denotes continuous staining of the tumour cell membrane whereas discontinuous staining was interpreted as an "incomplete membrane" staining pattern. The expression levels of CD44 (complete membrane), CD44v6 (complete membrane), CD98hc (complete membrane), ALDH1A1 (cytoplasmic), SOX2 (nuclear) and OCT4A (nuclear) were evaluated by deriving the HScore, which was calculated as $\Sigma P_i\,(i+1)$, where P_i is the fraction of stained tumour cells (0–100%) at each intensity, and i is the staining intensity (on a scale of 0–3). The continuous scale of HScores ranged from 0 to 300. Discrepancies (cases with HScore difference ≥50) were jointly resolved by both pathologists.

Statistical analyses

Categorical data are presented as frequencies and percentages, whereas continuous data are expressed as median and range or interquartile range (IQR). Spearman's rank test was used to determine correlations between continuous variables [36]. Pearson's chi-square test was used to determine the association between categorical variables. Progression-free survival (PFS) was the primary endpoint, and loco-regional control (LRC), and overall survival (OS) were secondary endpoints. PFS, LRC and OS were measured from the date of randomisation to the date of progression, date of loco-regional failure and date of death respectively. PFS, LRC and OS were estimated using the Kaplan–Meier method and compared using log-rank tests. The prognostic impact of each biomarker on clinical outcomes was analysed using a univariate Cox regression model by deriving hazard ratios (HRs) and 95% confidence intervals (CIs). Multivariate analysis using the backward likelihood ratio method was then applied to assess the independent prognostic significance after adjusting for potential confounders (clinical characteristics including age, sex, clinical stage and tumour site, associated with PFS, LRC or OS with $P < 0.20$). To assess the predictive significance of biomarkers, Cox models were fit that included treatments (NCRT versus CRT), biomarker status (low versus high) and the interaction between treatment effect and biomarker status [37, 38]. Internal validation of prognostic and predictive models was achieved using the bootstrap resampling method (1,000 samples), and concordance indices (c-indices) were calculated. All statistical analyses were performed using SPSS software version 21 (IBM, Armonk, NY, USA), except for the creation of Forest plots, and the bootstrap method for which STATA version 14 (StataCorp, College Station, TX, USA) was used. All reported P-values were two-sided, and $P \le 0.05$ was considered statistically significant. P-values were not corrected for multiple testing. Some biomarker subgroups had small sample sizes owing to the limited availability of tumour tissue samples.

RESULTS

Patients

The baseline characteristics of the 404 study patients were balanced between the two treatment groups and were representative of the trial cohort ($n = 536$), as reported previously [9]. Demographic details of the patients included in this study are listed in Supplementary Table 1 [9]. The median follow-up duration was 39.13 months. Of the 404 patients, 155 experienced loco-regional failure, 188 experienced disease progression and 195 patients died. The treatment outcomes in the biomarker subgroup ($n = 404$) were previously reported [9].

Expression patterns of CSC markers

We carried out biomarker analysis in 404 HPV-negative cases out of which 206 received CRT and 198 received NCRT treatment. The overall frequency distribution of all biomarkers was comparable between the two treatment arms (Supplementary Fig. 1). We observed complete membrane expression of CD44, CD44v6 and CD98hc in approximately 74%, 92% and 77% of the HNSCC tumours, respectively. Expression of CD44 and CD98hc was also observed in the immune cells [24, 39]. However, these cells did not express CD44v6 [39]. Representative images of IHC staining of complete membrane expression of CD44, CD44v6 and CD98hc are presented in Fig. 2a-c. ALDH1A1 expression was predominantly observed in the cytoplasm of the tumour cells. Approximately 51.4% of cases showed ALDH1A1 expression. Nuclear SOX2 staining was evident in approximately 73.6% of the cases. Representative IHC staining images showing cytoplasmic ALDH1A1 and nuclear SOX2 expression are presented in Supplementary Fig. 2A, B. 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 (Supplementary Fig. 3).

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]. Interestingly, we observed mutually exclusive expressions of CD98hc and ALDH1A1 (Supplementary Fig. 4). Next, we studied the association between CSC marker expression and clinicopathological parameters. We did not observe any significant associations between the clinicopathological parameters and different CSC markers (Supplementary Table 3).

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 (HScore = 40) or CD44v6 (HScore = 180) 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 (Supplementary Table 4A, 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

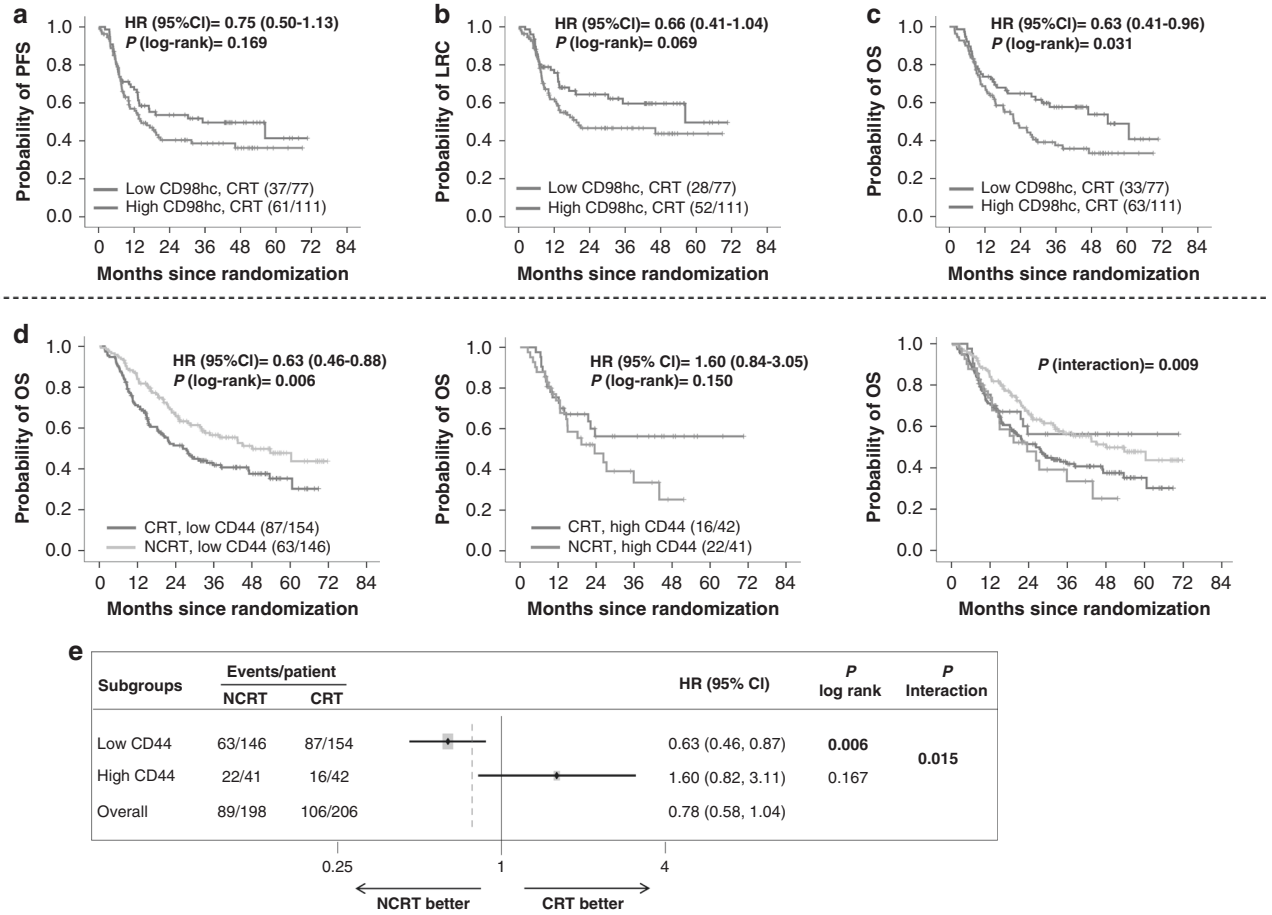

Fig. 3 Prognostic and predictive association of CD98hc and CD44, respectively, in HPV-negative LA-HNSCC patients. Kaplan–Meier plots showing **a** progression-free survival, **b** loco-regional control and **c** overall survival according to the CD98hc expression status (dichotomised at HScore of 40). **d** Kaplan–Meier plots showing overall survival for LA-HNSCC patients in both the treatment groups at low or high expression status of CD44 (dichotomised at HScore of 150). **e** Forest plot showing results of bootstrap resampling validation confirming the predictive effect of CD44. A hazard ratio of <1 indicates a benefit from NCRT. The dotted line represents the hazard ratio (HR) for the overall study population. CI confidence interval, CRT cisplatin-radiation therapy, NCRT nimotuzumab plus cisplatin-radiation therapy.

expressing high CD98hc (Supplementary Table 4C). Univariate Cox analysis revealed that low CD98hc expression defined using the cut-off of 40 ($n = 77$) 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) (Fig. 3a-c).

We did not observe any prognostic association of ALDH1A1 expression at any of the studied cut-offs (Supplementary Table 4D). 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 (Supplementary Table 4E).

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, $P = 0.028$) (Supplementary Table 5). 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) (Table 1) [9]. CD98hc did not emerge as an independent prognostic factor for LRC and OS in this multivariable model. Low-HIF1α expression was strongly associated with improved PFS (HR = 0.64, 95% CI = 0.42–0.97), LRC (HR = 0.57, 95% CI = 0.36–0.89) and OS (HR = 0.61, 95% CI = 0.41–0.93) when compared to high HIF1α expression subgroup ($n = 91$). 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.

Predictive association of CD44 and CD44v6

Predictive associations of CD44 and CD44v6 were studied at different possible HScore cut-offs, including the respective median cut-off. HRs for disease progression, loco-regional failure and death were significantly lower at many cut-offs for patients with low CD44 expression, suggesting a treatment benefit from NCRT relative to that from CRT (Supplementary Table 6). HRs for disease progression and loco-regional failure were significantly lower when high CD44 status was defined using lower cut-offs (HScore 0–30). At high cut-offs (HScore 60–180) patients with low CD44 status showed significantly lower HRs for disease

Table 1. Prognostic significance of clinical parameters and biomarkers in 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 (below 60 vs 60 and above)	1.46 (0.94–2.28)	0.092	1.59 (0.97–2.62)	0.067
[b]Clinical stage (III vs IV)	0.48 (0.30–0.78)	0.003	0.43 (0.25–0.73)	0.002
Site of tumour (oropharynx vs others)	1.74 (1.19–2.56)	0.004	–	–
pEGFRY1068 (negative vs positive)	0.63 (0.40–1.0)	0.048	–	–
pEGFRY1173 (negative vs positive)	0.74 (0.48–1.14)	0.170	–	–
HIF1α (low vs high)	0.69 (0.47–1.01)	0.053	**0.64 (0.42–0.97)**	**0.033**
CD98hc (low vs high)	0.75 (0.50–1.13)	0.171	–	–
Loco-regional control (LRC)				
Age (below 60 vs 60 and above)	1.49 (0.91–2.43)	0.111	1.59 (0.92–2.73)	0.095
[b]Clinical stage (III vs IV)	0.43 (0.25–0.75)	0.003	0.37 (0.20–0.69)	0.002
Site of tumour (oropharynx vs others)	1.58 (1.05–2.40)	0.030	–	–
HIF1α (low vs high)	0.58 (0.38–0.89)	0.011	**0.57 (0.36–0.89)**	**0.014**
CD98hc (low vs high)	0.66 (0.41–1.04)	0.071	–	–
Overall survival (OS)				
Age (below 60 vs 60 and above)	1.59 (1.0–2.53)	0.049	1.57 (0.96–2.58)	0.075
[b]Clinical stage (III vs IV)	0.64 (0.40–1.00)	0.051	0.59 (0.36–0.96)	0.034
Site of tumour (oropharynx vs others)	1.62 (1.10–2.37)	0.014	–	–
HIF1α (low vs high)	0.62 (0.42–0.91)	0.016	**0.61 (0.41–0.93)**	**0.020**
CD98hc (low vs high)	0.63 (0.41–0.96)	0.032	–	–

Bold values indicate biomarkers with statistically significant association with treatment outcomes in Multivariable Cox regression analysis.
HR hazard ratio, *CI* confidence interval.
[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). (–) data not available
[b]According to AJCC-UICC system (8th edition).

progression, loco-regional failure and death. For OS, HRs were >1.0 at higher cut-offs (HScore 90–180), although the difference was not statistically significant (Supplementary Table 6). We observed a statistically significant qualitative interaction between CD44 status (low: cases with HScore ≤ cut-off HScore and high: cases with HScore >cut-off HScore) and treatment effect for OS when dichotomised at a cut-off of HScore 140 (P for interaction = 0.022) and HScore 150 (P for interaction = 0.009). At a cut-off of 150, low CD44 status was associated with longer OS with NCRT (n = 146) relative to CRT (n = 154) (HR = 0.63, 95% CI = 0.46–0.88) (Fig. 3d). Similar benefits with NCRT (n = 41) were not observed in patients with high CD44 status for OS (HR = 1.60, 95% CI = 0.84–3.05) when compared to CRT (n = 42) (Fig. 3d). Bootstrap resampling validation confirmed the predictive effect of CD44 status dichotomised at the cut-off of 150 for OS (P for interaction = 0.015; c index = 0.57, 95% CI = 0.53–0.61) (Fig. 3e). 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.

HRs for disease progression, loco-regional failure and death were statistically significantly <1.0 regardless of the cut-off used to define low CD44v6 expression, suggesting a treatment benefit from NCRT compared with that from CRT (Supplementary Table 7). HRs for disease progression and loco-regional failure for patients expressing high CD44v6 were also <1.0, although a statistically significant difference was observed in PFS and LRC only at the lower cut-offs (HScore 0–90). We observed a statistically significant qualitative interaction between CD44v6 status dichotomised at a cut-off of 40 and treatment effect for LRC (P for interaction = 0.023) and OS (P for interaction = 0.036), but not for PFS (P for interaction = 0.075). At this cut-off, low CD44v6 status was significantly associated with improved LRC (HR = 0.25, 95% CI = 0.10–0.62) and OS (HR = 0.38, 95% CI = 0.19–0.74) with NCRT (n = 40) relative to that with CRT (n = 33) (Fig. 4a, b). Similar improvement in LRC (HR = 0.77, 95% CI = 0.54–1.09) or OS (HR = 0.88, 95% CI = 0.64–1.21) with NCRT (n = 163) versus CRT (n = 161) was not observed in patients with high CD44v6 status (Fig. 4a, b). The predictive impact of CD44v6 for LRC was not significant in the bootstrap resampling validation (P for interaction = 0.152, data not shown). However, the predictive value of CD44v6 status for OS was confirmed using the bootstrap resampling (P for interaction = 0.041; c index = 0.56, 95% CI = 0.52–0.60) (Fig. 4c).

Combined predictive association of CD44, CD44v6, HIF1α and EGFR

We performed a combined predictive analysis for CD44 and CD44v6. Patients with low expression of both CD44 (cut-off 150) and CD44v6 (cut-off 40) performed significantly better with NCRT (n = 31) versus CRT (n = 39) concerning PFS (HR = 0.28, 95% CI = 0.12–0.63), LRC (HR = 0.16, 95% CI = 0.06–0.48) and OS (HR = 0.36, 95% CI = 0.18–0.71) than patients with high expression of either one or both markers (Table 2). CD44-CD44v6 status showed a statistically significant interaction with the treatment effect for PFS (P for interaction = 0.044), LRC (P for interaction = 0.016) and OS (P for interaction = 0.002).

In our previous report, HIF1α expression emerged as an important predictive biomarker in these patients. Therefore, we performed a combined analysis of CD44 (cut-off 150)—HIF1α (cut-off 90) and CD44v6 (cut-off 40)—HIF1α (cut-off 90) [9]. Patients with CD44 low and HIF1α high status showed significantly improved PFS (HR = 0.46, 95% CI = 0.28–0.75), LRC (HR = 0.46, 95% CI = 0.27–0.77) and OS (HR = 0.41, 95% CI = 0.25–0.66) with NCRT (n = 69) relative to CRT (n = 66). The other subgroups did

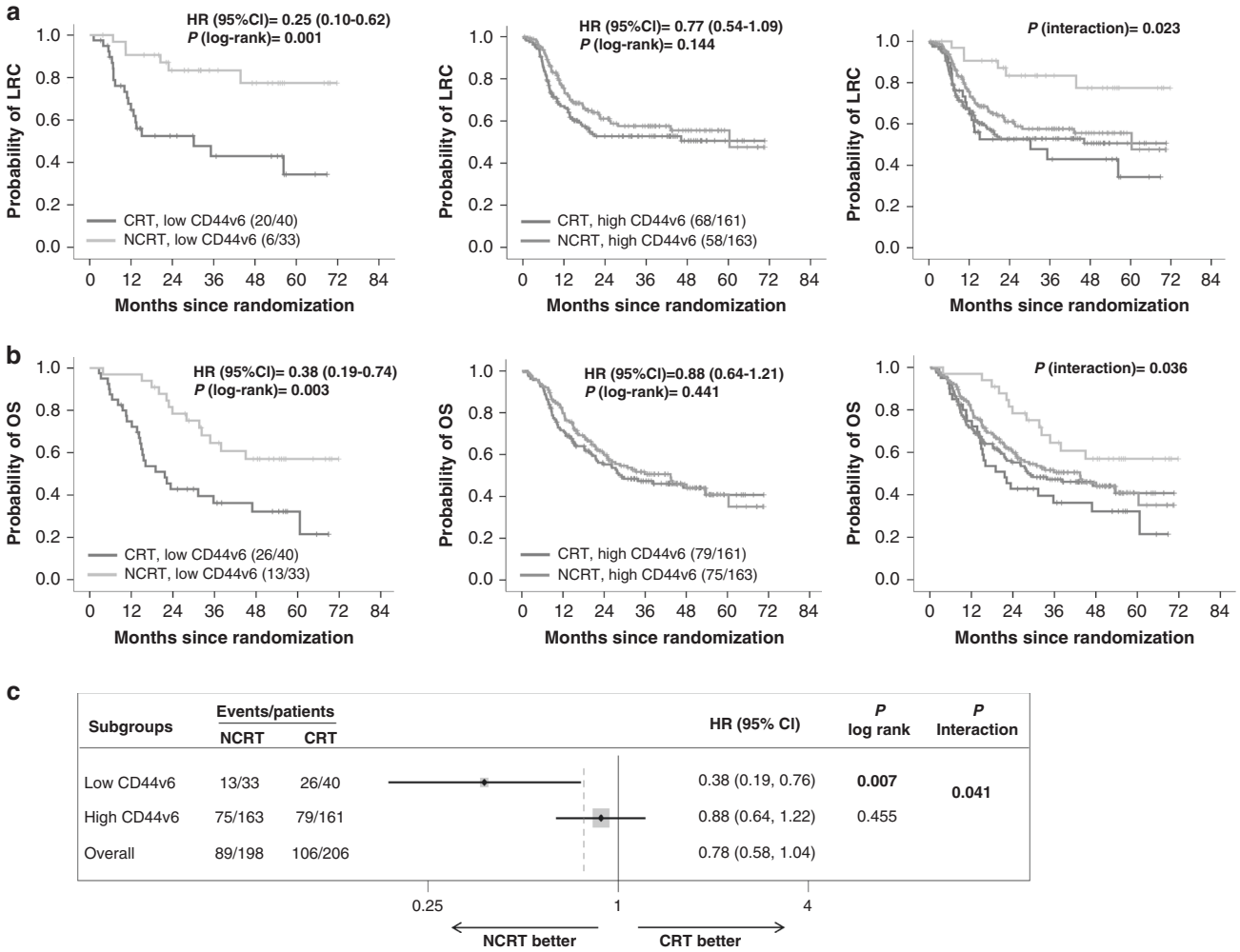

Fig. 4 Predictive association of CD44 and CD44v6 in HPV-negative LA-HNSCC patients. Complete membrane expression of CD44v6 showing a qualitative interaction with treatment effect for loco-regional control (**a**) and overall survival (**b, c**) in HPV-negative LA-HNSCC patients. **a** Kaplan–Meier plots showing overall survival for LA-HNSCC patients in both the treatment groups at low or high expression status of CD44v6 (dichotomised at HScore of 40). **b** Kaplan–Meier plots showing overall survival for LA-HNSCC patients in both the treatment groups at low or high expression status of CD44v6 (dichotomised at HScore of 40). **c** Forest plot showing results of bootstrap resampling validation confirming the predictive effect of CD44v6 for overall survival. HR < 1 indicates a benefit from NCRT. The dotted line represents HR for the overall study population. HR hazard ratio, CI confidence interval, CRT cisplatin-radiation therapy, NCRT nimotuzumab plus cisplatin-radiation therapy.

not show similar improvements to NCRT (Supplementary Table 8). However, we did not observe a significant interaction between CD44-HIF1α status and the treatment effect for any of the clinical endpoints. Combined analysis of CD44v6 and HIF1α showed that among different patient groups, patients with CD44v6 high and HIF1α low status did not show any improvement in PFS, LRC or OS with NCRT ($n = 68$) versus CRT ($n = 84$) (Supplementary Table 9). Patients with CD44v6 low-HIF1α high had significantly improved PFS (HR = 0.15, 95% CI = 0.03–0.69), LRC (HR = 0.18, 95% CI = 0.04–0.83) and OS (HR = 0.15, 95% CI = 0.03–0.67) with NCRT ($n = 10$) when compared to CRT ($n = 17$). We did not observe any significant interaction between CD44v6-HIF1α status and treatment interactions for PFS, LRC or OS. However, the sample size of this group was very small and therefore needs to be validated further to derive meaningful conclusions.

We also carried out the combined analysis of CD44-EGFR (Supplementary Table 10) and CD44v6-EGFR (Supplementary Table 11). Subgroup with CD44 low and EGFR high expression levels showed significantly improved PFS (HR = 0.50, 95% CI = 0.30–0.81), LRC (HR = 0.49, 95% CI = 0.28–0.83) and OS (HR = 0.53, 95% CI = 0.33–0.86) with NCRT ($n = 68$) compared to CRT ($n = 68$). Similar improvements were also observed in subgroup expressing low CD44v6 low-EGFR high, PFS (HR = 0.30, 95% CI = 0.10–0.92) and LRC (HR = 0.21, 95% CI = 0.06–0.74) with NCRT ($n = 16$) when compared to CRT ($n = 19$). However, we did not find significant interactions with any of the clinical endpoints.

Predictive association of CD98hc, ALDH1A1 and SOX2

Overall, the HRs for disease progression, loco-regional failure and death were <1.0, regardless of the cut-off used to define low or high CD98hc status (Supplementary Table 12). We did not observe any statistically significant interaction between CD98hc status and treatment effect at any of the studied cut-offs, including the median cut-off. Our results suggest that the treatment benefits of NCRT relative to CRT are independent of CD98hc expression status.

We next analysed the predictive role of ALDH1A1 expression dichotomised at different possible cut-offs. Low ALDH1A1 status was associated with improved PFS, LRC and OS, with lower HRs irrespective of the cut-off (HScore 20–160), suggesting a treatment benefit from NCRT relative to CRT (Supplementary Table 13). High ALDH1A1 status did not show a similar significant improvement in

Table 2. Combined predictive analysis of CD44 and CD44v6.

Biomarker combination	Events/*n* (NCRT)	Events/*n* (CRT)	4 YR survival (months)	HR (95% CI)	*P* [a]	*P* (interaction)
Progression-free survival (PFS)						
Both low	8/31	24/39	69.6 vs 32.9	0.28 (0.12–0.63)	**0.002**	**0.044**
Either one high	51/114	61/115	48.0 vs 42.2	0.73 (0.50–1.06)	0.100	
Both high	16/41	20/41	50.9 vs 43.6	0.88 (0.45–1.69)	0.691	
Loco-regional control (LRC)						
Both low	4/31	20/39	82.6 vs 41.8	0.16 (0.06–0.48)	**0.001**	**0.016**
Either one high	42/114	50/115	54.6 vs 50.6	0.75 (0.50–1.14)	0.179	
Both high	14/41	17/41	54.2 vs 48.9	0.89 (0.44–1.81)	0.747	
Overall survival (OS)						
Both low	12/31	26/39	57.2 vs 31.1	0.36 (0.18–0.71)	**0.003**	**0.002**
Either one high	51/114	61/115	49.6 vs 40.5	0.76 (0.53–1.11)	0.157	
Both high	22/41	16/41	25.2 vs 54.9	1.54 (0.81–2.94)	0.189	

NCRT nimotuzumab plus cisplatin-radiation, *CRT* cisplatin-radiation alone, *HR* hazard ratio, *CI* confidence interval.
[a]Univariate Cox regression analysis. H-score values used as the cutpoint for categorisation were [CD44 = 150 and CD44v6 = 40].

PFS, LRC or OS at any of the cut-offs. We did not find a statistically significant interaction between ALDH1A1 status and treatment effects for the studied clinical endpoints. However, at the cut-off of 70, we observed a trend for an interaction between ALDH1A1 status and treatment effect for LRC (*P* for interaction = 0.069) and OS (*P* for interaction = 0.089). Low ALDH1A1 status was significantly associated with improved LRC (HR = 0.58, 95% CI = 0.39–0.86, *P* = 0.006) and OS (HR = 0.68, 95% CI = 0.48–0.95, *P* = 0.024) with NCRT (*n* = 146) relative to CRT (*n* = 138). However, similar improvement with NCRT (*n* = 31) was not observed in patients with high ALDH1A1 status for LRC (HR = 1.29, 95% CI = 0.63–2.62, *P* = 0.489) or OS (HR = 1.35, 95% CI = 0.65–2.78, *P* = 0.421) compared to CRT (*n* = 43).

The HRs for disease progression, loco-regional failure and death were <1.0, for both low and high SOX2 expressing patients studied at different cut-offs (Supplementary Table 14). We did not observe any significant interaction between SOX2 status and treatment effect at any of the cut-offs, suggesting that SOX2 expression status is not predictive of treatment response to NCRT in these patients. In addition to molecular markers, we also analysed the predictive impact of baseline clinical and demographic characteristics that included age, sex, tumour site, disease stage and tobacco smoking. None of these characteristics showed any predictive effects (Supplementary Table 15).

Table 3. Summary of prognostic and predictive associations of different biomarkers studied alone or in combination.

Biomarker expression		Association shown
HIF1α	Nuclear	Prognostic and predictive [9]
EGFR	Membrane and cytoplasmic	None [9]
pEGFR dimers	Membrane	None [9]
EGFR gene copy number	NA	None [9]
CD44	Membrane	Predictive
CD44v6	Membrane	Predictive
CD98hc	Membrane	Prognostic
ALDH1A1	Cytoplasmic	None
SOX2	Nuclear	None
[a]CD44 + CD44v6	as mentioned above	Predictive
[a]CD44 + HIF1α	as mentioned above	Not predictive
[a]CD44v6 + HIF1α	as mentioned above	Not predictive

[a]Only predictive association was studied.

DISCUSSION

Our previous study demonstrated both prognostic and predictive roles of HIF1α expression status in HPV-negative LA-HNSCC patients treated with CRT or NCRT in a randomised setting [9]. In the current study, we evaluated the prognostic and predictive roles of putative CSC markers in HNSCCs in the same patient cohort. The prognostic and predictive associations of all the biomarkers studied alone or in combination are summarised in Table 3. The findings indicated that complete membrane expression of CD44 and CD44v6 could predict clinical benefit from the addition of nimotuzumab to CRT treatment when compared with that from CRT alone. These biomarkers can serve for identifying patients (low CD44 or CD44v6) requiring NCRT treatment for the improved clinical outcome as well as marking for patients (high CD44 or CD44v6) that may not benefit from NCRT thus reducing overtreatment. In addition, we also showed the prognostic role of complete CD98hc membrane expression. The prognostic significance of CD98hc in HNSCC has been reported in the literature [25, 40]. However, this is the first study demonstrating the predictive roles of CD44 and CD44v6 in HPV-negative LA-HNSCC patients for anti-EGFR-based treatment response [41, 42].

CD44 and its variant isoforms have been studied widely for their prognostic role in HNSCC. However, the findings have been inconsistent [21]. We did not observe any prognostic association of CD44 or CD44v6 at any of the studied HScore cut-offs. A lack of prognostic association of CD44 has been reported in HPV-DNA-negative HNSCC patients [41].

Studies have failed to detect any predictive value of EGFR expression in EGFR-targeting treatment response [10]. We have also analysed the predictive association of EGFR expression with OS, PFS and LRC in the same cohort in our earlier study; EGFR expression did not show any predictive role [9]. Interestingly, in the current study, CD44 and CD44v6 expression status showed a significant qualitative interaction with the treatment effect for OS, which remained significant in the bootstrap validation. The low expression status of CD44 or CD44v6 was significantly associated with better treatment response to NCRT relative to CRT. Patients with low CD44 or CD44v6 showed improved OS compared to patients with high expression in NCRT arm (CD44: 4 Year OS = 51.2 vs 25.2 months, log-rank *p* = 0.009 and CD44v6: 4 Year OS = 56.9 vs 45.8 months, *p* = 0.048, respectively). Our results also indicate that the high expression of CD44 or CD44v6 might be associated

with resistance to EGFR-based treatment. There are no comparable in vitro studies showing such association after treatment of nimotuzumab or cetuximab. However, different in vitro studies have reported that CD44 interacts with EGFR and erbB2 in different cancers, including HNSCC and this interaction might be playing a role in mediating resistance to EGFR-targeted therapies [43–45]. It has also been reported that the interaction of hyaluronan-CD44 promotes EGFR activation independent of EGF in HNSCC [46, 47].

The present study provides the first evidence of the role of CD44 and CD44v6 in predicting EGFR-based treatment response in HPV-negative HNSCC patients. Furthermore, our combined predictive analysis showed an enhanced predictive effect of CD44-CD44v6 on PFS, LRC and OS. Several studies have shown that CD44 expression is positively regulated by HIF1α [48, 49]. We observed a positive but weak correlation between CD44, CD44v6 and HIF1α. We previously described a predictive role for HIF1α expression, wherein high HIF1α expression (Hscore ≥ 90) was associated with NCRT treatment benefit relative to CRT [9]. Therefore, we presently performed a combined predictive analysis of CD44-HIF1α and CD44v6-HIF1α. No additional predictive effect of the combined biomarkers was evident. This might reflect the small sample size of the subgroups. Thus, the results need to be validated in a larger cohort.

A negative prognostic association of CD98hc (SLC3A2) gene expression in HPV-negative LA-HNSCC was recently reported [42]. In the current study, CD98hc protein expression was an independent negative prognostic biomarker of LRC and OS in HPV-negative LA-HNSCC patients. Hypoxia and activation of HIF1α signaling are important features of the tumour microenvironment and regulate the CSC phenotype [50, 51]. Interestingly, we observed that HIF1α expression was a stronger prognosticator for LRC and OS than CD98hc, which is a known putative CSC marker of HNSCC [22]. We did not observe any predictive role of CD98hc in these patients.

Low expression of ALDH1A1 was associated with a significantly better clinical response to NCRT than to CRT at all the analysed cut-offs. However, we did not observe any significant interaction between ALDH1A1 status and the treatment effect. In the present study, >45% ($n = 174$) of the cases were negative for ALDH1A1 expression, leading to small sample size in the subgroup with high ALDH1A1 expression status. The predictive effect of ALDH1A1 expression needs to be studied in a larger cohort. In addition, ALDH1A1 did not show any significant prognostic associations with any of the studied clinical endpoints. Further, the majority of the published data have shown a negative prognostic impact of SOX2 [30]. However, in contrast, a positive prognostic impact of SOX2 expression in HNSCCs, as well as other cancers, has been reported [30, 52, 53]. Currently, no prognostic or predictive associations with SOX2 expression have been found. Furthermore, nuclear expression of OCT4A was not evident in any of the tumour tissues. Similar observations were reported in 348 tumour tissues of HNSCC [54].

There are a few limitations of the current study, which need to be considered. One is the semi-quantitative assessment of IHC staining by pathologists, which is inherently subjective. There is no consensus regarding the method of evaluation of IHC staining for the studied biomarkers (percentage of tumour cells stained and/or intensity of staining) [21, 27, 41, 52, 54, 55]. Therefore, IHC staining was independently evaluated by two pathologists to derive the HScore. Cases showing discrepancies were resolved by the pathologists to reduce bias. As an automated IHC assessment is not yet widely available. Thus, the HScore is currently an acceptable and reasonable substitute for the evaluation of IHC markers desired for widespread applicability. For biomarker expression analysis with continuous data, selection of the appropriate cutpoint for dichotomising patients into expression subgroups remains a difficult decision, as there is no consensus among studies. The median value is often used as the cut-off, but it may not be optimal for all biomarkers. Using a pre-specified cut-off will increase the probability of failure in detecting important associations. Therefore, we dichotomised all biomarkers at different possible cut-offs and performed prognostic and predictive analyses. However, the use of multiple cut-offs for finding associations carries a risk of inflation of the type I error and overestimation of the results. Also, the results were not corrected for multiple testing and therefore, these results need to be validated in an independent study [56].

In summary, this is the first comprehensive study to show the predictive potential of CD44 and CD44v6 alone or in combination for NCRT treatment response in HPV-negative LA-HNSCC patients in a randomised setting. In addition, HIF1α has revealed a stronger prognostic biomarker than CD98hc. Investigating the correlation between HIF1α and putative CSC markers with clinical outcomes in HNSCC patients will greatly aid treatment decisions. After validation of the observations in a larger cohort, these biomarkers may help stratify HNSCC patients for conventional or EGFR-based targeted therapies.

DATA AVAILABILITY

All data generated or analysed during this study are included in this manuscript (and its supplementary information file). However, if required we can submit the clinical outcomes/follow-up data and biomarker data.

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ACKNOWLEDGEMENTS

We acknowledge all patients and investigators who contributed to the study. Additionally, we would like to thank Cactus Communications Pvt. Ltd., Mumbai, India for their assistance in English language editing.

AUTHOR CONTRIBUTIONS

Methodology: UP, SM, VS and MBM; scoring of IHC slides: SR, NM, PG and AP; data curation and formal analysis: UP and SK; project administration: UP and MBM; writing—original draft: UP and MBM; writing—review and editing: UP, MBM, SR, NM and SK; conducting the trial: AJ, VN, VMP and KP; conceptualisation and supervision: MBM; funding acquisition and resources: MBM. All authors approved the final paper.

FUNDING

Council of Scientific & Industrial Research, fellowship to Usha Patel, Grant/Award Number: 09/513(0099)/2014-EMR-I; Science and Engineering Research Board, Grant/Award Number: EMR/2015/001591; Tata Memorial Centre, Seed In Air grant, Grant/Award Number: TMC/SIA/2696.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the institutional ethics committee of Tata Memorial Center (IEC approval 50 of 2011) and was performed in accordance with the Declaration of Helsinki. All patients provided written informed consent.

CONSENT FOR PUBLICATION

NA

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41416-022-01730-9.

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