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Original Research Article

Independent validation of tumour volume, cancer stem cell markers and hypoxia-associated gene expressions for HNSCC after primary radiochemotherapy

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ABSTRACT

Objective: To independently validate the impact of tumour volume, p16 status, cancer stem cell (CSC) marker expression and hypoxia-associated gene signatures as potential prognostic biomarkers for patients with locally advanced head and neck squamous cell carcinoma (HNSCC), who underwent primary radiotherapy or radiochemotherapy (RCTx). These markers have previously been reported in a study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG) (Linge et al., 2016).
Materials and methods: In this retrospective monocentric study, 92 patients with locally advanced HNSCC were included. Univariable and multivariable logistic regressions and Cox models presented in the study of the DKTK-ROG were validated using the area under the curve (AUC) and the concordance index (ci), respectively. The primary endpoint of this study was loco-regional tumour control (LRC) after primary RCTx.
Results: Although both cohorts significantly differed in the proportion of the tumour subsites, the parameters tumour volume, p16 status and N stage could be validated regarding LRC and overall survival (OS) using multivariable Cox regression (LRC ci: 0.59, OS ci: 0.63). These models were slightly improved by combination with the putative CSC marker *CD44* (LRC ci: 0.61, OS ci: 0.69). The logistic regression model for 2-year LRC based on tumour volume, p16 status and CD44 protein was validated with an AUC of 0.64. The patient stratification based on hypoxia-associated gene signatures status was similar to the original study but without significant differences in LRC and OS.
Conclusions: In this validation study, the inclusion of the putative CSC marker CD44 slightly improved the prognostic performance of the baseline parameters tumour volume, p16 status and N stage. No

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improvement was observed when including expressions of the hypoxia-associated gene signatures. Prospective validation on a larger cohort is warranted to assess the clinical relevance of these markers.

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) are representing one of the ten most frequent tumours worldwide [2]. Primary radiochemotherapy (RCTx) is currently the considered treatment standard for patients with locally-advanced and functionally inoperable tumours, after several clinical trials showed a benefit over radiotherapy alone [3–8]. However despite treatment escalation by simultaneous radiochemotherapy, the outcome of radio(chemo)therapy is still unsatisfying with only about half of the patients being alive after 5 years [9]. Thus, the identification of patients who are very likely to have a poor treatment response is necessary and may be achieved by the complementation of well accepted clinical parameters by molecular biomarkers of the individual tumour. This may allow for inclusion of patients in treatment intensification trials, such as dose escalation or combination with novel systemic therapeutics [10,11].

Over the last one to two decades, the human papilloma virus (HPV) infection status has become one of the major risk factors for the development of HNSCC besides alcohol abuse and tobacco [12–14]. Several studies showed that patients with HPV-driven HNSCC show a favourable prognosis after primary or postoperative radiochemotherapy compared to those with HPV-negative tumours [1,15–18].

In a recent multicentre study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG), previous studies were confirmed showing that patients with an overexpression of the HPV-surrogate marker p16 have a better LRC and OS compared to patients with HPV-negative tumours. Importantly, patient stratification regarding LRC and OS could be further improved, when the expression of the putative cancer stem cell markers CD44 or *SLC3A2* was also considered. Further refinement of for the prediction of prognosis was achieved by the tumour volume, which was suggested by others before [10,19–22]. For patients with small tumours, tumour hypoxia as assessed by hypoxia-associated gene signatures was also found to be a prognostic biomarker.

The aim of the present study was to independently validate these results obtained within the DKTK-ROG [23] using a retrospective monocentric cohort of 92 patients with locally advanced HNSCC, who were treated by primary RCTx.

2. Materials and methods

2.1. Patients and study design

In the current publication, two independent patient cohorts who received curatively intended primary radio(chemo)therapy are being evaluated.

The retrospective primary HNSCC cohort of the DKTK-ROG served as the training cohort and included 158 patients with locally advanced and histologically proven HNSCC. Inclusion criteria have previously been described in detail [1]. All patients received primary RCTx with a median dose of 72.0 Gy (range 68.4–74.0 Gy) based on cisplatinum or mitomycin-C between 2005 and 2011 at one of six partner sites of the DKTK-ROG. The relation of the primary tumour volume, p16 status, the CSC markers CD44 and *SLC3A2* and hypoxia-associated gene signatures to LRC and OS were investigated in this cohort before [1].

To validate these results, an independent cohort of 92 patients with locally advanced HNSCC treated by curatively intended primary RCTx was considered in this manuscript. Out of these 92 patients, 43 patients were presented earlier in a prospective mono-centre single-arm non-randomised observational imaging trial, which was registered (www.clinicaltrials.gov, NCT00180180) and approved by the German Federal Radiation Protection Authority (Bundesamt für Strahlenschutz, Z5 – 22461/2 – 2004-061) and the local Ethics Committee (EK166082004) [24,25]. Briefly, these patients were treated between 2006 and 2013 at the DKTK partner site Dresden, had to be at least 18 years old with WHO performance status 0–2, were treated with primary RCTx and received a median dose of 72 Gy (range 69.0–72.0 Gy). The remaining 49 patients were also treated at the Dresden site between 1999 and 2006 or 2009 and 2015 and received primary RCTx with a median dose of 70.6 Gy (range 70.0–76.8 Gy). None of the patients of the validation cohort were included in the training cohort. Further inclusion criteria were the histologically confirmation of the presence of squamous cell carcinoma arising from the oropharynx, oral cavity, hypopharynx or larynx. For all patients, formalin-fixed paraffin-embedded (FFPE) tumour material in terms of pre-treatment biopsies, radiotherapy treatment plans, computed tomography (CT), magnetic resonance imaging (MRI) or positron emission tomography–CT (PET/CT) images of the location of the recurrent tumours as well as follow-up data of patients had to be available. The composition of the cohorts is presented in Fig. 1.

2.2. Segmentation and failure pattern analyses

The segmentations of the primary and of the nodal gross tumour volume (GTV) of all cases have retrospectively been performed in CT scans by one radiation oncologist (F.L.), who has expertise in the delineation of head and neck cancers. For segmentation, RayStation 6 (Raysearch Laboratories, Stockholm Sweden) and an in-house software solution has been used [24]. Disease status as well as the first site of relapse (e.g. loco-regional failure, distant failure or combined failure) have been evaluated. For each loco-regional failure, the radiotherapy treatment plan and radiological images of the recurrence (CT, MRI or PET–CT) were centrally reviewed by one experienced radiation oncologist (F.L.) in order ensure that the failure occurred within the irradiated volume.

2.3. Preparation of biomaterials for biomarker analysis

The preparation of FFPE tissue material was performed as described in [1]. Briefly, a fresh section of each FFPE block was first subjected to haematoxylin and eosin staining in order to histologically confirm the presence of squamous cell carcinoma. Afterwards, the FFPE material was further processed for immunohistochemistry or for preparation of genomic DNA or RNA under standardized conditions as described previously [1]. Briefly, for p16 immunohistochemistry the CINtec Histology kit (Roche mtm laboratories AG, Basel, CH) was used according to the manufacturers' instructions [1]. A moderate or strong overexpression of p16 in at least 70% of the tumour cells was considered as a p16 positive tumour [1]. For the immunohistochemical analysis of CD44 protein expression, the monoclonal mouse anti-human

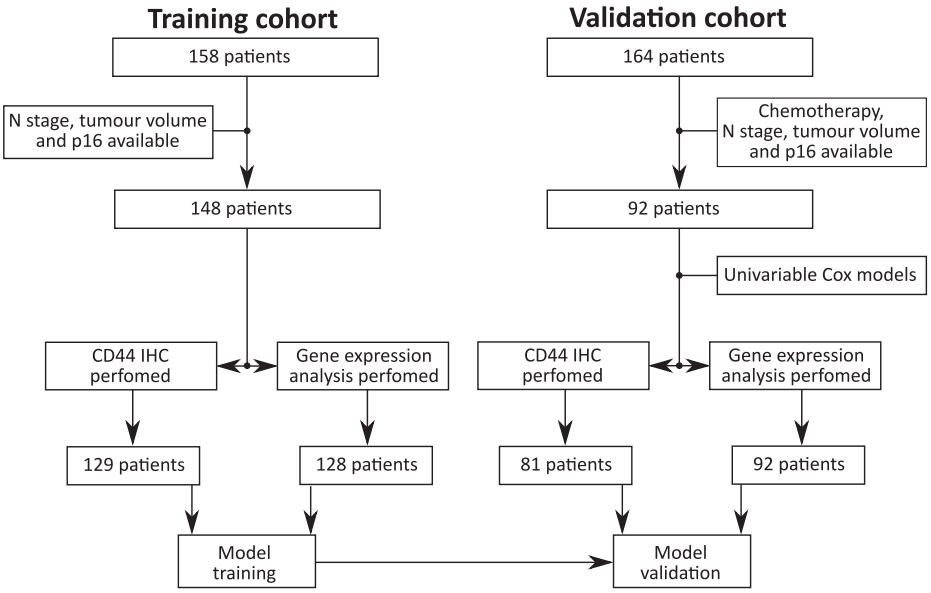

Fig. 1. Study design. The number of patients initially available is presented along with the analyses, which were performed. IHC = immunohistochemical staining.

CD44 antibody (Clone DF1485; Dako) was used. Negative control slides were incubated with the corresponding IgG antibody control (Dako). CD44 staining intensity was considered as positive if specific staining was observed in at least 5% of the tumour cells. Blinded samples were evaluated by two independent observers (AL and CvN) with an inter-observer variability of <5% for all immunohistochemical analyses.

DNA extraction and PCR-array based analyses of HPV status have been performed as described previously [26]. Briefly, genomic DNA was extracted from 5-μm FFPE sections using the QIAamp DNA FFPE tissue kit (Qiagen). HPV DNA analyses including genotyping were performed using the LCD-Array HPV 3.5 kit (CHIPRON GmbH, Berlin, DE) according to the manufacturer's instruction.

Gene expression analyses were performed using nanoString Elements technology (nanoString Technologies, Seattle, WA, USA) as described previously [1] and included the potential CSC markers *CD44* and *SLC3A2* as well as the 15-, 26-, and 30-gene hypoxia-associated signatures [27–31]. Briefly, the raw counts were logarithmised and then normalized to the mean of the internal level of reference genes *ACTR3*, *B2M*, *GNB2L1*, *NDFIP1*, *POLR2A*, *RPL11*, *RPL37A*. For the hypoxia-gene signatures, the corresponding reference genes were used [27,30,31]. Note that *DHX34* was not available. Thus only 29 genes of the original 30-gene signature [27,30,31] were evaluated.

2.4. Clinical endpoints and statistical analyses

The primary endpoint was loco-regional tumour control (LRC). Overall survival (OS) was the secondary endpoint. The corresponding times were calculated starting from the first day of radiotherapy to the date of event or censoring. The Kaplan-Meier method was used to estimate survival curves. The endpoints were compared between stratified groups using log-rank tests. Univariable and multivariable Cox regression was applied to estimate the impact of potential prognostic variables on the endpoints. The concordance index (ci) was used to validate the performance of the multivariable Cox models defined in the training cohort [1]. While a ci of 1.0 represents a perfect prediction, a ci of 0.5 is obtained for a non-informative model. The 95% confidence interval (95% CI) of the ci and the p-value of the corresponding model were estimated by 1000 bootstrap samples of the particular cohort. The validation was considered successful when the lower boundary of the 95% CI was above 0.5. On the training cohort a multivariable logistic regression model was developed, predicting 2-year LRC [1]. This model was validated using the area under the receiver operating characteristics curve (AUC). As for the ci, a non-informative model leads to an AUC of 0.5 and a perfect model to an AUC of 1.0 [32]. Differences between the cohorts were evaluated by Mann-Whitney-U tests (continuous variables) and chi-squared tests (categorical variables). Hypoxia classification was performed on the validation cohort using two cluster centres for every gene signature, which were determined on the training cohort by k-means clustering [1]. The analyses were performed using SPSS 25 (IBM Corporation, Armonk, NY, USA), R-Statistics (R Foundation for Statistical Computing [33]) and Python (Python Software Foundation. Python Language Reference, version 2.7). For all analyses, two-sided tests were performed and p-values <0.05 were considered statistically significant.

3. Results

In comparison to the training cohort [1], the patients of the validation cohort had significantly larger primary tumour volumes (p = 0.003). The proportion of tumours arising from the oropharynx was much higher in the training cohort (50.6%) than in the validation cohort (26.1%), while oral cavity tumours were more common in the validation cohort (32.6%) than in the training cohort (17.1%). The 2-year rates of LRC were similar between both cohorts (LRC: 62.6% vs 64.1%, p = 0.62), while, as a statistical trend, OS was higher in the training cohort (59.6% vs 53.2%, p = 0.084). Characteristics of both cohorts are compared and summarized in Table 1. The corresponding Kaplan-Meier estimates are shown in Supplementary Fig. 1.

Univariable Cox models were built on the validation cohort using the same parameters as were applied for the training cohort in [1] (Table 2). In contrast to the training cohort, the logarithmised primary tumour volume (training (t): HR = 1.44, p = 0.028; validation (v): HR = 1.30, p = 0.24, Fig. 2C, D) could not be validated as a significant prognostic factor for LRC, while the total volume of primary tumour and lymph nodes showed borderline significance for LRC in the validation set (t: HR = 1.57, p = 0.008; v: HR = 1.61, p = 0.050). N stage (t: HR = 1.86, p = 0.048; v: HR = 3.42,

Table 1
Patient characteristics of the training and validation cohort. 95% confidence intervals (95% CI) are marked by #. The hypoxia signatures, marked by *, stratified the patients into groups with more and less hypoxic tumours based on the gene expression of the 15-, 26-, and 30-gene hypoxia-associated signatures [26–30]. LN = lymph nodes; pts = patients.

Characteristics		Training cohort (158) Median (range)		Validation cohort (92) Median (range)		p-value
Follow-up (months)		54.4 (10.9–81.1)#		59.0 (7,7–131.9)#		**0.064**
Age (years)		58.6 (39.2–81.9)		56 (39.8–82.1)		0.30
Dose (Gy)		72.0 (68.4–74.0)		72.0 (69.0–76.6)		**0.007**
Volume Tumour (cm^3)		26.8 (4.4–175.8)		33.9 (5.1–322.6)		**0.003**
Volume LN (cm^3)		8.2 (0–300.0)		7.2 (0–272.6)		0.94
Volume total (Tumour + LN) (cm^3)		41.0 (5.6–351.7)		53.0 (7.9–344.7)		**0.042**
CD44		0.60 (-0.79–3.36)		0.38 (-1.20–1.64)		**0.008**
SLC3A2		−3.17 (-5.86-(-1.27))		−2.56 (-4.19-(-1.26))		0.83
		Number of pts	(%)	Number of pts	(%)	
Gender	Male	133	84.2	76	82.6	
	Female	25	15.8	16	17.4	0.75
Never smoker	Yes	21	13.3	7	7.6	
	No	137	86.7	85	92.4	0.17
Localization	Oropharynx	80	50.6	24	26.1	
	Oral cavity	27	17.1	30	32.6	
	Hypopharynx	51	32.3	30	32.6	
	Larynx	0	0	8	8.7	**<0.001**
T stage	1	0	0	1	1.1	
	2	18	11.4	4	4.3	
	3	41	25.9	22	23.9	
	4	99	62.7	65	70.7	0.13
N stage	0	28	17.7	12	13.0	
	1	7	4.4	7	7.6	
	2	115	72.8	68	73.9	
	3	8	5.1	5	5.4	0.60
Chemotherapy	Yes	158	100.0	92	100.0	
	No	0	0			–
HPV16 DNA	Negative	137	86.7	78	89.7	
status	Positive	20	12.7	9	10.3	
	Missing	1	0.6	5	5.4	0.58
p16 protein	Negative	125	79.1	80	87.0	
	Positive	24	15.2	12	13.0	
	Missing	9	5.7	0	0	0.52
CD44 protein	Negative	28	17.7	5	5.4	
	Positive	108	68.4	76	82.6	
	Missing	22	13.9	11	12.0	**0.004**
15-gene hypoxia	Negative	55	34.8	41	44.6	
signature*	Positive	83	52.5	51	55.4	
	Missing	20	12.7	0	0	0.48
26-gene hypoxia	Negative	47	29.7	24	26.1	
signature*	Positive	91	57.6	68	73.9	
	Missing	20	12.7	0	0	0.20
30-gene hypoxia	Negative	53	33.5	49	53.3	
signature*	Positive	85	53.8	43	46.7	
	Missing	20	12.7	0	0	**0.026**

Table 2
Univariable Cox regression on the validation cohort. The hazard ratios (HR) and the corresponding 95% confidence interval (95% CI) are shown for the endpoints loco-regional tumour control and overall survival. The parameters which were significant in the training cohort are marked by a *. Not converging models are marked by †. GTV = gross tumour volume; Ln = natural logarithm; LN = lymph nodes.

Parameter	Loco-regional tumour control		Overall survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Oral cavity vs others	1.36 (0.64–2.92)	0.43	1.76 (1.02–3.03)	**0.040**
N stage (0,1 vs 2,3)	3.63 (1.10–12.00)	**0.030**	3.42 (1.46–7.99)	**0.005***
p16	0.78 (0.24–2.57)	0.68*	0.85 (0.36–1.98)	0.71*
HPV16 DNA	1.13 (0.34–3.74)	0.85	1.19 (0.51–2.79)	0.68
Ln(GTV)	1.30 (0.84–2.03)	0.24*	1.53 (1.11–2.10)	**0.009**
Ln(LN)	1.38 (1.06–1.80)	**0.020**	1.21 (1.01–1.46)	**0.040**
Ln(GTVtot)	1.61 (1.00–2.60)	0.050*	1.75 (1.25–2.46)	**0.001**
CD44 protein	–†	–*	2.24 (0.54–9.25)	0.26*
CD44	1.30 (0.73–2.33)	0.38	1.80 (1.16–2.79)	**0.009***
MET	1.05 (0.66–1.68)	0.83	0.85 (0.60–1.20)	0.35
SLC3A2	1.29 (0.66–2.5)	0.46*	1.41 (0.86–2.31)	0.17
15-gene hypoxia signature	1.89 (0.90–3.99)	0.09	2.06 (1.19–3.58)	**0.010**
26-gene hypoxia signature	1.56 (0.64–3.81)	0.33	1.13 (0.62–2.07)	0.69
30-gene hypoxia signature	1.68 (0.82–3.47)	0.16	1.18 (0.70–1.99)	0.55

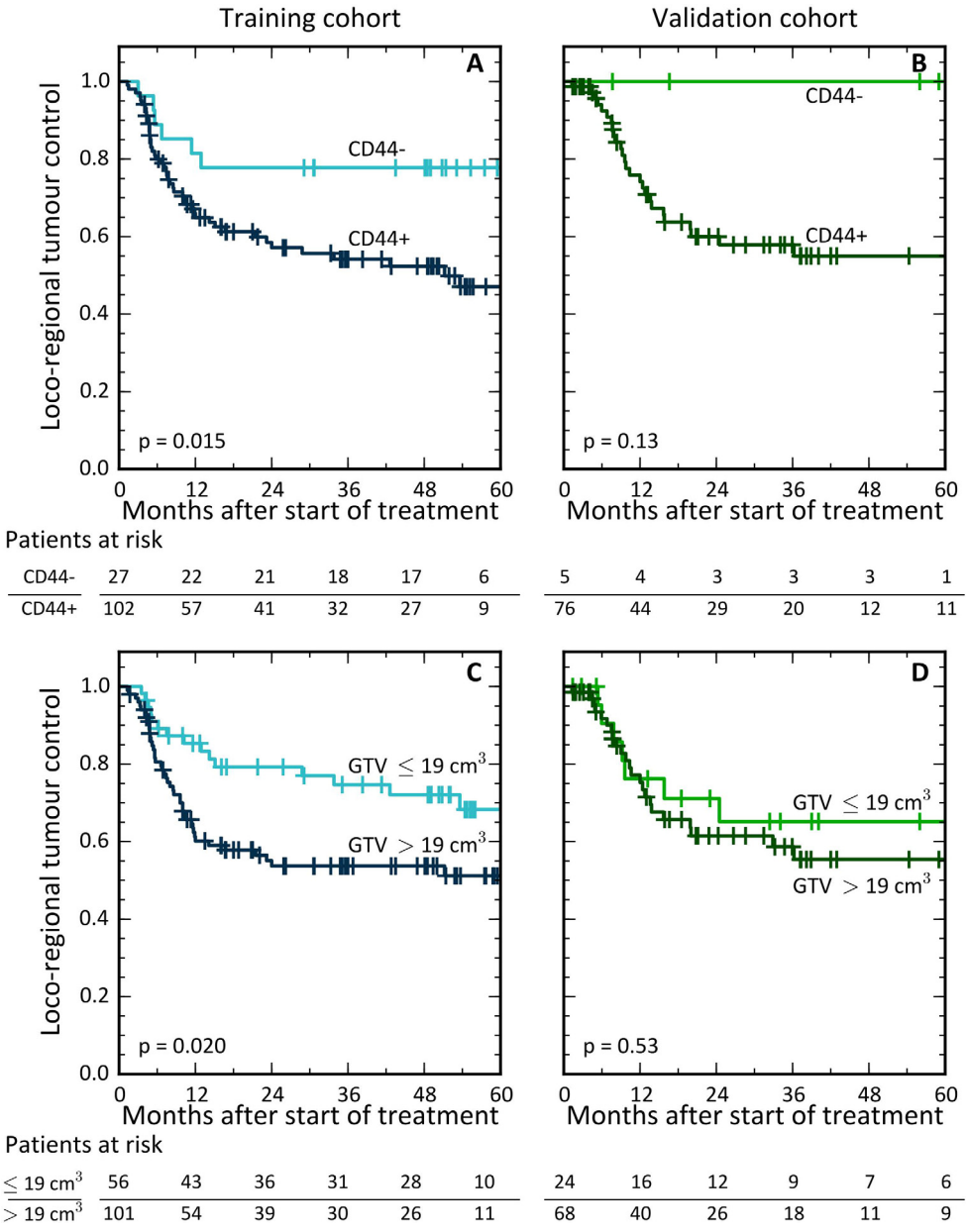

Fig. 2. Kaplan-Meier estimates for loco-regional tumour control in the training cohort (A, C) and the validation cohort (B, D) stratified based on the CD44 protein status (A, B) and the tumour volume (C, D). The p-values are based on log-rank tests.

p = 0.005), the primary tumour volume (t: HR = 1.63, p = 0.001; v: HR = 1.53, p = 0.009) and the gene expression of *CD44* (t: HR = 1.81, p = 0.006; v: HR = 1.80p = 0.009) were significantly associated with the secondary endpoint OS. Only 5 patients with a CD44 protein negative tumour were included in the validation cohort. None of them showed a loco-regional recurrence (Fig. 2A, B). While less hypoxic tumours showed higher LRC for all hypoxia-associated gene signatures (Supplementary Fig. 2), these differences were not significant.

Similar results were obtained for the validation of the multivariable Cox models (Table 3). The baseline model containing the logarithmised primary tumour volume, p16 status and N stage was derived from our multicentre study [1]. It showed a validation ci of 0.59 (95% confidence interval (CI): 0.49–0.70) for LRC and of 0.63 (0.55–0.71) for OS, which was only slightly lower than the results on the training cohort. The performance of these baseline models could be slightly improved on the validation cohort by including the expression of *CD44* (LRC: 0.61 (0.50–0.72); OS: 0.69 (0.61–0.75)), CD44 protein (LRC: 0.62 (0.50–0.72); OS: 0.65 (0.56–0.73)) and *SLC3A2* (OS: 0.65 (0.57–0.73)). None of the hypoxia-associated gene signatures could improve the performance of the baseline model, which is similar to the training cohort.

Two logistic regression models were developed previously to predict 2-year LRC [1]. The first univariable regression included the primary tumour volume only, while the second multivariable regression combined the primary tumour volume with p16 and CD44 protein status. The AUC of the univariable and multivariable regressions on the training cohort were 0.65 and 0.73, respectively. The same models were applied to the validation cohort leading to AUC = 0.58 (0.43–0.74) and to AUC = 0.64 (0.49–0.79), respectively.

Table 3
Training and validation of different multivariable Cox models for the endpoints loco-regional tumour control and overall survival. The concordance index (ci) and its 95% confidence interval (95% CI) are shown for the trained models and their independent validation. Bold values present two sided p-values <0.05 which were considered statistically significant. The baseline model (BL) consisting of N stage, p16 status and the logarithmised primary tumour volume (lnGTV) is supplemented by the additional putative CSC markers (*CD44* gene or CD44 protein; *SLC3A2*) or hypoxia classifiers based on the 15- and 30-gene hypoxia-associated signatures [26,27,30].

	Training cohort		Validation cohort	
	ci (95% CI)	p-value	ci (95% CI)	p-value
Loco-regional tumour control				
Baseline (BL): N stage, p16, lnGTV	0.64 (0.56–0.71)	**<0.01**	0.59 (0.49–0.70)	0.09
BL, CD44	0.66 (0.59–0.75)	**<0.01**	0.62 (0.50–0.73)	**0.046**
BL, *CD44*	0.64 (0.58–0.72)	**<0.01**	0.61 (0.50–0.72)	**0.046**
BL, *SLC3A2*	0.65 (0.59–0.73)	**<0.01**	0.58 (0.49–0.67)	0.09
BL, 15-gene hypoxia signature, 15-gene hypoxia signature * lnGTV	0.63 (0.58–0.73)	**<0.01**	0.54 (0.43–0.65)	0.50
BL, 30-gene hypoxia signature, 30-gene hypoxia signature * lnGTV	0.62 (0.58–0.73)	**<0.01**	0.59 (0.48–0.69)	0.12
BL, 15-gene hypoxia signature, 15-gene hypoxia signature * lnGTV, *SLC3A2*	0.66 (0.62–0.75)	**<0.01**	0.56 (0.45–0.66)	0.29
BL, 30-gene hypoxia signature, 30-gene hypoxia signature * lnGTV, *SLC3A2*	0.66 (0.61–0.75)	**<0.01**	0.60 (0.49–0.70)	0.07
Overall survival				
Baseline (BL): N stage, p16, lnGTV	0.68 (0.62–0.75)	**<0.01**	0.63 (0.55–0.71)	**<0.01**
BL, CD44	0.71 (0.65–0.78)	**<0.01**	0.65 (0.56–0.73)	**<0.01**
BL, *CD44*	0.68 (0.62–0.75)	**<0.01**	0.69 (0.61–0.76)	**<0.01**
BL, *SLC3A2*	0.67 (0.62–0.74)	**<0.01**	0.65 (0.57–0.73)	**<0.01**
BL, 15-gene hypoxia signature, 15-gene hypoxia signature * lnGTV	0.68 (0.62–0.75)	**<0.01**	0.60 (0.52–0.68)	**0.02**
BL, 30-gene hypoxia signature, 30-gene hypoxia signature * lnGTV	0.69 (0.63–0.75)	**<0.01**	0.63 (0.54–0.71)	**<0.01**
BL, 15-gene hypoxia signature, 15-gene hypoxia signature * lnGTV, *CD44*	0.68 (0.63–0.76)	**<0.01**	0.66 (0.58–0.74)	**<0.01**
BL, 30-gene hypoxia signature, 30-gene hypoxia signature * lnGTV, *CD44*	0.69 (0.64–0.76)	**<0.01**	0.70 (0.61–0.77)	**<0.01**

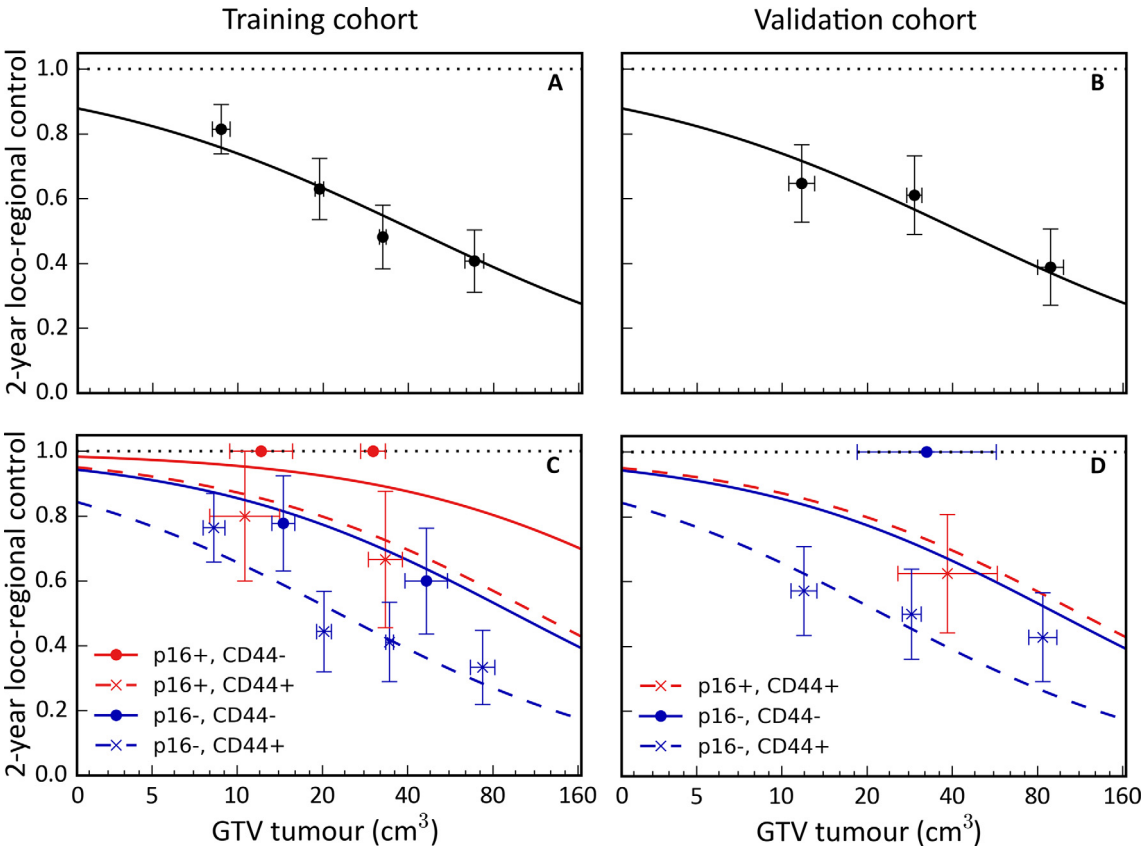

Fig. 3. Logistic regression regarding 2-year loco-regional tumour control. The results of the training cohort (A, C) and the validation cohort (B, D) are shown. A and B show the univariable logistic regression solely based on the primary tumour volume, while C and D show the multivariable logistic regression, which was additionally based on the p16 and CD44 protein status. Since none of the patients within the validation cohort presented with a p16 positive and simultaneous CD44 negative tumour, the regression for the corresponding model is not shown in D.

The logistic regressions are shown in Fig. 3 for the training and the validation cohort.

4. Discussion

In our previous study we demonstrated the prognostic value of the primary tumour volume, the potential CSC markers CD44 protein, *CD44* and *SLC3A2* gene expression as well as hypoxia-associated gene signatures [27,31] for patients with locally advanced HNSCC who were treated with primary RCTx. In the current study, an independent cohort including 92 patients with locally advanced HNSCC, who received primary RCTx between 1999 and 2015 was used to validate these results. The inclusion of the putative CSC marker *CD44* and CD44 protein resulted in slight improvements of the prognostic value of the baseline model containing primary tumour volume, p16 status and N stage for the endpoints LRC and OS. The logistic regression model for 2-year LRC based on the primary tumour volume could be improved by including the p16 status and the CD44 protein status, which is in line with the previous training cohort [1].

The volume of the primary tumour is a widely accepted prognostic biomarker for the outcome of radiotherapy in patients with HNSCC [20,22,34] and showed a significant correlation to LRC in the training cohort. In validation, the affected lymph node volume was significantly related to LRC, while the volume of the primary tumour showed no significant association. Since many patients presented with large tumours, the distinction between primary tumour and lymph nodes may be difficult, which may also lead to differences in delineation between radiation oncologists and thereby to differences in the dose prescription in the irradiated volume, respectively. Considering the total volume of both primary tumour and lymph nodes, a significant association to LRC was obtained for the training cohort and a statistical trend ($p = 0.050$) was shown for the validation cohort, underlining the importance of the tumour volume as a biomarker.

In contrast to the training cohort, the p16 status was not significantly related to LRC in the validation cohort, which may be explained by a substantially smaller proportion of oropharyngeal tumours in the latter cohort (26% in the validation cohort vs. 51% in the training cohort) and by the cohort size as such. In the validation cohort, 5 out of 24 patients with oropharyngeal tumours presented with a p16 positive tumour with 2 of them being also positive for HPV16 DNA. In contrast, the training cohort included 16 patients with p16-positive oropharyngeal tumours with 11 being also positively tested for HPV16 DNA. Thus, the numbers of HPV-driven positive oropharyngeal tumours, as characterized by the simultaneous positivity for p16 and HPV16 DNA [35], were very low.

In the validation cohort, CD44 protein slightly improved the performance of the multivariable Cox models for LRC and OS, and it improved the logistic regression model for the prognosis of 2-year LRC. Furthermore, the only two Cox models that could be successfully validated for LRC ($p < 0.05$) were the models including CD44, either at its protein or at its gene expression level. This underlines the importance of CD44 for outcome prediction of HNSCC after radio(chemo)therapy and is in line with earlier findings, e.g. it was shown that the expression of *CD44* and the CD44 protein obtained from pre-treatment biopsies of laryngeal cancer patients were correlated with the endpoint local recurrence [36,37].

In the training cohort, the impact of the hypoxia-associated gene signatures on LRC was significant for tumours smaller than 19 cm^3 [1]. For the complete cohort, significant patient stratifications based on the hypoxia status were not achieved, even though less hypoxic tumours showed a slightly better outcome [1]. The results obtained in the validation cohort, comparing subgroups with more or less hypoxic tumours, were similar to the training cohort for all three considered gene-signatures (Supplementary Fig. 2). Due to the smaller validation cohort and significantly larger tumours, however, the power was not sufficient to allow for significant stratifications. In addition, the ratio of more and less hypoxic tumours, identified by the 15- and 26-gene signatures, were similar in training and validation, indicating that hypoxia classifications by these gene signatures may be considered as robust biomarkers. The evaluation of the prognostic impact of hypoxia-associated gene expressions on LRC requires a larger cohort of patients undergoing primary radiochemotherapy, and will be performed in the prospective HNprädBio study of the DKTK-ROG, which has completed patient accrual.

The prognostic performance of the multivariable Cox models defined in [1] was slightly higher in training than in validation, with a ci between 0.63 and 0.74 in training and 0.56–0.68 in validation. Interestingly, Cox models including the most features (6 features) showed a lower ci in validation than in training. This may be explained by overfitting, which occurs if the number of parameters in a multivariable model is too large, compared to the number of events [32].

5. Conclusions

In this study an independent validation of earlier identified potential biomarkers for treatment outcome of patients with locally advanced HNSCC treated with primary RCTx was performed. The importance of the clinical parameters tumour volume and N stage, as well as the putative CSC markers CD44 and *SLC3A2* could be confirmed for OS as well as the importance of CD44 for LRC. However, the clinical utility of the observed slight improvements in prognostic power in this study has yet to be addressed in large data sets. While the stratification based on the hypoxia status was similar as in training, a significant impact on LRC and OS was not observed. Since several limitations are associated with the retrospective nature of this study, further prospective validation is required to assess the clinical relevance of the biomarkers. This will be performed in the currently ongoing prospective multicentre HNprädBio study (www.clinicaltrials.gov; NCT02059668) within the DKTK-ROG, which completed patient accrual in 2018.

Conflict of interest

Volker Gudziol is a member of the advisory board of Bristol, Myers Squibb and received speaking fees from Roche Company.

In the past 5 years, Dr. Baumann attended an advisory board meeting of MERCK KGaA (Darmstadt), for which the University of Dresden received a travel grant. He further received funding for his research projects and for educational grants to the University of Dresden by Teutopharma GmbH (2011–2015), IBA (2016), Bayer AG (2016–2018), Merck KGaA (2016–2030), Medipan GmbH (2014–2018).

Dr. Baumann, as former chair of OncoRay (Dresden) and present CEO and Scientific Chair of the German Cancer Research Center (DKFZ, Heidelberg), signed/s contracts for his institute(s) and for the staff for research funding and collaborations with a multitude of companies worldwide.

For the German Cancer Research Center (DKFZ, Heidelberg) Dr. Baumann is on the supervisory boards of HI-STEM gGmbH (Heidelberg).

For the present study, Dr. Baumann confirms that none of the above mentioned funding sources were involved in the

study design or materials used, nor in the collection, analysis and interpretation of data nor in the writing of the paper.

The other authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ctro.2019.03.002.

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