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Original article

HPV status, cancer stem cell marker expression, hypoxia gene signatures and tumour volume identify good prognosis subgroups in patients with HNSCC after primary radiochemotherapy: A multicentre retrospective study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG)

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ABSTRACT

Objective: To investigate the impact of the tumour volume, HPV status, cancer stem cell (CSC) marker expression and hypoxia gene signatures, as potential markers of radiobiological mechanisms of radioresistance, in a contemporary cohort of patients with locally advanced head and neck squamous cell carcinoma (HNSCC), who received primary radiochemotherapy (RCTx).

Materials and Methods: For 158 patients with locally advanced HNSCC of the oral cavity, oropharynx or hypopharynx who were treated at six DKTK partner sites, the impact of tumour volume, HPV DNA, p16 overexpression, p53 expression, CSC marker expression and hypoxia-associated gene signatures on outcome of primary RCTx was retrospectively analyzed. The primary endpoint of this study was loco-regional control (LRC).

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Results: Univariate Cox regression revealed a significant impact of tumour volume, p16 overexpression, and *SLC3A2* and CD44 protein expression on LRC. The tumour hypoxia classification showed a significant impact only for small tumours. In multivariate analyses an independent correlation of tumour volume, *SLC3A2* expression, and the 15-gene hypoxia signature with LRC was identified (CD44 protein n/a because of no event in the CD44-negative group). Logistic modelling showed that inclusion of CD44 protein expression and p16 overexpression significantly improved the performance to predict LRC at 2 years compared to the model with tumour volume alone.
Conclusions: Tumour volume, HPV status, CSC marker expression and hypoxia gene signatures are potential prognostic biomarkers for patients with locally advanced HNSCC, who were treated by primary RCTx. The study also supports that the individual tumour volumes should generally be included in biomarker studies and that panels of biomarkers are superior to individual parameters.
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Head and neck squamous cell carcinoma (HNSCC) is the 6th leading cancer worldwide. Although the treatment has been technically and medically improved, the 5-year overall survival (OS) rate is still stagnating at about 50% [1]. Currently, most patients with functionally inoperable HNSCC are treated with primary radiochemotherapy (RCTx), after randomized trials showed superior OS [2,3] and superior loco-regional control (LRC) as well as freedom of distant metastases [4] in patients who received RCTx compared to radiotherapy alone. However, concomitant chemotherapy is leading to increased toxicity of the treatment [3]. Furthermore, tumours are responding heterogeneously. Therefore, biomarkers are urgently needed to stratify patients for individualized escalation or de-escalation schemes based on their tumour biology in addition to established clinical parameters [5].

HNSCC and specifically oropharyngeal tumours are increasingly driven by human papilloma virus (HPV) infection [6]. In a number of investigations, it has been shown that HPV positivity is a strong prognosticator for LRC and OS after primary R(C)Tx [7–10]. The German Cancer Consortium Radiation Oncology Group (DKTK-ROG) recently demonstrated that this also applies to postoperative radiochemotherapy (PORT-C) [11]. Therefore, positive HPV infection status may be a suitable biomarker for patient stratification towards de-escalation treatment regimens, which is currently being tested in a number of clinical studies (clinicaltrials.gov; e.g. NCT01530997, NCT02281955, NCT01687413, NCT01088802).

However, additional biomarkers are needed for further stratification of patients with HPV-negative tumours [12]. In our multicentre retrospective evaluation of patients with resectable, locally advanced HNSCC, who received PORT-C, we have shown that besides the HPV type 16 deoxyribonucleic acid (HPV16 DNA) infection status, the expression of cancer stem cell (CSC) markers as well as tumour hypoxia-associated gene signatures are prognostic parameters for treatment outcome (DKTK-ROG) [13].

We report here the results of biomarker studies in the retrospective cohort of patients, who received primary RCTx in the multicentre retrospective-prospective trial of the DKTK-ROG. The purpose was to test whether the same biomarkers which have been identified as prognostic in the PORT-C cohort also have prognostic value for treatment of macroscopic tumours and whether the prognostic value is independent of tumour volume.

Material and methods

Patients

Patients meeting the following criteria were included in this retrospective study: histologically proven squamous cell carcinoma arising from the oral cavity, oropharynx or hypopharynx; treatment between 2005 and 2011 with primary RCTx based on cisplatin or mitomycin-C in curative intention according to standard protocols covering the tumour region and regional lymph nodes and including a boost to the tumour region and involved regional lymph nodes. Hyperfractionated accelerated radiotherapy up to 72 Gy was used in 69 patients, normofractionated treatment up two 70 Gy was used in 86 patients and a simultaneously integrated boost technique in 3 patients.

For patients without progressive disease a minimum follow-up time of 24 months was required. Additionally, formalin-fixed paraffin-embedded (FFPE) tumour material, 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. It was aimed to include 40 patients per DKTK partner centre (i.e. 320 patients in total). However, FFPE material was only available in six of the eight partner sites, restricting enrolment into the primary cohort to those six centres (Berlin, Dresden, Essen, Frankfurt, Munich, Tübingen). As the incidence of HPV-positive HNSCC has been increasing in recent years, patients were included backwards from 2011 to 2005. Finally, 158 patients were found to meet all requirements and were included in this study. Pathological tumour tissue specimens, radiotherapy treatment plans, radiological images of recurrent tumours and follow-up data of patients were centrally collected at the RadPlanBio Platform at the DKTK partner site Dresden [14].

Ethical approval for multicentre retrospective analyses of clinical and biological data was obtained from the Ethics Committees of all DKTK partner sites.

Segmentation

Primary and nodal gross tumour volume (GTV) segmentations have retrospectively been performed in CT scans by two radiation oncologists (FL and CV) with expertise in delineating head and neck cancers. For segmentation, an in-house software solution has been used [15].

Failure pattern analyses

Disease status and first site of relapse (loco-regional failure, distant failure or combined failure) have been evaluated by the respective treating institution. If loco-regional recurrence and distant metastases occurred within six weeks, the event was counted as combined failure. To ensure that the failure occurred within the irradiated volume, the radiotherapy treatment plan and radiological images of the recurrence (CT, MRI or PET–CT) were centrally reviewed by one experienced radiation oncologist (FL) for each loco-regional failure. For rigid image fusion the in-house software solution was used [15].

Preparation of biomaterials for biomarker analyses

From all DKTK partner sites, FFPE blocks of the primary tumour biopsies (before any tumour-specific treatment) were centrally

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collected at the DKTK partner site Dresden. All FFPE blocks were first subjected to haematoxylin and eosin staining to histologically confirm the presence of squamous cell carcinoma. Afterwards, they were processed under standardized procedures for further biomarker investigations, which are currently ongoing at the different DKTK partner sites. This includes central preparation of slides for immunohistochemistry, extraction of genomic DNA and total RNA as well as preparation of cDNA. Analyses of HPV DNA, p16, p53, tumour hypoxia-associated genes and CSC expression reported in this paper were performed and evaluated at the DKTK partner site Dresden.

Immunohistochemical staining of p16 protein

For p16 staining, 149 samples were evaluable. Immunohistochemical staining was performed using the CINtec Histology kit (Roche mtm laboratories AG, Basel, CH) according to the instructions of the manufacturer. Overexpression of p16 (also termed p16 positivity) was defined as $\geqslant$70% intense tumour staining. Blinded samples were scored by two independent observers (AL and CvN) with an inter-observer variability of <5%.

Immunohistochemical staining of p53 and CD44 protein

Immunohistochemical analyses of p53 and CD44 were performed as described previously [11,13]. Briefly, 3-µm sections of the primary tumour biopsies were prepared. The sections were incubated with monoclonal mouse anti-human p53 antibody (Clone CO-7; Dako) or with monoclonal mouse anti-human CD44 antibody (Clone DF1485; Dako). Negative control slides were incubated with the corresponding IgG antibody control (Dako). Blinded samples were evaluated by two independent observers (AL and CvN) with an inter-observer variability of <5%. For p53 and CD44 analyses, 153 and 136 samples were evaluable, respectively.

DNA extraction and PCR-array based analyses of HPV status

DNA extraction and PCR-array based analyses of HPV status have been performed as described previously [11]. 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. One hundred and fifty-seven samples were evaluable for HPV DNA analyses.

nanoString RNA analyses

Gene expression analyses were performed using nanoString Elements technology (nanoString Technologies, Seattle, WA, USA) including the genes of three hypoxia gene signatures (Supplementary Table 1) as well as potential CSC markers *CD44*, *SLC3A2* and *MET*. nanoString analyses have been performed as described previously [13]. Raw counts were logarithmized and then normalized to the mean of the internal level of Ref. genes *ACTR3*, *B2M*, *GNB2L1*, *NDFIP1*, *POLR2A*, *RPL11*, *RPL37A*. For the hypoxia-gene signatures, the corresponding Ref. genes were used (Supplementary Table 1), respectively [16–18]. Twenty samples had to be omitted from nanoString analyses due to insufficient tumour material or due to too low RNA yield, thus 138 samples were evaluable.

Clinical endpoints and statistical analysis

The primary endpoint was LRC and secondary endpoints were freedom from distant metastases (DM) and OS. All endpoints were calculated from the first day of radiotherapy to the date of event or censoring. Survival curves were estimated by the Kaplan–Meier method. The impact of potential prognostic variables on the endpoints was evaluated using the univariate Cox-regression model. Significant parameters were included in multivariate Cox regression. To predict 2-year LRC multivariate logistic regression was performed. To compare patient groups stratified by p16 status, CSC marker expression and hypoxia status, Log-rank tests were employed. For the stratification of the patient cohort, CSC markers and tumour volume were binarized according to the bootstrapping procedure outlined in [13]. This results in the cut-off values 1.400 (power 68%), -2.135 (power 96%) and 19 ccm (power 75%) for *CD44*, *SLC3A2* and tumour volume, respectively. For stratification with respect to hypoxia-induced gene expression, tumours were assigned to a less and a more hypoxia class, according to low or high expression levels of the corresponding hypoxia gene signature, using two-class k-means clustering based on the Euclidian distance. Due to the more advanced disease state of the patient cohort, this procedure was chosen instead of the classification methods originally used in Toustrup et al. [16], Eustace et al. [17], and Lendahl et al. [18]. To assess correlations between continuous variables the Pearson correlation coefficient was used. Between binary parameters the mean square contingency coefficient (phi coefficient) was employed. Differences in continuous parameters between two groups were evaluated by the Mann–Whitney-*U* test. The bootstrapping procedure was performed by STATA 11 (StataCorp LP, College Station, TX, USA) and the other analyses by SPSS 23 software (IBM Corporation, Armonk, NY, USA). For all analyses, two-sided tests were performed and *p*-values <0.05 were considered statistically significant.

Results

In this retrospective multicentre study, a total of 158 patients with locally advanced HNSCC treated with contemporary primary RCTx were evaluated. Patient characteristics and treatment parameters are summarized in Table 1. Isolated loco-regional failure was observed in 61 patients, isolated distant failure occurred in 29 patients and combined failures occurred in 11 patients. For the total patient population, actuarial rates of LRC, freedom from DM and OS were 62.6%, 81.8% and 59.6% after two years, respectively.

In univariate analyses (Table 2), the logarithm of the primary tumour volume (HR 1.44, $p = 0.028$) as well as the total tumour volume compromising primary tumour volume and involved lymph node volume (HR 1.57, $p = 0.008$) were found to be significant prognosticators for LRC (Fig. 1A). No significant impact on LRC was found for gender, T stage, N stage, UICC stage, tumour localization, smoking or alcohol consumption.

In this patient cohort 12.7% of the tumours were tested positive for HPV16 DNA. For HPV16 DNA positive tumours, a trend for improved LRC (HR 0.39, $p = 0.072$) was observed (Fig. 1B). Overexpression of p16, a surrogate marker for HPV infection, was found in 15.2% of the patients and showed a significant impact on LRC (HR 0.30; $p = 0.021$) in univariate analysis (Fig. 1C). Overexpression of p53 had no impact on LRC (Table 2).

In the previously reported postoperative cohort, the expression of the potential CSC markers *CD44*, *SLC3A2* and *MET* as well as CD44 protein had been identified as significant prognosticators for LRC [13]. Here, in univariate analysis, *SLC3A2* and CD44 protein expression were found to have a significant impact on LRC (*SLC3A2*: HR 1.72, $p = 0.007$, Fig. 1D; CD44: HR 2.31, $p = 0.040$, Fig. 1E; Table 2), while *CD44* (Fig. 1F) was also important for the secondary endpoints. When stratified for tumour volume ($\leqslant$19 ccm vs. >19 ccm), CSC marker expression analyses revealed similar results in patients with small tumours compared to all patients (Table 2). In contrast, CSC marker expression was not found to be signifi-

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Table 1
Patient characteristics. IHC = immunohistochemistry.

Variable		Of 158	Fraction (%)
Gender	Male	133	84.2
	Female	25	15.8
Tumour localization	Oral cavity	27	17.1
	Oropharynx	80	50.6
	Hypopharynx	51	32.3
T stage	2	18	11.4
	3	41	25.9
	4	99	62.7
N stage	0	28	17.7
	1	7	4.4
	2	115	72.8
	3	8	5.1
UICC stage	III	13	8.2
	IVa, b	145	91.8
Never smoker	Yes	21	13.3
	No	137	86.7
Never drinker	Yes	63	39.9
	No	88	55.7
	Missing	7	4.4
Chemotherapy	Mitomycin C	29	18.4
	Cisplatin	129	81.6
HPV16 DNA	Negative	137	86.7
	Positive	20	12.7
	Missing	1	0.6
p16 (IHC)	Negative	125	79.1
	Positive	24	15.2
	Missing	9	5.7
p53 (IHC)	Negative	100	63.3
	Positive	53	33.5
	Missing	5	3.2
CD44 analyses (IHC)	Performed	136	86.1
	Missing	22	13.9
nanoString analyses	Performed	138	87.3
	Missing	20	12.7
Centre	Berlin	35	22.2
	Dresden	34	21.5
	Essen	30	19.0
	Frankfurt	23	14.6
	Munich	7	4.4
	Tübingen	29	18.4
Variable	Median (Range)		
Age	58.6 (39.2–81.9)		
Volume Tumour (ccm)	26.8 (4.4–175.8)		
Volume LN (ccm)	8.1 (0.0–300.0)		
Volume total (ccm)	41.1 (5.6–351.7)		
Dose (Gy)	72.0 (68.4–74.0)		
Treatment time (days)	48.0 (38.0–71.0)		

cantly associated with LRC in patients with large tumours. This might potentially be explained by the fact that in the advanced tumours the hierarchical organization driven by CSC is often be lost [19].

Tumour hypoxia has been assessed using three different hypoxia-related gene signatures [16–18] (Supplementary Table 1) using nanoString technology. In univariate analyses of all tumours, gene expression status did not show a significant impact on LRC or on the secondary endpoints (Table 2; Fig. 2A, C, E). When stratified for primary tumour volume, the 15-gene signature [16] and the 30-gene signature [18] showed a significant association with LRC, but only in small tumours (HR 7.35, $p = 0.009$ and HR 5.52, $p = 0.025$; Table 2; Fig. 2B, D, F).

In order to study a potential interaction between the hypoxia-associated gene expression signatures and CSC marker expression and to evaluate their association with tumours' radio(chemo)resistance, correlation analyses between CSCs and the hypoxia-related gene signatures were conducted. Overall, only low correlations were observed (Supplementary Table 2). Furthermore, correlation analyses between expression status of hypoxia-associated genes, CSC marker expression and p16 status were performed. p16-negative tumours were found to have a higher hypoxia-related gene expression than p16-positive tumours (Supplementary Table 3A). In addition, p16-negative tumours were associated with increased CD44 expression, while p16-positive tumours on average showed low expression of *CD44* and *SLC3A2* (Supplementary Table 3B).

Hypoxia classifiers and CSC markers that showed a significant impact on LRC in univariate Cox regression were included in multivariate models together with N stage, tumour volume (lnGTV) and p16 status (Table 3). The 15- and 30-gene hypoxia classifiers revealed a statistical trend for the association of tumours classified as "hypoxic" with poor LRC (HR 10.7, $p = 0.068$; HR 12.8, $p = 0.071$) and for the interaction of tumour volume and hypoxia status (HR 0.51, $p = 0.069$; HR 0.49, $p = 0.074$). For the CSC markers, a strong association of both CD44 (HR 2.42, $p = 0.045$) and *SLC3A2* (HR 1.59, $p = 0.035$) with LRC was found.

Finally, to predict 2-year LRC two logistic regression models were developed, one containing only tumour volume and the other containing p16, tumour volume and the CSC marker CD44 protein, i.e. the parameters providing the highest statistically significant hazard ratios in multivariate analyses (Table 3, Supplementary Table 4). The area under the receiver operating characteristics curve was 0.65 and 0.73, respectively. CD44 protein and p16 status significantly improved the performance compared to the model with tumour volume alone (likelihood-ratio test, $p = 0.007$). Fig. 3 shows the regression curves for 2-year LRC in dependence of tumour volume for all patients (A) and stratified by p16 and CD44 protein status (B). While no recurrences occurred in the patient group with p16 positive and CD44 negative tumours, the patient group with p16 negative and CD44 positive tumours showed the lowest LRC.

Discussion

Our previous retrospective multicentre study in patients with locally advanced HNSCC, who were treated by PORT-C after radical surgery, showed that positive HPV status, low expression of putative CSCs markers and tumour hypoxia related gene signatures play a prognostic role for loco-regional tumour control [11,13]. The present study extends these investigations to patients with inoperable locally advanced HNSCC, who received primary RCTx.

In the postoperative cohort, almost no recurrences were found in high-risk patients after PORT-C, whose tumours were positive for infection for HPV16 DNA [11], which is associated with increased radiosensitivity [20,21]. This is well in line with the current study, showing that p16-overexpressing tumours are associated with significantly better LRC. For HPV16 DNA positive tumours a statistical trend was observed. The magnitude of the effect of the HPV infection status on LRC was smaller than in the PORT-C cohort, which might be due to a lower number of oropharyngeal tumours and therefore a lower HPV positivity rate in the current patient cohort.

The putative CSC markers CD44, *SLC3A2* and *MET* have been identified as prognosticators for LRC of HNSCC in the PORT-C cohort [13]. Recently, CD44 and *SLC3A2* could be confirmed as prognosticators in a validation cohort, consisting of 152 patients with locally advanced HNSCC who received PORT or PORT-C [22]. In a previous study, de Jong et al. showed that increased expression of the potential CSC marker CD44 (both *CD44* mRNA and CD44 protein) predicts local recurrence in early-stage laryngeal cancer after primary radiotherapy [23]. Rietbergen et al. showed that the expression of the potential CSC marker CD98, whose heavy chain is encoded by *SLC3A2*, is prognostic for OS and progression-free survival in patients with HPV-positive oropharyngeal carcinomas; and increased expression of CSC markers such as CD44 and CD98 was significantly associated with decreased LRC [24]. In the study

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Table 2
Univariate analyses for all tumours and stratified for tumour volume ($V \leqslant 19$ ccm and $V > 19$ ccm). HR = hazard ratio; 95% CI = 95% confidence interval.

Variable	Loco-regional control		Distant metastases		Overall survival	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Gender	1.35 (0.70–2.60)	0.37	0.44 (0.11–1.87)	0.27	1.22 (0.66–2.26)	0.53
Age	0.99 (0.96–1.01)	0.28	1.02 (0.98–1.06)	0.28	1.00 (0.97–1.02)	0.66
Oral cavity	1.68 (0.92–3.05)	0.089	0.39 (0.09–1.63)	0.19	1.43 (0.82–2.47)	0.21
Oropharynx	0.83 (0.50–1.38)	0.47	1.05 (0.51–2.18)	0.89	1.06 (0.68–1.65)	0.80
Hypopharynx	0.86 (0.50–1.48)	0.59	1.40 (0.67–2.94)	0.37	0.74 (0.45–1.20)	0.22
T stage (<4 vs 4)	1.25 (0.73–2.14)	0.41	1.04 (0.49–2.19)	0.93	1.48 (0.91–2.41)	0.11
N stage (0,1 vs 2,3)	1.32 (0.70–2.49)	0.39	4.39 (1.04–18.5)	**0.043**	1.86 (1.00–3.44)	**0.048**
UICC stage (III vs IV)	3.30 (0.81–13.5)	0.097		*	4.39 (1.08–17.8)	**0.039**
Treatment time	0.98 (0.94–1.02)	0.26	1.00 (0.95–1.06)	0.97	0.98 (0.95–1.01)	0.22
Dose	0.90 (0.71–1.15)	0.41	0.81 (0.57–1.15)	0.23	0.93 (0.75–1.16)	0.53
Never smoker	0.49 (0.20–1.22)	0.12	0.66 (0.20–2.19)	0.50	0.65 (0.31–1.36)	0.25
Never drinker	0.68 (0.40–1.16)	0.16	1.26 (0.60–2.66)	0.54	0.83 (0.52–1.32)	0.43
Chemotherapy	0.94 (0.49–1.80)	0.84	1.42 (0.61–3.32)	0.42	0.63 (0.32–1.22)	0.17
p53	1.06 (0.62–1.79)	0.84	1.36 (0.64–2.90)	0.43	1.16 (0.73–1.86)	0.53
p16	0.30 (0.11–0.83)	**0.021**	0.31 (0.07–1.30)	0.11	0.39 (0.18–0.84)	**0.017**
HPV16 DNA	0.39 (0.14–1.09)	0.072	0.43 (0.10–1.81)	0.25	0.62 (0.30–1.28)	0.19
lnGTV (V in ccm)	1.44 (1.04–1.99)	**0.028**	1.48 (0.94–2.34)	0.093	1.63 (1.23–2.16)	**0.001**
lnGTVtot (V in ccm)	1.57 (1.12–2.19)	**0.008**	2.16 (1.33–3.48)	**0.002**	1.97 (1.45–2.67)	**<0.001**
CD44	2.31 (1.04–5.13)	**0.040**	4.27 (1.01–18.1)	**0.049**	2.38 (1.17–4.80)	**0.016**
CD44	1.40 (0.90–2.12)	0.14	3.28 (1.56–6.90)	**0.002**	1.81 (1.18–2.76)	**0.006**
MET	0.98 (0.73–1.32)	0.90	1.57 (0.95–2.59)	0.078	1.01 (0.77–1.33)	0.94
SLC3A2	1.72 (1.16–2.53)	**0.007**	1.75 (0.98–3.13)	0.058	1.33 (0.94–1.88)	0.11
15-gene signature	1.34 (0.77–2.34)	0.30	1.27 (0.56–2.87)	0.57	1.24 (0.77–2.01)	0.37
26-gene signature	1.39 (0.77–2.52)	0.28	1.00 (0.44–2.27)	1.00	1.15 (0.70–1.89)	0.58
30-gene signature	1.48 (0.84–2.62)	0.18	1.21 (0.53–2.74)	0.65	1.08 (0.67–1.74)	0.75
Volume ⩽ 19 ccm						
CD44	5.55 (0.71–43.3)	0.10		*	3.16 (0.72–13.8)	0.13
CD44	1.57 (0.58–4.29)	0.38	4.42 (1.26–15.5)	**0.021**	3.20 (1.44–7.13)	**0.004**
MET	1.07 (0.57–2.01)	0.83	2.70 (0.91–8.06)	0.075	1.03 (0.60–1.78)	0.92
SLC3A2	2.89 (1.24–6.72)	**0.014**	2.67 (0.92–7.70)	0.070	1.56 (0.76–3.20)	0.23
15-gene signature	7.35 (1.65–32.7)	**0.009**	3.86 (0.80–18.7)	0.093	4.88 (1.61–14.8)	**0.005**
26-gene signature	2.47 (0.70–8.78)	0.16	1.30 (0.32–5.20)	0.71	1.89 (0.68–5.26)	0.22
30-gene signature	5.52 (1.24–24.6)	**0.025**	6.90 (0.86–55.4)	0.069	3.60 (1.19–10.9)	**0.023**
Volume > 19 ccm						
CD44	1.75 (0.73–4.18)	0.21	2.58 (0.58–11.4)	0.21	2.12 (0.95–4.74)	0.067
CD44	1.22 (0.74–2.03)	0.44	2.66 (1.04–6.78)	**0.041**	1.27 (0.77–2.10)	0.35
MET	0.92 (0.65–1.31)	0.65	1.24 (0.68–2.25)	0.49	0.96 (0.70–1.31)	0.79
SLC3A2	1.43 (0.93–2.20)	0.11	1.41 (0.70–2.84)	0.34	1.21 (0.82–1.78)	0.34
15-gene signature	0.67 (0.36–1.27)	0.22	0.61 (0.23–1.66)	0.33	0.62 (0.36–1.09)	0.096
26-gene signature	1.16 (0.58–2.32)	0.68	0.80 (0.29–2.20)	0.66	0.94 (0.53–1.69)	0.85
30-gene signature	1.01 (0.52–1.94)	0.98	0.52 (0.20–1.41)	0.20	0.68 (0.39–1.18)	0.17

Bold values present *p*-values <0.05, and were considered statistically significant.
* As there were no events in one group, the Cox model did not converge.

presented here, both CD44 and *SLC3A2* expression were found to be significant prognostic biomarkers for LRC after primary RCTx, confirming previous results [13]. In contrast to our results for PORT-C, the *MET* expression level did not have any prognostic impact on LRC after primary RCTx. It may be speculated that *MET* expression plays a role in lymphatic spread and thus for the burden of undetected CSC remaining in the neck after surgery. These, after complete removal of the primary tumour and macroscopic lymph node metastases, are a major cause of loco-regional recurrence in patients who received PORT-C. In contrast patients, who were treated with primary RCTx are more likely to develop recurrences primarily at the site of the primary tumour or macroscopic lymph node metastases, i.e. at the sites of the largest tumour burden. Therefore, an increased risk of spread of few cells from *MET* expressing tumours to volumes of the neck that are adjuvantly treated could easily be overseen. To further explore this hypothesis we currently investigate lymphatic spread and recurrence risk in patients whose tumours were undergoing resection alone. Interestingly, in the present study the CSC marker expression (*CD44* mRNA, CD44 protein, and *SLC3A2*) on average was found to be lower in HPV positive tumours compared to HPV negative tumours. This is in line with a recent report by Vlashi et al., who showed that HPV positive HNSCC are associated with a lower proportion of CSCs. Furthermore, they demonstrated that HPV positive cell lines are not only characterized by a higher cellular radiosensitivity but also have significantly lower plating efficiencies [25], suggesting a smaller proportion of less radioresistant CSCs in HPV induced tumours [24].

It is well recognized from radiobiological and clinical studies in several tumour entities, that the tumour volume is as a strong parameter affecting the outcome of primary radio(chemo)therapy [26–28]. The absolute number of CSCs is expected to increase with increasing tumour volume [27,29,30], leading to a poorer treatment outcome. All other radiobiological factors being identical, the radiation dose necessary to achieve LRC is anticipated to increase with the logarithm of the tumour volume [26,29,31,32]. The EORTC 22811 randomized trial reported that the tumour volume is significantly influencing LRC in HNSCC and has to be considered for the interpretation of treatment results [33]. However, the impact of the tumour volume may also depend on the tumour site as demonstrated by Mendenhall et al. [34]. They showed that the tumour volume has a significant impact for laryngeal tumours but is less important for oropharyngeal tumours. The latter may be due to a higher rate of HPV positivity and thereby increased

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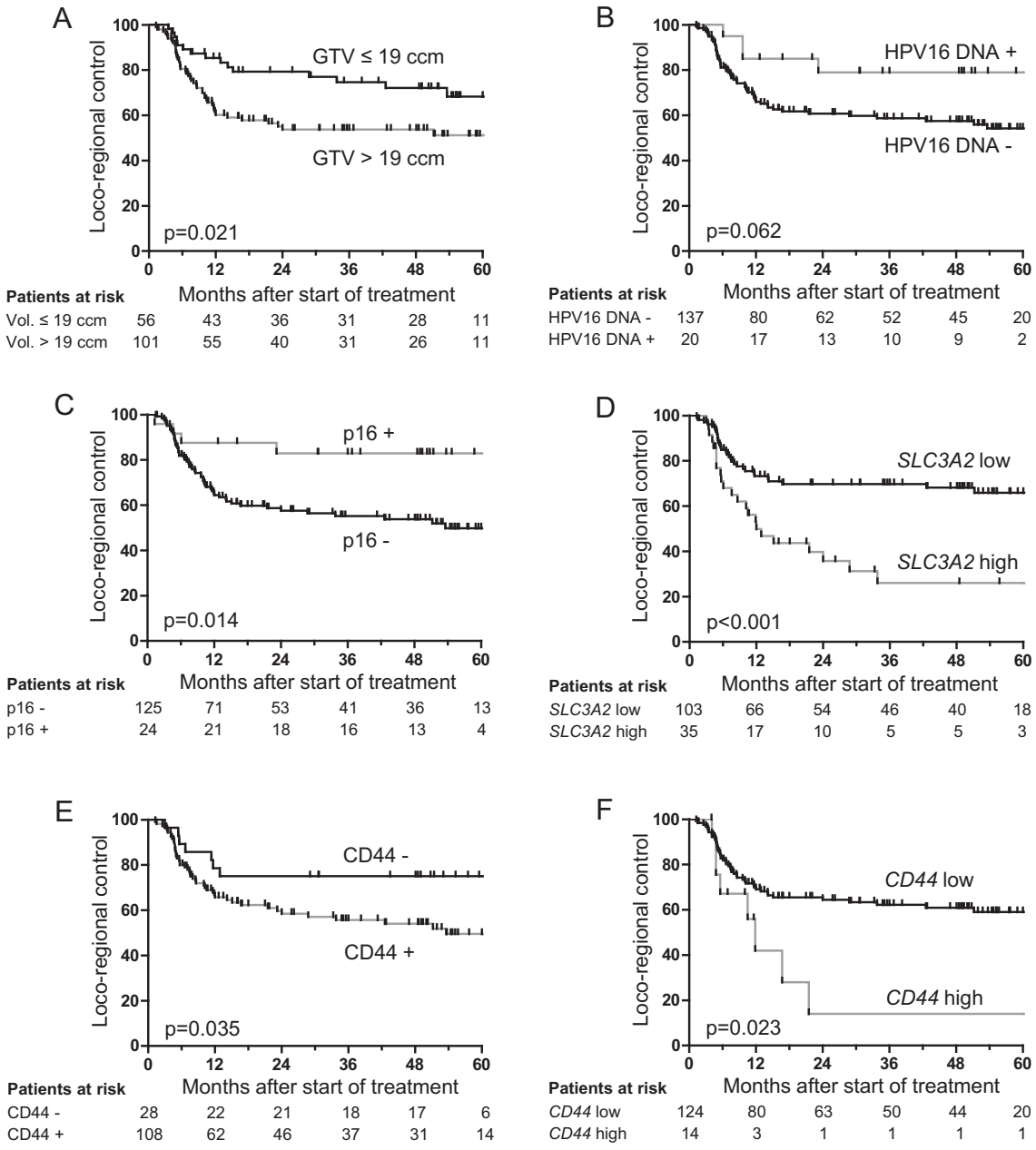

Fig. 1. Kaplan–Meier estimates of loco-regional control (LRC) for patients regarding (A) their tumour volume, (B,C) their HPV status assessed with (B) HPV DNA and (C) p16 overexpression, (D-F) potential cancer stem cell (CSC) markers expression of (D) *SLC3A2*, (E) CD44 protein and (F) *CD44* mRNA.

radiosensitivity. This study shows, that in patients with locally advanced HNSCC, the tumour volume is significantly and in its magnitude importantly associated with LRC in carcinomas of the oral cavity, oropharynx and hypopharynx. This important finding is well in line with earlier publications, which reported on the tumour volume in head and neck cancers [26,31,35–38]. Here, small tumours were found to be significantly associated with improved LRC compared to large tumours at the same radiation dose, independently of the HPV status.

The significant impact of tumour volume on LRC after primary RCTx leads to the question whether the biomarkers investigated in the study presented here improve the prognostic power of radiobiological models compared to the evaluation of the volume of the primary tumour alone. Our results show a significant improvement of the performance of the logistic model to predict 2-year LRC by inclusion of CD44 protein and p16 status.

The negative impact of hypoxia on the likelihood to achieve local tumour control after primary radio- or radiochemotherapy of HNSCC has been demonstrated in many preclinical experiments and clinical studies [39–43]. Tumour hypoxia may decrease the cellular radiosensitivity [44,45] and has also been shown to contribute to an increased clonogenic potential [41,46,47] which may increase radioresistance of HNSCC. To assess the hypoxia status of the tumours non-invasively, several gene signatures have been developed, which allow to determine the response to tumour hypoxia on the molecular level. The signature by Toustrup et al. consists of genes correlated with tumour hypoxia in experimental studies und have later on been used for patient stratification for the effect of hypoxic cell sensitizers [16,48]. The hypoxia gene signature by Eustace et al. [17] is based on genes which were initially derived from gene expression analysis compromising 99 genes whose *in vivo* expression is known to be correlated with 10 well-

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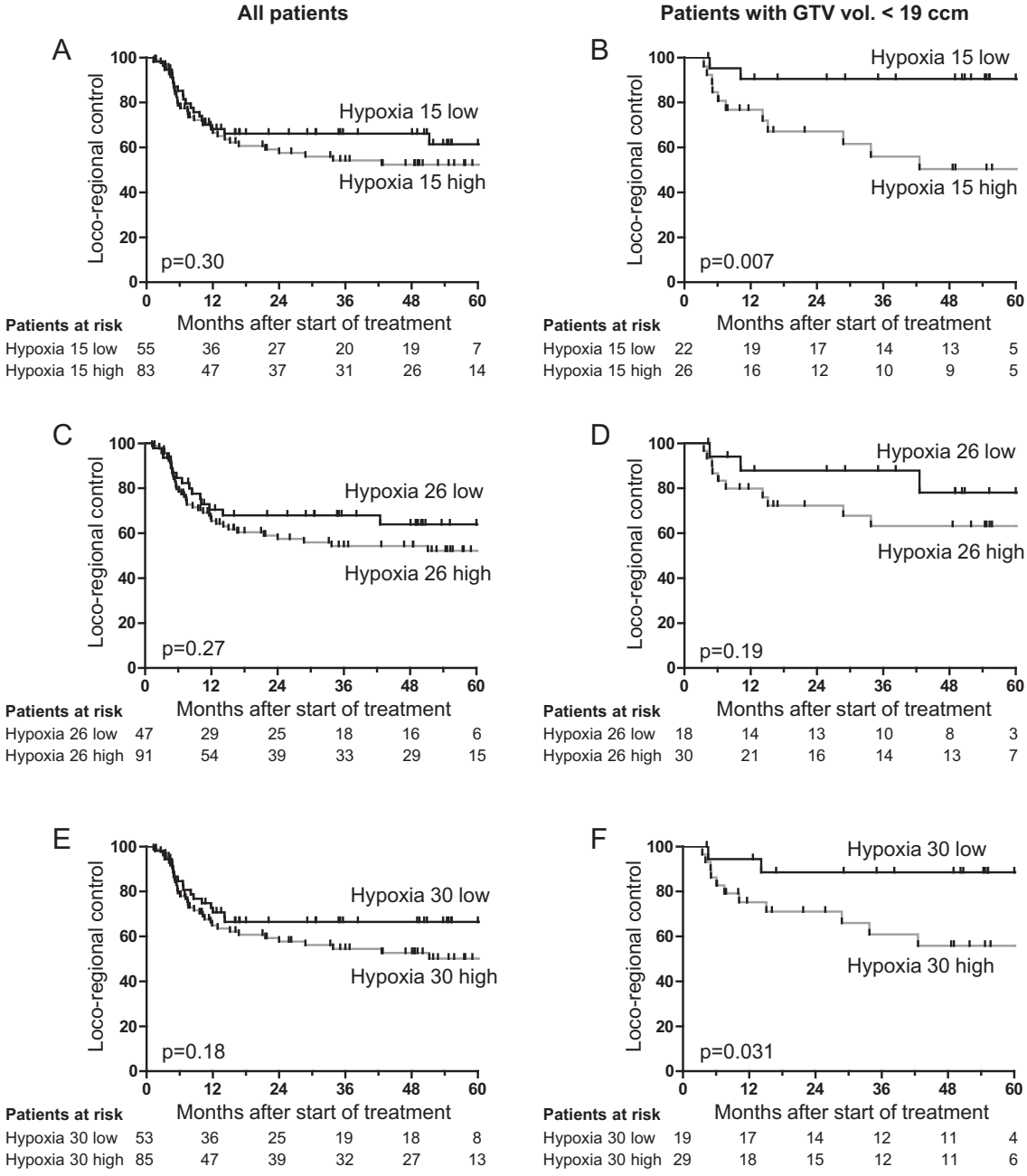

Fig. 2. Kaplan-Maier estimates of loco-regional control (LRC) stratified for tumour hypoxia status for (A, C, E) all patients and (B, D, F) patients with small tumours.

known hypoxia regulated genes [49]. The third signature applied here was developed by Lendahl et al. and is based on an *in silico* meta-analysis on data sets of the NCBI Gene Expression Omnibus public microarray repository [18]. In the present study, small tumours with high expression of hypoxia-associated genes [16,18] in pre-treatment biopsies were found to have lower LRC rates compared to small, less hypoxic tumours. In contrast, no such effect was found in large tumours. Possible reasons for this observation, which needs to be validated in further studies, include (1) limited statistical power due to the limited sample size and the heterogeneous patient cohort, and (2) masking of the impact of hypoxia by other radiobiological parameters driving early recurrence, in particular high CSC numbers in the most advanced tumours.

Taken together the results of our study provide evidence for a complex interplay between tumour volume and different biomarkers for local tumour control. It has previously been hypothesized that the combination of determinations of the tumour volume, as a surrogate of average CSC number, and the expression of putative CSC markers, as a surrogate for CSC density in an individual tumour, might be a powerful and clinically applicable predictive parameter for local tumour control [30,50,51]. The results of our study support this hypothesis and further extent the model (see Fig. 3). Patients with HPV positive, CD44 negative tumours showed superior LRC, while patients with HPV negative, CD44 positive tumours are presenting a group with poor prognosis, which has recently also been described by Motegi et al. [52] for oropharyngeal tumours. The intermediate group (HPV positive, CD44 negative and *vice versa*) demonstrates, that HPV positivity and CD44 expression seems to be of similar importance. Strikingly, this figure also demonstrates that the patients cannot only be stratified into three risk groups regarding their biomarker profile, but that their

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Table 3
Multivariate analyses of CSC markers or hypoxia-gene signatures and additional prognostic factors. HR = hazard ratio; 95% CI = 95 percent confidence interval.

	Loco-regional control		Distant metastases		Overall survival	
	HR (95% CI)	*p*-Value	**HR (95% CI)**	*p*-Value	**HR (95% CI)**	*p*-Value
N stage 0,1 vs 2,3	1.49 (0.69–3.21)	0.31	3.05 (0.72–13.0)	0.13	1.62 (0.80–3.29)	0.18
p16	0.30 (0.09–0.99)	**0.047**	0.41 (0.10–1.78)	0.24	0.46 (0.20–1.07)	0.072
lnGTV	1.43 (1.03–1.98)	**0.031**	1.34 (0.84–2.13)	0.22	1.62 (1.21–2.17)	**0.001**
CD44	2.42 (1.02–5.75)	**0.045**	4.00 (0.93–17.1)	0.062	2.70 (1.28–5.70)	**0.009**
N stage 0,1 vs 2,3	1.32 (0.65–2.66)	0.45	4.42 (1.03–19.0)	**0.046**	1.73 (0.90–3.32)	0.10
p16	0.28 (0.08–0.92)	**0.035**	0.66 (0.14–3.01)	0.59	0.48 (0.20–1.15)	0.099
lnGTV	1.32 (0.94–1.85)	0.11	1.16 (0.70–1.91)	0.57	1.51 (1.12–2.04)	**0.007**
CD44	1.12 (0.68–1.87)	0.66	3.58 (1.58–8.11)	**0.002**	1.63 (1.01–2.63)	**0.046**
N stage 0,1 vs 2,3	1.39 (0.69–2.81)	0.36	3.69 (0.86–15.9)	0.080	1.64 (0.85–3.16)	0.14
p16	0.34 (0.10–1.15)	0.082	0.47 (0.10–2.15)	0.33	0.43 (0.18–1.02)	0.056
lnGTV	1.31 (0.94–1.83)	0.11	1.28 (0.79–2.10)	0.32	1.59 (1.18–2.14)	**0.002**
SLC3A2	1.59 (1.03–2.44)	**0.035**	1.64 (0.86–3.15)	0.14	1.25 (0.85–1.84)	0.26
N stage 0,1 vs 2,3	1.17 (0.58–2.37)	0.66	3.12 (0.73–13.4)	0.13	1.50 (0.78–2.88)	0.23
p16	0.29 (0.09–0.95)	**0.041**	0.41 (0.09–1.81)	0.24	0.41 (0.17–0.95)	**0.038**
lnGTV	2.12 (1.15–3.92)	**0.017**	2.47 (0.96–6.32)	0.060	2.59 (1.50–4.49)	**0.001**
15-gene signature	10.7 (0.84–136)	0.068	21.9 (0.42–1137)	0.13	10.4 (1.04–104)	**0.047**
15-gene signature * lnGTV	0.51 (0.25–1.06)	0.069	0.42 (0.14–1.25)	0.12	0.50 (0.26–0.96)	**0.037**
N stage 0,1 vs 2,3	1.16 (0.57–2.36)	0.67	3.23 (0.75–13.9)	0.12	1.58 (0.82–3.06)	0.17
p16	0.32 (0.10–1.06)	0.061	0.43 (0.09–1.93)	0.27	0.38 (0.16–0.90)	**0.028**
lnGTV	2.28 (1.15–4.52)	**0.019**	2.85 (1.09–7.49)	**0.033**	2.60 (1.49–4.54)	**0.001**
30-gene signature	12.8 (0.81–202)	0.071	31.3 (0.53–1861)	0.099	6.78 (0.66–70.1)	0.11
30-gene signature * lnGTV	0.49 (0.23–1.07)	0.074	0.36 (0.12–1.09)	0.070	0.52 (0.27–1.01)	0.054
N stage 0,1 vs 2,3	1.34 (0.66–2.74)	0.42	5.22 (1.14–23.9)	**0.033**	1.78 (0.91–3.48)	0.094
p16	0.38 (0.11–1.26)	0.11	0.72 (0.16–3.26)	0.67	0.52 (0.22–1.24)	0.14
lnGTV	2.05 (1.14–3.66)	**0.016**	2.58 (1.01–6.59)	**0.047**	2.61 (1.52–4.48)	**<0.001**
15-gene signature	7.78 (0.66–91.4)	0.10	26.1 (0.50–1371)	0.11	11.3 (1.14–112)	**0.038**
15-gene signature * lnGTV	0.52 (0.26–1.05)	0.070	0.33 (0.11–1.01)	0.052	0.46 (0.24–0.88)	**0.019**
SLC3A2	1.63 (1.03–2.57)	**0.037**				
CD44			4.34 (1.85–10.1)	**0.001**	1.81 (1.12–2.94)	**0.016**
N stage 0,1 vs 2,3	1.30 (0.64–2.67)	0.47	5.43 (1.17–25.1)	**0.031**	1.88 (0.95–3.71)	0.069
p16	0.38 (0.11–1.28)	0.12	0.69 (0.14–3.34)	0.64	0.46 (0.19–1.14)	0.094
lnGTV	2.08 (1.07–4.02)	**0.030**	2.77 (1.02–7.53)	**0.047**	2.47 (1.42–4.31)	**0.001**
30-gene signature	7.76 (0.51–118)	0.14	29.8 (0.43–2076)	0.12	6.14 (0.59–63.9)	0.13
30-gene signature * lnGTV	0.54 (0.25–1.15)	0.11	0.31 (0.10–1.00)	**0.049**	0.52 (0.27–0.99)	**0.048**
SLC3A2	1.54 (0.98–2.42)	0.061				
CD44			4.39 (1.87–10.3)	**0.001**	1.80 (1.11–2.94)	**0.018**

Bold values present *p*-values <0.05, and were considered statistically significant.

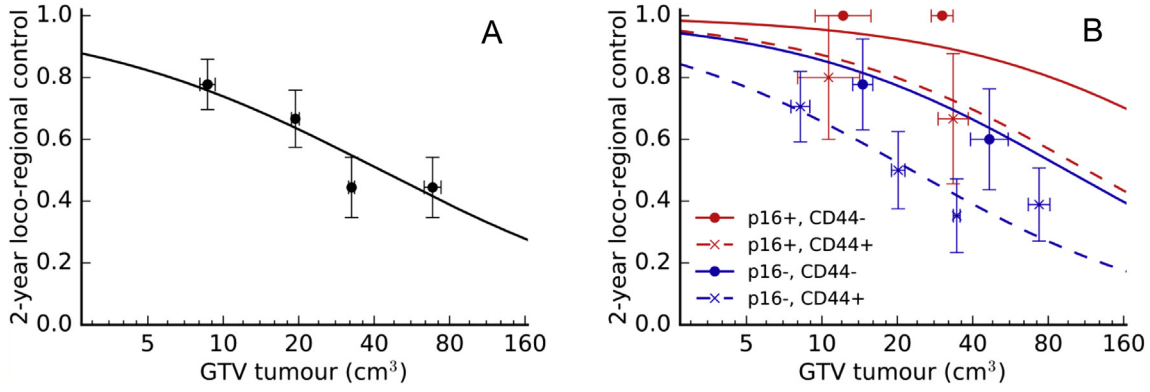

Fig. 3. Logistic regression of loco-regional control (LRC) two years after primary RCTx including (A) tumour volume only and (B) p16 status, CD44 protein status and tumour volume. Regression curves are shown as solid and dashed lines (A) for all patients and (B) for patients stratified by p16 and CD44 status. Outcome of the corresponding patients is shown as data points with error bars representing the standard error of the mean. Patients were divided into groups of similar size based on their tumour volume.

individual risk for loco-regional failure is also being determined by the tumour volume. This implies for studies investigating new biomarkers and for trials using biomarkers as stratification parameters for primary RCTx, that the tumour volume should be determined for every individual patient for integration into the analysis. Furthermore, in addition to established clinical parameters, biomarker panels instead of single biomarkers appear to be superior for risk assessment and individualization of radio- or radiochemotherapy, which has been suggested by many researchers before [12,47,53,54]. For the PORT-C cohort further biomarker have been reported [55,56] which still await analyses in the primary RCTx cohort. Validation of the biomarkers and cut-offs presented here will be performed as demonstrated in [22] for the PORT-C cohort.

In summary, the present retrospective multicentre study confirmed the prognostic value of the HPV infection status, CSC expression and tumour hypoxia status in a contemporary cohort of patients with locally advanced HNSCC who received primary

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RCTx. In addition, this study provides evidence that, because of its strong impact on LRC, consideration of the tumour volume in biomarker-based radiobiological modelling of the risk of recurrence after radiotherapy is necessary. Our results indicate that CD44 protein and p16 status significantly improve the performance of prognostic models for primary RCTx compared to the impact of tumour volume alone. To validate our findings a prospective clinical trial of DKTK-ROG is currently being performed.

Conflict of interest

The authors declare no conflict of interest regarding the present manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2016.11.008.

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