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## Independent validation of the prognostic value of cancer stem cell marker expression and hypoxia-induced gene expression for patients with locally advanced HNSCC after postoperative radiotherapy



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a b s t r a c t

*Objective:* To validate the impact of HPV status, cancer stem cell (CSC) marker expression and tumour hypoxia status in patients with locally advanced head and neck squamous cell carcinoma (HNSCC), who received postoperative radiotherapy. The results of the exploration cohort have previously been reported by the German Cancer Consortium Radiation Oncology Group (DKTK-ROG; Lohaus et al., 2014; Linge et al., 2016).

*Materials and methods:* For 152 patients with locally advanced HNSCC the impact of HPV16 DNA status, CSC marker expression and hypoxia-associated gene signatures on outcome of postoperative radiother- apy were retrospectively analysed. Out of them, 40 patients received postoperative radiochemotherapy. Cox models presented in a previous study were validated using the concordance index as a performance measure. The primary endpoint of this study was loco-regional control. Results were compared to those previously reported by DKTK-ROG.

*Results:* Loco-regional control, freedom from distant metastases and overall survival were inferior to the previously reported cohort. Despite of this, the prognostic value of the combination of HPV infection sta- tus, CSC marker expression (*SLC3A2*) and tumour hypoxia status could be validated in univariate analyses using an independent validation cohort. For multivariate models, the concordance index was between

0.58 and 0.69 in validation, indicating a good prognostic performance of the models. The inclusion of *CD44* and the 15-gene hypoxia signature moderately improved the performance compared to a baseline model without CSC markers or hypoxia classifiers.

*Conclusions:* The HPV status, CSC marker expression of *CD44* and *SLC3A2* as well as hypoxia status are potential prognostic biomarkers for patients with locally advanced HNSCC treated by postoperative radiotherapy.

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Introduction

The 5-year overall survival rate of patients with head and neck squamous cell carcinoma (HNSCC) is about 50%, although treatment efficacy has been therapeutically improved in the last

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decade [[1–3]](#_bookmark20). Furthermore, the number of oropharyngeal cancers has increased worldwide over the last years [[4–6]](#_bookmark20). For patients with resected locally advanced HNSCC it has been shown in three randomized clinical trials [[7–9]](#_bookmark21) that loco-regional control can be improved by postoperative radiochemotherapy (PORT-C) com- pared to postoperative radiotherapy (PORT) alone. However, due to their biological characteristics tumours are responding very heterogeneously to this treatment. In addition to established clin- ical parameters, novel biomarkers are needed to identify patient groups, which require escalated or de-escalated treatment schedules.

Independent of exogenous carcinogens, e.g. tobacco and alcohol consumption, infection with high-risk human papilloma virus (HPV) has become a major risk factor for the development of HNSCC over the past decade [[10]](#_bookmark27). It has been shown that patients with HPV-related HNSCC are a distinct subgroup with better out- come of primary radiochemotherapy [[11–14]](#_bookmark29). Recently, in a multi- centre retrospective trial conducted by the German Cancer Consortium Radiation Oncology Group (DKTK-ROG) it was shown, that patients with HPV16 DNA positive oropharyngeal tumours have a high rate of loco-regional tumour control and overall sur- vival also after PORT-C compared to patients with HPV16 DNA neg- ative tumours [[15]](#_bookmark22). Furthermore, in a recent study it was shown that high expression of previously developed tumour hypoxia- associated gene signatures [[16,17]](#_bookmark22) and cancer stem cell (CSC) markers within the surgically removed primary tumour were asso- ciated with impaired loco-regional tumour control after PORT-C in HNSCC [[18]](#_bookmark22). These biomarkers were used to stratify patients with HPV16 DNA negative tumours into smaller subgroups with low and high probability of loco-regional recurrence. In order to apply this patient stratification scheme in future clinical trials regarding treatment de-escalation or intensification, these results have to be validated. Therefore, the present publication reports on the valida- tion results of the Cox models presented in [[18]](#_bookmark22) on HPV16 DNA sta- tus, CSC marker expression and tumour hypoxia-associated gene signatures, using an independent validation cohort of HNSCC patients treated by PORT or PORT-C.

Material and methods

*Patients*

In this publication, two patient cohorts are being considered: a training cohort previously reported by DKTK-ROG and a monocen- tric validation cohort. For the training cohort, inclusion criteria, data collection, handling and analyses of biomaterial were previ- ously described in detail [[15,18]](#_bookmark22). Briefly, 221 patients, who received PORT-C between 2004 and 2012 at nine partner sites of the DKTK-ROG, were included to identify potential prognosticators for loco-regional control after PORT-C, such as CSCs and tumour hypoxia [[18]](#_bookmark22). In the retrospective validation cohort, patients trea- ted at the Department of Radiation Oncology of the University Hospital Dresden meeting the following criteria were included: not included in the previous DKTK-ROG cohort, histologically pro- ven squamous cell carcinoma arising from the oral cavity, orophar- ynx, hypopharynx or larynx; treatment between 1999 and 2006 with PORT or PORT-C in curative intention according to standard radiotherapy protocols (2 Gy/fraction, 5 fractions/week), covering the former tumour region and regional lymph nodes (50 Gy) and a boost (10–16 Gy) to the former tumour region and to the regions of involved lymph nodes. Before surgery, all patients had under- gone staging (computed tomography or magnetic resonance imag- ing, chest X-ray and abdominal ultrasound), and only patients without evidence of distant metastases were included. Formalin- fixed paraffin-embedded (FFPE) tumour material as well as

follow-up data of patients had to be available. Finally, 152 patients meeting all these criteria were included in the validation cohort. Follow-up data of patients were collected using the RadPlanBio Platform at the DKTK partner site Dresden [[19]](#_bookmark23). Ethical approval for retrospective analyses of clinical and biological data was obtained from the local ethics committee.

*Preparation of biomaterials for biomarker analyses*

FFPE blocks of the primary tumour specimens (removed by sur- gery) were first subjected to haematoxylin and eosin staining to histologically confirm the presence of squamous cell carcinoma. Afterwards, they were processed under standardised procedures for biomarker investigations described below.

*Immunohistochemical staining of p16 and CD44*

Immunohistochemical (IHC) staining of p16 was performed using the CINtec Histology kit (Roche mtm laboratories AG, Basel, CH) according to the manufacturer’s instructions. Overexpression of p16 was defined as P70% intense tumour staining. For p16 staining, 148 samples were evaluable. For CD44 protein expression, 145 patients of the validation cohort could be analysed. Immuno- histochemical staining was performed as described in [[18]](#_bookmark22). CD44 staining intensity was scored (0, +, ++, +++) and tumours with a minimum of 10% staining were considered as positive. Blinded samples were scored by two independent observers (AL and CK) with an inter-observer variability of <5%.

*DNA extraction and PCR-array based analyses of HPV status*

DNA extraction and PCR-array based analyses of HPV status were performed as described previously [[15]](#_bookmark22). Briefly, genomic DNA was extracted from 5-lm FFPE sections using the QIAamp DNA FFPE tissue kit (Qiagen). HPV DNA analyses including geno-

typing were performed using the LCD-Array HPV 3.5 kit (CHIPRON GmbH, Berlin, DE) according to the instructions of the manufac- turer. In total, 148 samples were evaluable for HPV DNA analyses.

*nanoString RNA analyses*

For the validation cohort, gene expression analyses have been performed using nanoString technology as described previously [[18]](#_bookmark22), including two hypoxia-associated gene signatures (see Sup- plementary Table 1) as well as potential CSC markers *CD44*, *SLC3A2* and *MET*. Briefly, total RNA as well as reporter and capture probes specific to the genes of interest were mixed and incubated at 62 °C for 22 h. Samples were then kept at 4 °C for a maximum of 18 h and subjected to the nCounter system. Raw counts were logarithmised and then normalised to the mean of the internal level of reference genes *ACTR3*, *B2M*, *GNB2L1*, *NDFIP1*, *POLR2A*, *RPL11, RPL37A*, or to

the reference genes of the corresponding hypoxia-gene signatures (Supplementary Table 1), respectively [[16,17]](#_bookmark22). Seven samples had to be omitted from nanoString analyses due to insufficient tumour material or due to too low RNA yield, thus 145 samples were evalu- able. The *CD44* probe design, which was incorrectly designed for the training cohort [[18]](#_bookmark22), has been corrected and included in the validation cohort.

*Clinical endpoints and statistical analysis*

The primary endpoint was loco-regional tumour control (LRC) and secondary endpoints were freedom from distant metastases (DM) and overall survival (OS). The endpoints were calculated from the first day of radiotherapy to the date of event or censoring and survival curves were estimated by the Kaplan-Meier method. To

compare patient groups stratified by HPV16 DNA status, CSC marker expression and hypoxia classification, Log-rank tests were used. For the stratification of the validation cohort with respect to CSC marker expression, the cut-offs from the training cohort reported in [[18]](#_bookmark22) were applied. Hypoxia classification on the valida- tion cohort was performed by k-means clustering (Euclidian distance) according to the cluster centres of the training cohort (Supplementary Table 2). The impact of potential prognostic variables on the endpoints was evaluated using univariate Cox-regression for both cohorts. To evaluate the prognostic perfor- mance of the multivariate Cox models presented in [[18]](#_bookmark22) for the validation cohort, the concordance index (ci) was calculated, which is equal to 0.5 for non-prognostic models and equals 1 for perfectly predicting models [[20]](#_bookmark24). Bootstrap resampling with 10,000 samples was used to estimate the confidence intervals (CI) of the concor- dance index. From these bootstrap samples a *p*-value for the hypothesis ci = 0.5 was calculated. To evaluate differences between the two cohorts Mann-Whitney-*U* tests were used for continuous variables and chi-squared tests for categorical variables. The boot- strapping procedure and ci calculation were implemented in Python. For all of the other analyses, SPSS 23 software (IBM Corpo- ration, Armonk, NY, USA) was used. In this study, two-sided tests were performed and *p*-values < 0.05 were considered statistically significant.

Results

In this retrospective study, the prognostic value of HPV16 DNA status, CSC markers and hypoxia classifiers presented in

[[18]](#_bookmark22) for 221 patients (training cohort) should be validated on an independent validation cohort of 152 patients. The patient data, treatment parameters and tumour characteristics of the training cohort have been described previously [[15,18]](#_bookmark22). They are summarised in [Table 1](#_bookmark12) together with the values of the vali- dation cohort and the comparison between both cohorts. Patients in the validation cohort were treated between 1999 and 2006 with PORT (*N* = 112) or PORT-C (*N* = 40), while all patients of the training cohort received PORT-C as the standard treatment (*p* < 0.001). Furthermore, patients in the retrospective validation cohort had a significantly lower age (*p* = 0.016), deliv- ered dose (*p* = 0.001), N stage (*p* < 0.001), R status (*p* < 0.001), extracapsular extension (ECE) status (*p* < 0.001) and also showed a different distribution in their tumour localisations (*p* < 0.001). The fraction of oropharyngeal tumours and tumours of the oral cavity were almost reverse in training and validation cohort (oropharynx: 57% vs 30%, oral cavity: 27% vs 55%). In the valida- tion cohort HPV infection occurred less frequently than in the (more recently treated) training cohort (HPV16 DNA positivity: 14% vs. 33%, *p* < 0.001). For the considered endpoints, patients in the validation cohort showed lower LRC and OS, while the occurrence of DM was not significantly different, see [Fig. 1](#_bookmark13). Actu- arial rates of LRC, freedom from DM and OS for the training and validation cohort were 90% vs 78% (*p* = 0.002), 85% vs 84%

(*p* = 0.21) and 83% vs 68% (*p* < 0.001), after two years,

respectively.

[Table 2](#_bookmark14) shows the results of univariate Cox regression with clin- ical parameters and HPV status for the considered endpoints of the validation cohort. Significantly lower LRC and OS was found for higher T stage (LRC: HR 2.46, *p* = 0.008; OS: HR 2.14, *p* = 0.001)

and tumours of the oral cavity (LRC: HR 2.68, *p* = 0.005; OS: HR 2.22, *p* < 0.001), while oropharyngeal tumours (LRC: HR 0.38, *p* = 0.020; OS: HR 0.40, *p* = 0.001) and HPV16 DNA positive tumours (LRC: HR 0.25, *p* = 0.060; OS: HR 0.35, *p* = 0.015) showed

improved LRC and OS. These results are consistent with the find- ings of the training cohort [[18]](#_bookmark22). [Fig. 2](#_bookmark16) A-B shows Kaplan-Meier

curves of the validation cohort stratified for HVP16 DNA status and use of chemotherapy. Patients with HPV16 DNA positive tumours showed significantly higher loco-regional control rates compared to patients with HPV16 DNA negative tumours using the Log-rank test; a statistical trend was obtained by Cox regres- sion. The higher loco-regional control rates in the small patient group receiving PORT-C did not reach statistical significance.

In [[18]](#_bookmark22) a significant impact of the cancer stem-cell markers *CD44*, *SLC3A2* and *MET* on LRC was found using their expression values dichotomised at the cut-offs 0.2, -3.135 and -4.135, respectively. These cut-offs were used for patient stratification of the validation cohort. The resulting Kaplan-Meier curves are depicted in [Fig. 2](#_bookmark16)D–F. In univariate analyses, the impact of *CD44* (HR 2.14, *p* = 0.049) and *SLC3A2* (HR 2.45, *p* = 0.047) on LRC could

be confirmed with lower hazard ratios, while *MET* expression showed no correlation with LRC ([Table 3](#_bookmark17)). Also the significant impact of CD44 protein on LRC could be confirmed (*p* = 0.027, [Fig. 2](#_bookmark16)C). For the secondary endpoints only *CD44* showed a signifi- cant correlation with freedom of DM. The prognostic value of *MET* and *SLC3A2* for OS and DM could not be confirmed.

For hypoxia-related gene classification based on the 15- and 26-gene signature [[16,17]](#_bookmark22), k-means clustering was used in [[18]](#_bookmark22). Both classifiers were able to stratify patients into a group with high hypoxia-related gene expression and low LRC and a group with low hypoxia-related gene expression and better LRC. For the validation cohort, the hypoxia classification was performed using the cluster centres of the training cohort. Based on this classification, [Fig. 2](#_bookmark16)G and [2](#_bookmark16)H present Kaplan-Meier curves of tumours with low and high hypoxia-related gene expression for the validation cohort. A statistical trend for differences in LRC between both groups was obtained for both signatures (15-gene signature: HR 2.32, *p* = 0.061; 26-gene signature: HR 3.36, *p* = 0.097; [Table 3](#_bookmark17)). Considering only patients with HPV16 DNA negative tumours, this trend remained for the 15-gene sig- nature (HR 2.13, *p* = 0.093) but not for the 26-gene signature. In

[[18]](#_bookmark22) low-risk patients (HPV16 DNA positive or *SLC3A2* negative or low hypoxia) showed favourable LRC compared to the remaining high-risk patients. Here, this classification also yields a significant difference in LRC (*p* = 0.010, [Fig. 2](#_bookmark16)I).

In [[18]](#_bookmark22) several multivariate Cox models were presented, which included HPV16 DNA status, one CSC marker and one hypoxia-associated gene classifier as well as the clinical param- eters ECE status, tumour localisation oropharynx and hypophar- ynx. For the primary endpoint LRC, these models were applied to the validation cohort without adapting the model parameters. The prognostic performance of these models was then evaluated by the concordance index (ci). To reveal an additional impact of the CSC markers or hypoxia classifiers, the model performance was compared to a baseline model, which did not contain any CSC marker or hypoxia classifier. In [Table 4](#_bookmark19) the results of the training cohort and the validation cohort are presented for all patients and for patients with HPV16 DNA negative tumours. For the training cohort, the baseline model showed a ci of 0.76 for all patients and of 0.66 for patients with HPV16 DNA nega- tive tumours. Using additional CSC markers and hypoxia classi- fiers improved the ci up to 0.81 and 0.76, respectively, revealing their significant impact. For the validation cohort the baseline model showed ci = 0.66 for all patients and ci = 0.63 for patients with HVP16 DNA negative tumours. Including addi- tional CSC markers or hypoxia classifiers improved these models only for *CD44* and the 15-gene hypoxia classifier up to ci = 0.69 and ci = 0.65, respectively. Validation on the patient subgroup which received PORT-C led to higher ci values for all CSC mark- ers and hypoxia classifiers, however, due to the low patient number with large confidence intervals.

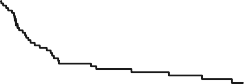
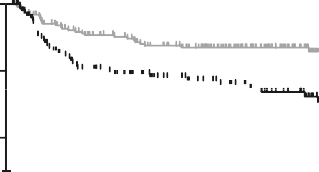
Table 1

Comparison of the patient and tumour characteristics of the training and validation cohort.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Characteristics | Training cohort (2004–2012) |  | Validation cohort (1999–2006) |  | *p*-Value |
|  | Median (range) |  | Median (range) |  |  |
| Follow-up (months) | 46.2 (2.5–100.1) |  | 43.1 (1.8–153) |  | 0.29 |
| Age (years) | 57.0 (24.0–75.2) |  | 52.7 (36.3–71.0) |  | 0.016 |
| Dose (Gy) | 64 (56–68) |  | 64 (60–66) |  | 0.001 |
|  | Number of pts | (%) | Number of pts | (%) |  |
| Gender |  |  |  |  |  |
| Male/Female | 180/41 | (81/19) | 127/25 | (84/16) | 0.60 |
| Clinical tumour (T) stage  cT1/cT2/cT3/cT4 | 41/98/47/35 | (19/44/21/16) | 40/69/24/19 | (26/45/16/13) | 0.20 |
| Clinical nodal (N) stage |  |  |  |  |  |
| cN0/cN1/cN2/cN3 | 22/31/138/30 | (10/14/62/14) | 15/31/104/2 | (10/20/68/1) | <0.001 |
| Stage (UICC 7th edition) |  |  |  |  |  |
| I/II/III/IV | 0/8/33/180 | (0/4/15/81) | 2/3/34/113 | (1/2/23/74) | 0.069 |
| R status |  |  |  |  |  |
| 0/1/unknown | 125/94/2 | (57/42/1) | 109/30/13 | (72/20/8) | <0.001 |
| ECE status |  |  |  |  |  |
| 0/1/unknown | 103/118/0 | (47/53/0) | 104/47/1 | (68/31/1) | <0.001 |
| Localisation |  |  |  |  |  |
| Oropharynx/oral cavity/hypopharynx/larynx | 126/60/35/0 | (57/27/16/0) | 46/83/15/8 | (30/55/10/5) | <0.001 |
| Grading |  |  |  |  |  |
| 1/2/3/unknown | 5/123/89/4 | (2/56/40/2) | 3/78/71/0 | (2/51/47/0) | 0.55 |
| Chemotherapy |  |  |  |  |  |
| yes/no | 221/0 | (100/0) | 40/112 | (26/74) | <0.001 |
| Smoking during therapy |  |  |  |  |  |
| yes/no/unknown | 186/20/15 | (84/9/7) | 103/16/33 | (68/11/22) | 0.30 |
| Alcohol during therapy |  |  |  |  |  |
| yes/no/unknown | 100/30/91 | (45/14/41) | 99/19/34 | (65/13/22) | 0.17 |
| p16 status |  |  |  |  |  |
| negative/positive/unknown | 135/79/7 | (61/36/3) | 128/20/4 | (84/13/3) | <0.001 |
| HPV16 DNA status |  |  |  |  |  |
| negative/positive/unknown | 143/72/6 | (65/33/2) | 126/22/4 | (83/14/3) | <0.001 |
| CD44 protein |  |  |  |  |  |
| negative/positive/unknown | 44/151/26 | (20/68/12) | 15/130/7 | (10/85/5) | 0.003 |
| *CD44* (log2-normalised expression) |  |  |  |  |  |
| 60.2 / > 0.2 / unknown | 78/118/25 | (35/54/11) | 55/90/7 | (36/59/5) | 0.73 |
| *SLC3A2* (log2-normalised expression) |  |  |  |  |  |
| 6-3.135 />-3.135 / unknown | 77/119/25 | (35/54/11) | 43/102/7 | (28/67/5) | 0.066 |
| *MET* (log2-normalised expression) |  |  |  |  |  |
| 6-4.135 />-4.135 / unknown | 94/102/25 | (43/46/11) | 47/98/7 | (31/64/5) | <0.001 |
| 15-gene hypoxia signature |  |  |  |  |  |
| low hypoxic/highly hypoxic/unknown | 79/117/25 | (36/53/11) | 41/104/7 | (27/68/5) | 0.021 |
| 26-gene hypoxia signature |  |  |  |  |  |
| low hypoxic/highly hypoxic/unknown | 72/124/25 | (33/56/11) | 22/123/7 | (14/81/5) | <0.001 |
| Loco-regional recurrences | 29 | (13) | 38 | (25) | 0.003 |
| Distant metastases | 42 | (19) | 24 | (22) | 0.43 |
| Deaths | 70 | (32) | 86 | (57) | <0.001 |

Bold numbers indicate significant *p*-Values with *p* < 0.05.

**100**



A

Training cohort

Validation cohort

Loco-regional control

**80**

**60**

**100**

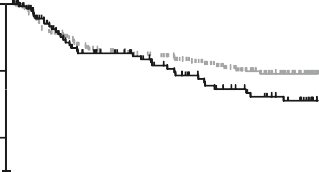
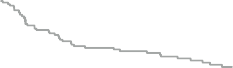
**80**

Freedom distant metastases

**60**

B

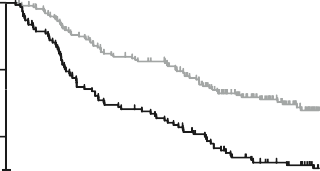
Training cohort



Validation cohort

**100**

**80**



C

Training cohort

Validation cohort

Overall survival

**60**

**Pts. at risk**

**0**

**0 12 24 36 48 60**



p=0.002

Months after start of treatment

**Pts. at risk**

**0**

**0 12 24 36 48 60**



p=0.21

Months after start of treatment

**Pts. at risk**

**0**

**0 12 24 36 48 60**



p<0.001

Months after start of treatment

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Training 221 191 | 173 | 148 | 107 | 63 | Training 221 | 187 | 167 | 144 | 103 | 61 | Training 221 | 198 | 178 | 153 | 111 64 |
| Validation 152 109 | 92 | 76 | 64 | 44 | Validation 152 | 110 | 95 | 77 | 64 | 45 | Validation 152 | 120 | 102 | 88 | 70 50 |

Fig. 1. (A–C) Kaplan-Meier estimates of (A) loco-regional control (LRC), (B) freedom of distant metastases (DM) and (C) overall survival (OS) for the training and validation cohort.

Table 2

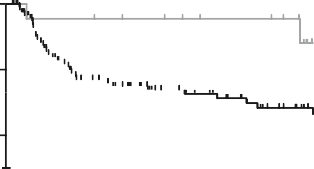
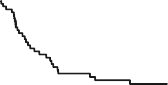
Univariate Cox regression with clinical variables as well as HPV status for the endpoints loco-regional control, freedom of distant metastases and overall survival for the validation cohort. Shown is the hazard ratio (HR) with 95% confidence interval (CI) and the *p*-value testing the hypothesis HR = 1.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Loco-regional control |  |  | Distant metastases |  |  | Overall survival |  |  |
| Variable | HR (95% CI) | *p*-Value |  | HR (95% CI) | *p*-Value |  | HR (95% CI) | *p*-Value |
| Age (years) | 0.97 (0.94–1.01) | 0.16[\*](#_bookmark15) |  | 0.99 (0.95–1.03) | 0.56 |  | 1.01 (0.99–1.04) | 0.31 |  |
| Dose (Gy) | 1.07 (0.94–1.24) | 0.31 |  | 1.21 (1.02–1.42) | 0.022 |  | 1.07 (0.98–1.18) | 0.15 |  |
| Gender | 0.36 (0.11–1.16) | 0.087 |  | 0.04 (0.00–1.33) | 0.071 |  | 0.59 (0.31–1.15) | 0.12 |  |
| T stage (1,2 vs 3,4) | 2.46 (1.26–4.79) | 0.008[\*](#_bookmark15) |  | 2.50 (1.24–5.05) | 0.010[\*](#_bookmark15) |  | 2.14 (1.35–3.38) | 0.001[\*](#_bookmark15) |  |
| N stage (0,1 vs 2,3) | 0.87 (0.44–1.74) | 0.69 |  | 0.99 (0.47–2.09) | 0.98[\*](#_bookmark15) |  | 0.85 (0.54–1.34) | 0.48 |  |
| UICC stage (I–III vs IV) | 1.13 (0.53–2.40) | 0.75 |  | 1.03 (0.48–2.21) | 0.95 |  | 0.99 (0.62–1.60) | 0.97 |  |
| R status | 0.86 (0.39–1.89) | 0.71 |  | 1.38 (0.64–3.00) | 0.41 |  | 1.29 (0.78–2.11) | 0.32 |  |
| ECE status | 1.60 (0.82–3.11) | 0.17 |  | 1.75 (0.87–3.49) | 0.11[\*](#_bookmark15) |  | 1.41 (0.90–2.21) | 0.13[\*](#_bookmark15) |  |
| Oropharynx | 0.38 (0.17–0.86) | 0.020[\*](#_bookmark15) |  | 0.41 (0.18–0.94) | 0.034[\*](#_bookmark15) |  | 0.40 (0.23–0.68) | 0.001[\*](#_bookmark15) |  |
| Oral cavity | 2.68 (1.35–5.33) | 0.005[\*](#_bookmark15) |  | 1.55 (0.78–3.08) | 0.21[\*](#_bookmark15) |  | 2.22 (1.43–3.46) | <0.001[\*](#_bookmark15) |  |
| Hypopharynx | 0.91 (0.32–2.60) | 0.86 |  | 1.87 (0.77–4.53) | 0.17 |  | 0.89 (0.43–1.84) | 0.74 |  |
| Larynx | 0.40 (0.05–2.89) | 0.36 |  | 1.05 (0.31–3.59) | 0.94 |  | 1.02 (0.44–2.35) | 0.96 |  |
| Grading (1,2 vs 3) | 0.97 (0.50–1.86) | 0.92 |  | 1.81 (0.91–3.61) | 0.094 |  | 1.12 (0.74–1.72) | 0.59 |  |
| Chemotherapy | 0.55 (0.23–1.32) | 0.18 |  | 1.42 (0.69–2.93) | 0.34 |  | 0.77 (0.45–1.31) | 0.33 |  |
| Smoking | 1.19 (0.36–3.98) | 0.78 |  | 2.20 (0.52–9.30) | 0.28 |  | 1.22 (0.55–2.68) | 0.63 |  |
| Alcohol | 0.87 (0.33–2.31) | 0.78 |  | 2.83 (0.67–12.0) | 0.16 |  | 1.26 (0.62–2.55) | 0.53 |  |
| p16 | 0.46 (0.14–1.49) | 0.19[\*](#_bookmark15) |  | 0.46 (0.14–1.50) | 0.20[\*](#_bookmark15) |  | 0.40 (0.17–0.92) | 0.031[\*](#_bookmark15) |  |
| HPV16 DNA | 0.25 (0.06–1.06) | 0.060[\*](#_bookmark15) |  | 0.40 (0.12–1.33) | 0.13[\*](#_bookmark15) |  | 0.35 (0.15–0.82) | 0.015[\*](#_bookmark15) |  |

Bold numbers indicate significant *p*-Values with *p* < 0.05.

\* *p* < 0.05 in published training cohort.

**100**



A

HPV16 DNA +

HPV16 DNA -

**100**

**100**

**80 80 80**

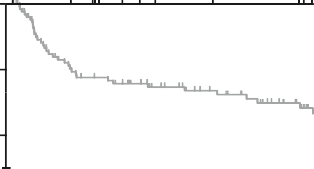
Loco-regional control

Loco-regional control

Loco-regional control

**60 60 60**

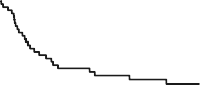
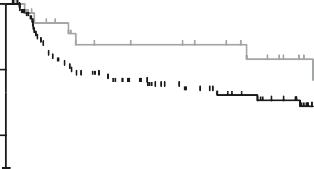
**0 0**



C

CD44 protein -

CD44 protein+



B

PORT-C

PORT



p=0.042



p=0.18

**0 12 24 36 48 60 0 12 24 36 48 60**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 85 | 71 | 57 | 47 | 35 | PORT-C | 40 | 29 | 25 | 23 | 18 | 13 | CD44 IHC - | 15 | 14 | 9 | 7 | 6 | 3 |
| 21 | 19 | 17 | 16 | 8 | PORT | 112 | 80 | 67 | 53 | 46 | 31 | CD44 IHC + | 130 | 91 | 79 | 65 | 56 | 39 |

**0**

**0 12 24 36 48 60**



p=0.027

**Pts. at risk** HPV16 DNA - HPV16 DNA +

126

22

Months after start of treatment

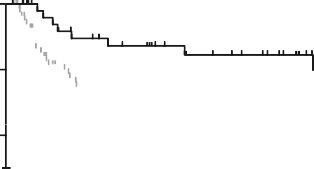
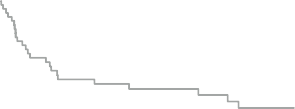
**Pts. at risk**

Months after start of treatment

**Pts. at risk**

Months after start of treatment

**100**



D

*CD44* -

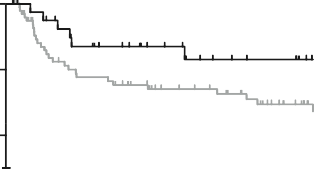
*CD44* +

Loco-regional control

**80**

**100**

**80**



*SLC3A2* -

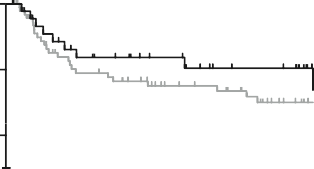
E

*SLC3A2* +

Loco-regional control

**100**

**80**



F

*MET* -

*MET* +

Loco-regional control

**60 60 60**

**0 0**



p=0.034



p=0.040

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **0** | **12** | **24** | **36** | **48** |
| **.**  *CD44*≤0.2 | 55 | 43 | 37 | 30 | 27 |
| *CD44*>0.2 | 90 | 61 | 52 | 43 | 34 |

**60 0 12 24 36 48 60**

**.** Months after start of treatment **.**

**0**

**0 12 24 36 48 60**

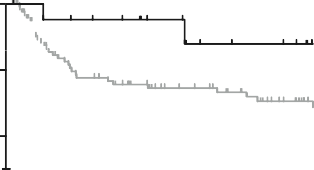


p=0.35

Months after start of treatment

18 *SLC3A2*≤-3.135 43 35

24 *SLC3A2*>-3.135 102 69



H

Hypoxia 26 low

Hypoxia 26 high

28 20 16

61 53 45

11 *MET*≤-4.135 47 36 31

31 *MET*>-4.135 98 68 58

24 20 12

49 41 30

**100**



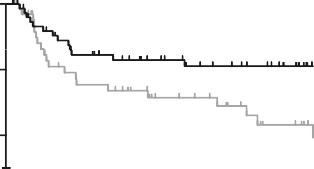
G

Hypoxia 15 low

Hypoxia 15 high

**100 100**

I

HPV16 DNA + or *SLC3A2 -*

or Hypoxia 15 low

**80 80 80**

Loco-regional control

Loco-regional control

Loco-regional control

**60 60**

**60** HPV16 DNA - and *SLC3A2 +*

and Hypoxia 15 high

**0**



p=0.054

**0 12 24 36 48 60**

**0**

**0 12 24 36 48 60**



p=0.078

**0**

**0 12 24 36 48 60**



p=0.010

**Pts. at risk** Months after start of treatment

**Pts. at risk**

Months after start of treatment

**Pts. at risk**

Months after start of treatment

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Hypoxia15 low 41 35 | 29 | 21 | 16 | 11 Hypoxia26 low 22 20 | 17 | 11 | 9 | 4 | Risk low | 78 61 | 52 | 43 | 38 | 26 |
| Hypoxia15 high 104 69 | 60 | 52 | 45 | 31 Hypoxia26 high 123 84 | 72 | 62 | 52 | 38 | Risk high | 66 43 | 37 | 30 | 23 | 16 |

Fig. 2. (A–I) Kaplan-Maier estimates of loco-regional control (LRC) for the validation cohort stratified for (A) HPV16 DNA status, (B) chemotherapy, (C–F) cancer stem cell (CSC) marker expression, (G and H) hypoxia-related gene classification and (I) combined risk. *Gene names are indicated using italics*.

Table 3

Training (cohort 1) and validation (cohort 2) of univariate Cox models containing cancer stem cell marker expression or hypoxia classifiers for the endpoints loco-regional control, freedom of distant metastases and overall survival. Shown is the hazard ratio (HR) with 95% confidence interval (CI) and the *p*-value testing the hypothesis HR = 1.

Loco-regional control Distant metastases Overall survival

HR (95% CI) *p*-Value HR (95% CI) *p*-Value HR (95% CI) *p*-Value

Cohort

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| *All patients* |  | | | | | | |
| CD44 protein | 1 | 9.09 (1.24–66.8) | 0.030 | 2.29 (0.90–5.86) | 0.084 | 1.78 (0.91–3.50) | 0.093 |
|  | 2 |  | [\*](#_bookmark18) | 1.80 (0.43–7.54) | 0.42 | 1.16 (0.53–2.53) | 0.71 |
| *CD44* > 0.2 | 1 | 3.56 (1.36–9.35) | 0.010 | 1.69 (0.83–3.43) | 0.15 | 1.57 (0.92–2.67) | 0.098 |
|  | 2 | 2.14 (1.00–4.56) | 0.049 | 2.30 (1.04–5.08) | 0.040 | 1.20 (0.76–1.89) | 0.44 |
| *SLC3A2* > -3.135 | 1 | 6.54 (1.97–21.7) | 0.002 | 4.17 (1.74–10.0) | 0.001 | 2.37 (1.36–4.13) | 0.002 |
|  | 2 | 2.45 (1.01–5.91) | 0.047 | 1.78 (0.77–4.10) | 0.18 | 1.19 (0.73–1.93) | 0.48 |
| *MET* > -4.135 | 1 | 5.19 (1.97–13.7) | 0.001 | 3.41 (1.61–7.23) | 0.001 | 2.74 (1.60–4.70) | <0.001 |
|  | 2 | 1.43 (0.67–3.07) | 0.35 | 1.18 (0.56–2.47) | 0.67 | 0.93 (0.58–1.47) | 0.74 |
| 15-gene signature | 1 | 3.55 (1.35–9.35) | 0.010 | 1.27 (0.65–2.46) | 0.49 | 1.47 (0.88–2.47) | 0.14 |
|  | 2 | 2.32 (0.96–5.59) | 0.061 | 1.06 (0.50–2.22) | 0.89 | 1.20 (0.74–1.96) | 0.46 |
| 26-gene signature | 1 | 9.37 (2.22–39.5) | 0.002 | 2.15 (1.01–4.55) | 0.046 | 2.48 (1.39–4.42) | 0.002 |
|  | 2 | 3.36 (0.81–14.0) | 0.097 | 2.16 (0.66–7.07) | 0.21 | 1.26 (0.66–2.38) | 0.48 |
| *Patients with HPV16 DNA-negative tumours* | | | | | | | |

CD44 protein 1 [\*](#_bookmark18)

|  |  |  |  |
| --- | --- | --- | --- |
| 2.20 (0.53–9.18) | 0.28 | 1.45 (0.58–3.64) | 0.43 |
| 0.79 (0.19–3.35) | 0.75 | 0.68 (0.29–1.57) | 0.36 |

2 [\*](#_bookmark18)

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| *CD44* > 0.2 | 1 | 4.15 (1.25–13.8) | 0.020 | 1.69 (0.72–3.94) | 0.23 | 1.40 (0.76–2.58) | 0.28 |
|  | 2 | 1.85 (0.86–3.96) | 0.11 | 2.54 (1.04–6.22) | 0.041 | 1.17 (0.72–1.90) | 0.52 |
| *SLC3A2* > -3.135 | 1 | 9.23 (1.25–68.1) | 0.029 | 3.56 (1.08–11.7) | 0.037 | 1.53 (0.79–2.98) | 0.21 |
|  | 2 | 1.87 (0.77–4.55) | 0.17 | 1.80 (0.69–4.73) | 0.23 | 1.13 (0.67–1.93) | 0.64 |
| *MET* > -4.135 | 1 | 3.74 (1.29–10.9) | 0.015 | 2.82 (1.16–6.87) | 0.023 | 2.42 (1.27–4.61) | 0.007 |
|  | 2 | 0.97 (0.45–2.08) | 0.94 | 0.75 (0.34–1.66) | 0.48 | 0.71 (0.43–1.16) | 0.17 |
| 15-gene signature | 1 | 4.66 (1.60–13.5) | 0.005 | 1.54 (0.73–3.21) | 0.26 | 1.88 (1.06–3.34) | 0.031 |
|  | 2 | 2.13 (0.88–5.15) | 0.093 | 1.37 (0.59–3.21) | 0.47 | 1.38 (0.80–2.37) | 0.25 |
| 26-gene signature | 1 | 11.3 (1.53–83.7) | 0.017 | 1.51 (0.65–3.51) | 0.34 | 2.05 (1.03–4.08) | 0.040 |
|  | 2 | 1.62 (0.39–6.78) | 0.51 | 1.36 (0.32–5.73) | 0.68 | 0.91 (0.42–1.99) | 0.81 |

Bold numbers indicate significant *p*-Values with *p* < 0.05.

\* No convergence due to no events in the CD44 protein negative group.

Table 4

Training and validation of multivariate Cox models for the endpoint loco-regional control containing cancer stem cell marker expression and hypoxia classifiers in addition to HPV16 DNA status and the clinical parameters ECE status, localisation oropharynx and hypopharynx. Shown is the concordance index (ci) with 95% confidence interval (CI) and the *p*-value testing the hypothesis ci = 0.5. Validation was also performed for the subgroup of patients who received radiochemotherapy (RCT).

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Training cohort |  |  | Validation cohort |  |  | Validation cohort, RCT | only |  |
| Included variables | ci (95% CI) | *p*-Value |  | ci (95% CI) | *p*-Value |  | ci (95% CI) | *p*-Value |
| *All patients* |  |  |  |  |  |  |  |  |  |
| Baseline (clinical parameters, HPV16 DNA status) | 0.76 (0.67–0.83) | <0.01 |  | 0.66 (0.58–0.75) | <0.01 |  | 0.69 (0.40–0.91) | 0.15 |  |
| *CD44* > 0.2 | 0.76 (0.68–0.83) | <0.01 |  | 0.69 (0.60–0.77) | <0.01 |  | 0.77 (0.55–0.93) | 0.02 |  |
| *SLC3A2* > -3.135 | 0.77 (0.68–0.84) | <0.01 |  | 0.66 (0.56–0.76) | <0.01 |  | 0.72 (0.44–0.92) | 0.10 |  |
| *MET* > -4.135 | 0.78 (0.69–0.85) | <0.01 |  | 0.64 (0.54–0.74) | 0.01 |  | 0.75 (0.50–0.92) | 0.04 |  |
| 15-gene signature | 0.80 (0.73–0.87) | <0.01 |  | 0.68 (0.58–0.77) | <0.01 |  | 0.74 (0.44–0.93) | 0.10 |  |
| 26-gene signature | 0.79 (0.71–0.85) | <0.01 |  | 0.67 (0.58–0.75) | <0.01 |  | 0.71 (0.43–0.91) | 0.11 |  |
| *CD44* > 0.2, 15-gene signature | 0.80 (0.72–0.87) | <0.01 |  | 0.69 (0.60–0.78) | <0.01 |  | 0.78 (0.53–0.94) | 0.04 |  |
| *SLC3A2* > -3.135, 15-gene signature | 0.81 (0.73–0.87) | <0.01 |  | 0.67 (0.58–0.75) | <0.01 |  | 0.76 (0.48–0.95) | 0.07 |  |
| *MET* > -4.135, 15-gene signature | 0.81 (0.74–0.88) | <0.01 |  | 0.66 (0.56–0.75) | <0.01 |  | 0.75 (0.47–0.93) | 0.07 |  |
| *Patients with HPV16 DNA negative tumours* |  |  |  |  |  |  |  |  |  |
| Baseline (clinical parameters) | 0.66 (0.55–0.77) | 0.01 |  | 0.63 (0.54–0.71) | 0.01 |  | 0.60 (0.21–0.87) | 0.49 |  |
| *CD44* > 0.2 | 0.68 (0.58–0.77) | <0.01 |  | 0.63 (0.53–0.73) | 0.01 |  | 0.70 (0.41–0.90) | 0.13 |  |
| *SLC3A2* > -3.135 | 0.69 (0.58–0.79) | <0.01 |  | 0.62 (0.51–0.71) | 0.03 |  | 0.63 (0.26–0.89) | 0.38 |  |
| *MET* > -4.135 | 0.71 (0.60–0.81) | <0.01 |  | 0.58 (0.47–0.70) | 0.16 |  | 0.66 (0.31–0.90) | 0.25 |  |
| 15-gene signature | 0.74 (0.65–0.82) | <0.01 |  | 0.64 (0.54–0.74) | 0.01 |  | 0.64 (0.25–0.91) | 0.36 |  |
| 26-gene signature | 0.71 (0.62–0.80) | <0.01 |  | 0.62 (0.53–0.71) | 0.01 |  | 0.61 (0.26–0.88) | 0.42 |  |
| *CD44* > 0.2, 15-gene signature | 0.74 (0.65–0.82) | <0.01 |  | 0.65 (0.54–0.74) | 0.01 |  | 0.69 (0.36–0.91) | 0.19 |  |
| *SLC3A2* > -3.135, 15-gene signature | 0.76 (0.68–0.84) | <0.01 |  | 0.62 (0.52–0.72) | 0.03 |  | 0.68 (0.33–0.92) | 0.24 |  |
| *MET* > -4.135, 15-gene signature | 0.76 (0.68–0.84) | <0.01 |  | 0.61 (0.50–0.71) | 0.05 |  | 0.66 (0.29–0.91) | 0.29 |  |

Discussion

In our previous multicentre retrospective study, we showed that HPV status, tumour expression of CSC markers and hypoxia-related genes play a prognostic role in patients with locally advanced HNSCC, who were treated by PORT-C [[15,18]](#_bookmark22). In the current study, these results were validated on an earlier,

independent cohort with patients who received PORT or PORT-C between 1999 and 2006. Despite significant differences in patient characteristics and treatment outcome between the two cohorts, the prognostic ability of the CSC markers CD44 protein, *CD44* and *SLC3A2* could be confirmed in univariate analyses; for the 15-gene hypoxia classifier a statistical trend was obtained. This underlines the robustness of the evaluated marker set.

Furthermore, it could be shown that *CD44* and the 15-gene hypoxia classifier moderately improved the ci of the published multivariate Cox models. However, this improvement did not reach statistical significance.

The validation cohort consisted of patients treated between 1999 and 2006. These patients showed favourable clinical param- eters with less R1-resected tumours and less lymphnodes showing extracapsular extension. However, LRC and OS were significantly lower compared to the later treated training cohort [[15]](#_bookmark22). This may be explained by the lower number of oropharyngeal cancers in this validation cohort, which show a significantly improved out- come due to a high radiosensitivity associated with positive HPV infection status [[21,22]](#_bookmark25) and the higher number of tumours in the oral cavity that generally show poorer outcome. From the litera- ture, there is also conflicting data on the value of HPV as a prognos- tic factor for non-oropharyngeal cancers. Lassen et al. showed that the radiotherapy outcome in the subgroup of non-oropharyngeal tumours does not differ by their p16 status [[12]](#_bookmark31). In contrast, Chung et al. demonstrated, that HPV infection may also play a role in a subset of non-oropharyngeal cancers [[23]](#_bookmark26), suggesting that this needs further exploration. Furthermore, only 40 of the 152 patients received simultaneous radiochemotherapy since this had not been a standard treatment at that time. Technological advances in radio- therapy [[3]](#_bookmark20) and diagnostics may also have contributed to a better outcome of the patients in the training cohort. Despite these signif- icant differences a statistical trend for improved LRC for patients with HPV16 DNA positive tumours could be obtained, which is in agreement with our previous study [[15]](#_bookmark22).

Validation of the multivariate Cox models published in [[18]](#_bookmark22)

showed a generally lower prognostic performance for the valida- tion cohort than for the training cohort (training: ci 0.66–0.81, val- idation: ci 0.58–0.69). This difference is expected, as the models were adjusted to the training data. Furthermore, cut-offs for the continuous CSC marker expressions as well as the clustering proce- dure for hypoxia classification were optimised for the training cohort. However, with an average ci of 0.67 for the total validation cohort and of 0.62 for patients with HPV16 DNA negative tumours, the multivariate models were still prognostic with an acceptable performance. The large difference in ci between all patients and those patients with HPV16 DNA negative tumours shows that the HPV16 DNA status is a strong prognosticator for LRC. Still, *CD44* and the 15-gene hypoxia classifier were able to further improve the models, also in the validation cohort. At first glance, it is sur- prising that tumour hypoxia-associated gene expression of the resected tumour is associated with the outcome of postoperative radiotherapy. As discussed before [[18]](#_bookmark22), it is very unlikely that the low number of any remaining tumour cells after surgery differs in their hypoxia status. However, this suggests that hypoxia may have the potential to impact the outcome after postoperative radiotherapy also by other radiobiological mechanisms than by direct biochemical or radiobiological effects. In previous data from our laboratory, it was shown that pre-treatment hypoxia has an impact on local tumour control after radiotherapy also when radio- therapy was applied under homogenous anoxic conditions [[24]](#_bookmark28).

The prognostic ability of the putative CSC marker *MET* could

not be confirmed by the validation cohort. However, the inclusion of *MET* resulted in multivariate models with higher ci values if only patients of the validation cohort who received PORT-C were considered. These models were not significant, which could be due to (1) the lower incidence of HPV positive tumours, which has shown to be associated with a lower CSC frequency [[25]](#_bookmark30) and (2) the low number of patients receiving PORT-C in the vali- dation cohort. Furthermore, in the validation cohort, patients only received PORT-C within clinical trials, i.e. a selection of patients with more favourable clinical characteristics is likely, leading to a more homogeneous patient group compared to the total cohort.

In addition, diagnostics and radiotherapy treatment have been technologically improved over the last years, such that the con- sideration of biological aspects might become even more important.

It should be noted that in the previously reported DKTK-ROG cohort [[18]](#_bookmark22) *CD44* expression was determined by real-time PCR (RT-PCR) analysis because the nanoString *CD44* probe design was invalid. In the present validation cohort, *CD44* expression was mea- sured by valid nanoString probes. Therefore, the validation of *CD44* is challenging, as its expression in the two cohorts was determined by different methods and the cut-off value 0.2 obtained for RT-PCR might be different for nanoString. In general, many genes of the validation cohort showed significantly different mean expression values between the cohorts, i.e. an average shift. This is problem- atic if the expressions are dichotomized at cut-off values, as these cut-offs may lead to imbalanced patient groups for the validation cohort. The same holds for the hypoxia classifiers built by k- means clustering. In the validation cohort, this shift in gene expres- sions caused the hypoxia classifiers to identify significantly more hypoxic tumours compared to the training cohort. Renormalizing the validation data to the training data, as described in [[26]](#_bookmark32), gives the same fraction of more and less hypoxic tumours for both cohorts and even leads to a significant impact of hypoxia status on LRC, in contrast to the statistical trend shown in [Table 3](#_bookmark17). How- ever, this method is not applicable for individual patient prognosis, as required in clinical trials on treatment adaptation, and may war- rant the inclusion of reference samples in future analyses.

The model validation showed a better performance for the sub-

group of patients, which received PORT-C than for all patients. This indicates that the prognostic ability of CSC markers and hypoxia classifiers might be stronger for a patient cohort, which is more similar to the cohort originally used in [[18]](#_bookmark22). However, at the time of treatment of the validation cohort simultaneous radiochemotherapy was applied only within clinical studies [[7–](#_bookmark21) [9]](#_bookmark21). This potentially led to a higher homogeneity with better clinical performance of the patient subgroup treated with PORT-C, which is important for the interpretation of the biological heterogeneity of the tumours. Currently, a homogeneous patient cohort with locally advanced HNSCC treated with PORT-C is being recruited within a prospective clinical trial of the DKTK-ROG, which will allow for fur- ther validation of the models.

Taken together, this validation study confirmed the prognostic value of the HPV infection status, CSC marker expression of CD44 protein, *CD44* and *SLC3A2* and tumour hypoxia status presented in [[18]](#_bookmark22) for patients with locally advanced HNSCC receiving postop- erative radiotherapy or radiochemotherapy. While a lower perfor- mance of the prognostic models is expected due to the older validation dataset and several differences between the cohorts, the significant validation results indicate the robustness of these biomarkers. After further validation on a currently recruiting prospective clinical trial of the DKTK-ROG these models may help to stratify patients for individualised treatment de-escalation or intensification strategies.

Conflict of interest

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

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ctro.2016.10.002>.

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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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a b s t r a c t

*Objective:* To independently validate the impact of tumour volume, p16 status, cancer stem cell (CSC) mar- ker 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 radio- therapy 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), respec- tively. 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 param- eters 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 com- bination 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 repre- senting one of the ten most frequent tumours worldwide [[2]](#_bookmark54). Pri- mary radiochemotherapy (RCTx) is currently the considered treatment standard for patients with locally-advanced and func- tionally inoperable tumours, after several clinical trials showed a benefit over radiotherapy alone [[3–8]](#_bookmark55). 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]](#_bookmark62). 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 indi- vidual tumour. This may allow for inclusion of patients in treat- ment intensification trials, such as dose escalation or combination with novel systemic therapeutics [[10,11]](#_bookmark63).

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]](#_bookmark66). 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]](#_bookmark56).

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 strat- ification 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 predic- tion of prognosis was achieved by the tumour volume, which was suggested by others before [[10,19–22]](#_bookmark63). 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]](#_bookmark57) using a retro- spective monocentric cohort of 92 patients with locally advanced HNSCC, who were treated by primary RCTx.

1. Materials and methods
   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 crite- ria have previously been described in detail [[1]](#_bookmark56). 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 mark- ers CD44 and *SLC3A2* and hypoxia-associated gene signatures to LRC and OS were investigated in this cohort before [[1]](#_bookmark56).

To validate these results, an independent cohort of 92 patients with locally advanced HNSCC treated by curatively intended pri- mary 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](http://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]](#_bookmark58). 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](#_bookmark48).

* 1. *Segmentation and failure pattern analyses*

The segmentations of the primary and of the nodal gross tumour volume (GTV) of all cases have retrospectively been per- formed in CT scans by one radiation oncologist (F.L.), who has expertise in the delineation of head and neck cancers. For segmen- tation, RayStation 6 (Raysearch Laboratories, Stockholm Sweden) and an in-house software solution has been used [[24]](#_bookmark58). Disease sta- tus as well as the first site of relapse (e.g. loco-regional failure, dis- tant failure or combined failure) have been evaluated. For each loco-regional failure, the radiotherapy treatment plan and radio- logical 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.

* 1. *Preparation of biomaterials for biomarker analysis*

The preparation of FFPE tissue material was performed as described in [[1]](#_bookmark56). Briefly, a fresh section of each FFPE block was first subjected to haematoxylin and eosin staining in order to histolog- ically confirm the presence of squamous cell carcinoma. After- wards, the FFPE material was further processed for immunohistochemistry or for preparation of genomic DNA or RNA under standardized conditions as described previously [[1]](#_bookmark56). Briefly, for p16 immunohistochemistry the CINtec Histology kit (Roche mtm laboratories AG, Basel, CH) was used according to the manufacturers’ instructions [[1]](#_bookmark56). A moderate or strong overex- pression of p16 in at least 70% of the tumour cells was considered as a p16 positive tumour [[1]](#_bookmark56). For the immunohistochemical analy- sis of CD44 protein expression, the monoclonal mouse anti-human

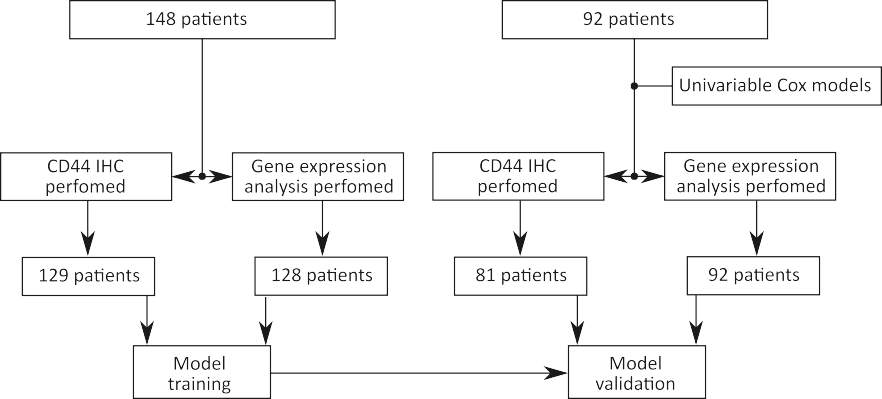
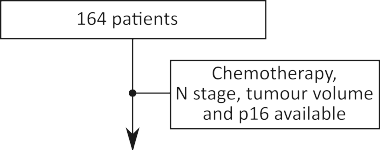


Fig. 1. Study design. The number of patients initially available is presented along with the analyses, which were performed. IHC = immunohistochemical staining.

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 speci- fic 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 immunohisto- chemical analyses.

DNA extraction and PCR-array based analyses of HPV status have been performed as described previously [[26]](#_bookmark60). Briefly, genomic DNA was extracted from 5-mm FFPE sections using the QIAamp DNA FFPE tissue kit (Qiagen). HPV DNA analyses including geno- typing 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]](#_bookmark56) and included the potential CSC markers *CD44* and *SLC3A2* as well as the 15-, 26-, and 30-gene hypoxia- associated signatures [[27–31]](#_bookmark61). Briefly, the raw counts were loga- rithmised 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 refer- ence genes were used [[27,30,31]](#_bookmark61). Note that *DHX34* was not avail- able. Thus only 29 genes of the original 30-gene signature [[27,30,31]](#_bookmark61) were evaluated.

* 1. *Clinical endpoints and statistical analyses*

The primary endpoint was loco-regional tumour control (LRC). Overall survival (OS) was the secondary endpoint. The correspond- ing times were calculated starting from the first day of radiother- apy to the date of event or censoring. The Kaplan-Meier method was used to estimate survival curves. The endpoints were com- pared 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 con- cordance index (ci) was used to validate the performance of the multivariable Cox models defined in the training cohort [[1]](#_bookmark56). 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]](#_bookmark56). 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]](#_bookmark64). Differences between the cohorts were evaluated by Mann- Whitney-U tests (continuous variables) and chi-squared tests (cat- egorical variables). Hypoxia classification was performed on the validation cohort using two cluster centres for every gene signa- ture, which were determined on the training cohort by k-means clustering [[1]](#_bookmark56). The analyses were performed using SPSS 25 (IBM Corporation, Armonk, NY, USA), R-Statistics (R Foundation for Sta- tistical Computing [[33]](#_bookmark65)) 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.

1. Results

In comparison to the training cohort [[1]](#_bookmark56), the patients of the val- idation 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 valida- tion 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](#_bookmark49). The corre- sponding 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]](#_bookmark56) ([Table 2](#_bookmark50)). In contrast to the training cohort, the logarithmised primary tumour volume (training (t): HR = 1.44, p = 0.028; valida- tion (v): HR = 1.30, p = 0.24, [Fig. 2](#_bookmark51)C, D) could not be validated as a significant prognostic factor for LRC, while the total volume of pri- mary 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]](#_bookmark60). 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 (cm3) | 26.8 (4.4–175.8) |  | 33.9 (5.1–322.6) |  | 0.003 |
|  | Volume LN (cm3) | 8.2 (0–300.0) |  | 7.2 (0–272.6) |  | 0.94 |
|  | Volume total (Tumour + LN) (cm3) | 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 y. 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) p16 | 3.63 (1.10–12.00)  0.78 (0.24–2.57) | 0.030  0.68\* |  | 3.42 (1.46–7.99)  0.85 (0.36–1.98) | 0.005\*  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 –y –\* 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 |

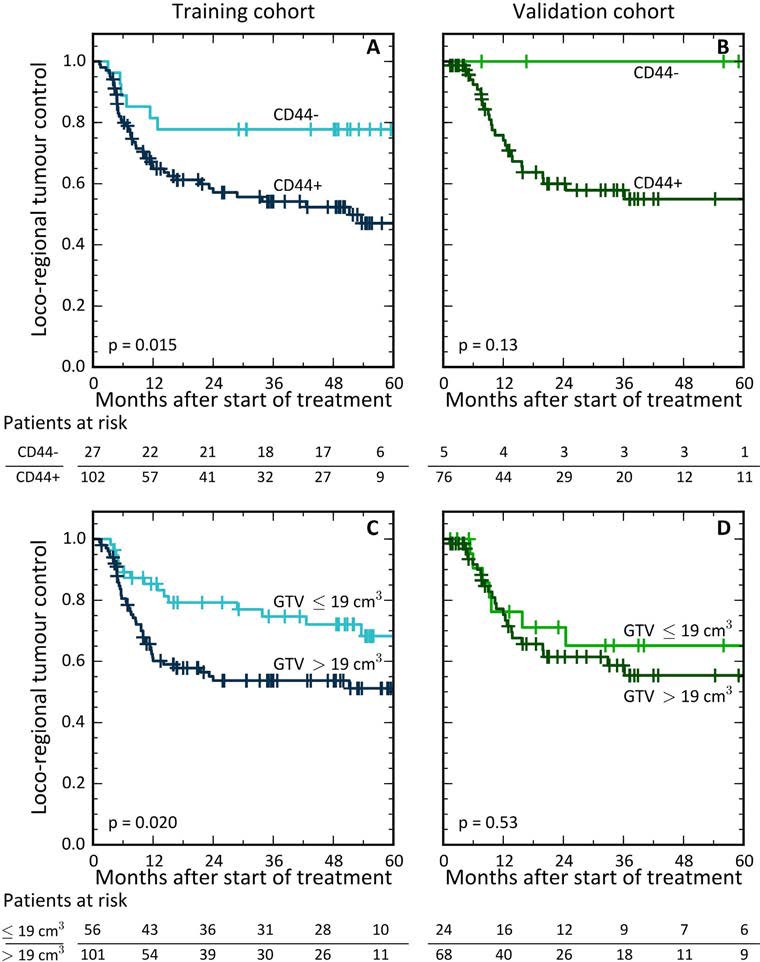


Fig. 2. Kaplan-Meier estimates for loco-regional tumour control in the training cohort (A, C) and the validation cohort (B, D) stratified based on the CD44 protein status (A, B) and the tumour volume (C, D). The p-values are based on log-rank tests.

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 asso- ciated 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. 2](#_bookmark51)A, 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 multi- variable Cox models ([Table 3](#_bookmark52)). The baseline model containing the logarithmised primary tumour volume, p16 status and N stage was derived from our multicentre study [[1]](#_bookmark56). 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 perfor- mance of the baseline model, which is similar to the training cohort.

Two logistic regression models were developed previously to predict 2-year LRC [[1]](#_bookmark56). 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]](#_bookmark60).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | 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, | 0.63 (0.58–0.73) | <0.01 |  | 0.54 (0.43–0.65) | 0.50 |  |
| 15-gene hypoxia signature \* lnGTV |  |  |  |  |  |  |
| BL, 30-gene hypoxia signature, | 0.62 (0.58–0.73) | <0.01 | 0.59 (0.48–0.69) | | 0.12 | |
| 30-gene hypoxia signature \* lnGTV |  |  |  |  |  |  |
| BL, 15-gene hypoxia signature, | 0.66 (0.62–0.75) | <0.01 | 0.56 (0.45–0.66) | | 0.29 | |
| 15-gene hypoxia signature \* lnGTV, *SLC3A2* |  |  |  |  |  |  |
| BL, 30-gene hypoxia signature, | 0.66 (0.61–0.75) | <0.01 | 0.60 (0.49–0.70) | | 0.07 | |
| 30-gene hypoxia signature \* lnGTV, *SLC3A2* |  |  |  |  |  |  |
| *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, | 0.68 (0.62–0.75) | <0.01 | 0.60 (0.52–0.68) | | 0.02 | |
| 15-gene hypoxia signature \* lnGTV |  |  |  |  |  |  |
| BL, 30-gene hypoxia signature, | 0.69 (0.63–0.75) | <0.01 | 0.63 (0.54–0.71) | | <0.01 | |
| 30-gene hypoxia signature \* lnGTV |  |  |  |  |  |  |
| BL, 15-gene hypoxia signature, | 0.68 (0.63–0.76) | <0.01 | 0.66 (0.58–0.74) | | <0.01 | |
| 15-gene hypoxia signature \* lnGTV, *CD44* |  |  |  |  |  |  |
| BL, 30-gene hypoxia signature, | 0.69 (0.64–0.76) | <0.01 | 0.70 (0.61–0.77) | | <0.01 | |
| 30-gene hypoxia signature \* lnGTV, *CD44* |  |  |  |  |  |  |

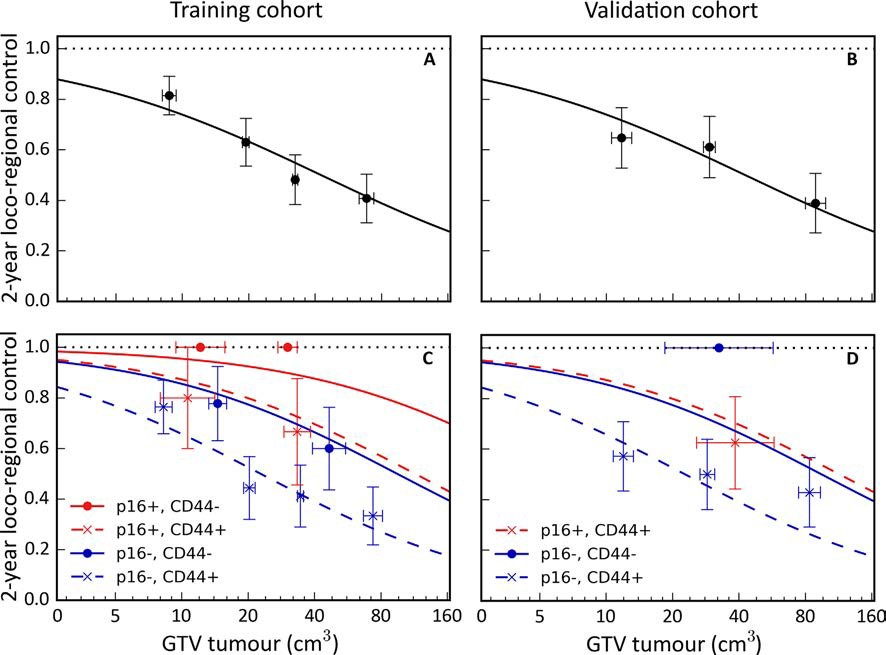


Fig. 3. Logistic regression regarding 2-year loco-regional tumour control. The results of the training cohort (A, C) and the validation cohort (B, D) are shown. A and B show the univariable logistic regression solely based on the primary tumour volume, while C and D show the multivariable logistic regression, 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](#_bookmark53) for the training and the validation cohort.

1. Discussion

In our previous study we demonstrated the prognostic value of the primary tumour volume, the potential CSC markers CD44 pro- tein, *CD44* and *SLC3A2* gene expression as well as hypoxia- associated gene signatures [[27,31]](#_bookmark61) for patients with locally advanced HNSCC who were treated with primary RCTx. In the cur- rent 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]](#_bookmark56).

The volume of the primary tumour is a widely accepted prog- nostic biomarker for the outcome of radiotherapy in patients with HNSCC [[20,22,34]](#_bookmark59) 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 vol- ume, 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 signif- icantly 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 valida- tion cohort, 5 out of 24 patients with oropharyngeal tumours pre- sented 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]](#_bookmark67), 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 suc- cessfully 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 find- ings, 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]](#_bookmark68).

In the training cohort, the impact of the hypoxia-associated gene signatures on LRC was significant for tumours smaller than 19 cm3 [[1]](#_bookmark56). For the complete cohort, significant patient stratifica- tions based on the hypoxia status were not achieved, even though less hypoxic tumours showed a slightly better outcome [[1]](#_bookmark56). 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 signif- icant stratifications. In addition, the ratio of more and less hypoxic tumours, identified by the 15- and 26-gene signatures, were simi- lar in training and validation, indicating that hypoxia classifica- tions 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 per- formed 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]](#_bookmark56) 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 vali- dation. 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]](#_bookmark64).

1. 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 per- formed. The importance of the clinical parameters tumour vol- ume 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 ongo- ing prospective multicentre HNprädBio study ([www.clinicaltri-](http://www.clinicaltrials.gov/) [als.gov](http://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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HPV in postoperative RT of oropharynx

## HPV16 DNA status is a strong prognosticator of loco-regional control after postoperative radiochemotherapy of locally advanced oropharyngeal carcinoma: Results from a multicentre explorative study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG)



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*Objective:* To investigate the impact of HPV status in patients with locally advanced head and neck squa- mous cell carcinoma (HNSCC), who received surgery and cisplatin-based postoperative radiochemother- apy.

*Materials and methods:* For 221 patients with locally advanced squamous cell carcinoma of the hypophar- ynx, oropharynx or oral cavity treated at the 8 partner sites of the German Cancer Consortium, the impact of HPV DNA, p16 overexpression and p53 expression on outcome were retrospectively analysed. The primary endpoint was loco-regional tumour control; secondary endpoints were distant metastases and overall survival.

*Results:* In the total patient population, univariate analyses revealed a significant impact of HPV16 DNA positivity, p16 overexpression, p53 positivity and tumour site on loco-regional tumour control. Multivar- iate analysis stratified for tumour site showed that positive HPV 16 DNA status correlated with loco- regional tumour control in patients with oropharyngeal carcinoma (*p* = 0.02) but not in the oral cavity carcinoma group. Multivariate evaluation of the secondary endpoints in the total population revealed a significant association of HPV16 DNA positivity with overall survival (*p* < 0.01) but not with distant metastases.

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*Conclusions:* HPV16 DNA status appears to be a strong prognosticator of loco-regional tumour control after postoperative cisplatin-based radiochemotherapy of locally advanced oropharyngeal carcinoma and is now being explored in a prospective validation trial.

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Head and neck squamous cell carcinoma (HNSCC) patients have a 5-year survival of approximately 50% [[1,2]](#_bookmark93). Within the last years, the number of oropharyngeal cancers has increased [[3–5]](#_bookmark94). Postop- erative radiotherapy (PORT) with concurrent cisplatin (PORT-C) has become standard for the adjuvant treatment of patients with resected locally advanced HNSCC after three randomised trials showing superior loco-regional control and improved survival rates compared to PORT alone [[6–8]](#_bookmark94). A meta-analysis of two of the above mentioned trials, the EORTC and the RTOG trials, con- cluded that patients with positive microscopic resection margins and/or extracapsular extension (ECE) from neck nodes benefit most from this combined treatment [[9]](#_bookmark94). However, all three trials also report increased toxicity after PORT-C vs. PORT [[6–8]](#_bookmark94).

In recent years, infection with high-risk human papillomavirus (HPV) has been implicated in the pathogenesis of HNSCC, indepen- dently of the risk factors smoking and alcohol consumption [[10]](#_bookmark94). On the molecular level, the HPV oncogenes E6 and E7 have been shown to drive carcinogenesis, which is accompanied by degrada- tion of p53 and pRB and overexpression of p16 due to loss of its transcriptional repression. In addition, HPV-related HNSCC show less p53 mutations than smoking and alcohol induced HNSCC [[11]](#_bookmark94). HPV-positive tumours most commonly originate from the oropharynx [[12,13]](#_bookmark95); high-grade histology and positive lymph nodes are frequent [[14]](#_bookmark96). Interestingly, a recent study observed that patients with HPV16 DNA positive oropharyngeal carcinoma and lower levels of comorbidity show a significantly better overall sur- vival compared to patients with HPV16 DNA negative tumours [[15]](#_bookmark97). Specifically for the field of Radiation Oncology, it has been shown that HPV-positivity is a strong prognostic marker for overall survival and/or local tumour control in patients treated with pri- mary radiochemotherapy [[16–20]](#_bookmark100). However, its impact on out- come after PORT-C is not well evaluated so far.

In an ongoing multicentre retrospective – prospective trial con- ducted by the Radiation Oncology Group of the German Cancer Consortium (DKTK-ROG), biomarkers for stratification of patients for dose of primary or postoperative radiochemotherapy of HNSCC are being evaluated. The present publication reports the impact of HPV infection on loco-regional tumour control and survival after PORT-C in the multicentre explorative cohort.

Material and methods

*Patients, treatment and tissue samples*

Patients meeting the following criteria were eligible for inclu- sion in this retrospective study: histologically proven squamous cell carcinoma arising from the hypopharynx, oropharynx or oral cavity, treatment between 2005 and 2010 with a curatively intended cisplatin-based PORT-C according to standard protocols covering the former tumour region and the neck nodes. All patients had to be judged as being at high risk for loco-regional recurrence due to locally advanced disease with a tumour stage pT4 and/or >3 positive lymph nodes and/or due to postoperative residual disease (positive microscopic resection margins and/or extracapsular spread). Minimum follow-up of patients without progressive dis- ease had to be 24 months. Additionally, formalin-fixed paraffin- embedded (FFPE) material, radiotherapy treatment plans, CT, MRI or PET–CT images of the localisation of the recurrent tumours as well as follow-up data of patients had to be available. Smoking sta-

tus and alcohol consumption were not consistently recorded for all patients and therefore could not be analysed. It was aimed to include 40 patients per DKTK partner centre (i.e., 320 patients in total). To enhance the proportion of HPV-positivity, patients were included consecutively backwards from 2010 towards 2005 in all centres, as HPV prevalence in HNSCC is increasing in recent years [[21]](#_bookmark105). Finally, 221 patients were found to meet all requirements. Those patients were evaluated in this study ([Table 2](#_bookmark83)). Pathological specimens, radiotherapy treatment plans, radiological images of recurrent tumours and follow-up data of patients were centrally collected in the DKTK RadPlanBio Platform [Skripcak et al., manu- script in preparation] at the DKTK partner site Dresden.

Ethical approval for multicentre retrospective analyses of clini- cal and biological data was obtained by the Ethics Committees of all DKTK partner sites.

*Failure pattern analysis*

Disease status and first site of relapse were evaluated by the treating institution (loco-regional failure, distant failure or com- bined failure). When loco-regional recurrence and distant metasta- ses occurred at the same time (maximally 6 weeks difference), the patient was counted as combined failure. For every reported loco- regional failure, the radiotherapy treatment plan and radiological images of the recurrence (CT, MRI or PET–CT) were centrally reviewed to ensure that failures originated from the irradiated volume.

*Preparation of biomaterials for biomarker analyses*

FFPE blocks of the primary tumours were centrally collected at the DKTK partner site Dresden where slides for immunohisto- chemistry were prepared and genomic DNA was extracted (vide infra). In parallel tissue microarrays, RNA isolates and cDNA were generated for further investigations of biomarkers, which are cur- rently ongoing at the different partner sites. HPV DNA, p16 and p53 reported in this article were evaluated at the DKTK partner site Dresden.

*Immunohistochemical staining of p16*

In all FFPE samples, the squamous cell carcinoma content was estimated from haematoxylin and eosin stained tissue sections and FFPE samples with <10% tumour content were excluded from p16 analysis. Two hundred and fourteen of the 221 tumour sam- ples (60 oral cavity, 121 oropharynx, 33 hypopharynx) were evalu- able for p16. Immunohistochemical staining was performed using the CINtec® Histology Kit (Roche mtm laboratories AG, Basel, CH) according to the manufacturer’s instruction. Overexpression of p16 was defined as P70% intense tumour staining [[16]](#_bookmark100). Blinded samples were evaluated semi-quantitatively by two independent observers (A.L. and C.v.N.) with an inter-observer variability of <5%.

*Immunohistochemical staining of p53*

FFPE material from all 221 patients was available for p53 anal- ysis. Following deparaffinisation and antigen retrieval in target retrieval solution (pH 9; Dako, Glostrup, DK) for 35 min at 630 W, immunohistochemical staining was performed. Endoge- nous peroxidase activity was blocked (Peroxidase Block, Dako).

Sections were then incubated with the monoclonal mouse anti- human p53 antibody (Clone DO-7; Dako) in Dako REAL Antibody Diluent for 30 min. Negative control slides were incubated with corresponding IgG antibody control (Dako). The staining was visu- alised by DAB immunostaining (Dako REAL EnVision Detection System, Peroxidase/DAB, Rabbit/Mouse). Blinded samples were evaluated semi-quantitatively by two independent observers (A.L. and C.v.N.) with an inter-observer variability of <5%. Percent- age of p53 staining [[22]](#_bookmark106) and staining intensity were scored (0, +, ++,

+++). Tumours with P70% positive nuclei and moderate (++) or strong (+++) staining intensities were considered as p53 positive.

*DNA extraction and PCR array-based analysis of HPV status*

Genomic DNA was extracted from 5 lm FFPE-sections using the QIAamp DNA FFPE tissue kit (Qiagen GmbH, Hilden, DE) according to the manufacturer’s instruction and stored at -20 °C until required. HPV DNA analyses including genotyping were carried out using the LCD-Array HPV 3.5 kit (CHIPRON GmbH, Berlin, DE) according to the manufacturer’s instruction. Briefly, PCR was per- formed using the provided Primer Mix A (My 11/09) and B (‘125’) and the HotStarTaq Plus Master Mix (Qiagen GmbH).

Hybridisation mix including 5 ll of each amplified PCR product *A*

and *B* were added to each field of the LCD-Array. After staining and washing, the hybridisation spots were scanned and analysed using the SlideReader Software (CHIPRON GmbH). For internal quality control purposes, a positive control (HPV33 DNA, UT-SCC- 45 xenograft) and a negative control (RNase free water; Qiagen GmbH) were included in each array. Six tissue samples had to be omitted from HPV DNA analysis due to too low DNA yield, thus 215 of the 221 tumour samples (58 oral cavity, 123 oropharynx, 34 hypopharynx) were evaluable for HPV-PCR array.

*Statistics*

The primary endpoint was loco-regional tumour control; free- dom from distant metastases and overall survival were evaluated as secondary endpoints. Loco-regional tumour control, distant metastases and overall survival were calculated from the first day of radiotherapy to the date of local or regional recurrence, date of metastases and date of death or last follow-up, respectively. All endpoints were estimated with the Kaplan–Meier method. The impact of potential prognosticators on the endpoints was evalu- ated using the Cox-regression model. Parameters found to be sig- nificant in univariate analysis were included in a multivariate Cox model. Statistical analyses were performed for all patients and for the subgroups of patients with oral cavity cancers as well as oropharyngeal cancers. Patients diagnosed with hypopharyn- geal cancers were excluded from this subgroup analysis due to the low number of cases. Sensitivity and specificity of p16 and HPV16 DNA for predicting loco-regional recurrence were deter- mined by cross tabulation. For all analyses, two-sided tests were used and *p*-values <0.05 were considered statistically significant. SPSS 21 software (IBM Corporation, Armonk, NY, USA) was used for the generation of Kaplan–Meier plots. STATA 11 (StataCorp LP, College Station, TX, USA) was used for Cox analyses.

Results

In total, 221 patients treated with PORT-C for locally advanced HNSCC were evaluated in this multicentre retrospective study. Patient characteristics, treatment parameters and the number of patients included at each of the 8 DKTK partner sites are summa- rised in [Tables 1 and 2](#_bookmark84).

Isolated loco-regional failure occurred in 21 patients, isolated distant failure in 31 patients and combined failures were observed

in 8 patients. In 2 patients loco-regional recurrence occurred after dis- tant progression and 4 patients developed distant progression after loco-regional recurrence. All loco-regional recurrences occurred in the treatment volume. Actuarial rates at two years for loco-regional control, freedom from distant metastases and overall survival for the total patient population were 89.6%, 85.1% and 83.4%.

The results of the biomarker analyses of HPV DNA, p16 and p53 and their occurrence at the different tumour sites are shown in [Table 3](#_bookmark87). According to the International Agency for Research in Cancer (IARC), HPV16 DNA positive HNSCC are currently being considered as HPV associated [[23]](#_bookmark98) and only this parameter was used for further analysis. HPV16 DNA positivity was observed in 34% of the tumours. Overexpression of p16 was found in 37% of all tumours, and 53% of the oral cavity tumours were positive for p53. The majority (86%) of HPV16 DNA positive tumours were found to be p53 negative.

Only two loco-regional recurrences occurred in patients suffer- ing from HPV16 DNA positive tumours: the first in a R0-resected, ECE positive, pT2 pN2b oral cavity carcinoma (floor of mouth) after 23 months in the boost volume (66 Gy), the second in a R0 resected, ECE positive pT2 pN2b oropharyngeal cancer (tonsil) after 26 months at the margin of the boost volume (64 Gy) to the adju- vant volume (54 Gy). Univariate analyses revealed a significant impact on loco-regional tumour control for HPV16 DNA positivity (HR 0.13; *p* < 0.01; [Fig. 1](#_bookmark89)A, Supplementary Table S1). This effect was seen at all 8 treatment centres ([Fig. 2](#_bookmark90)). Overexpression of p16 (HR 0.24; *p* < 0.01), p53 positivity (HR 3.36; *p* < 0.01) and tumour site (oral cavity vs. all other tumour sites, HR 3.86; *p* < 0.01; oropharynx vs. all other tumour sites, HR 0.38; *p* = 0.01) also showed a significant impact on loco-regional tumour control. No significant impact was found for sex, UICC stage, R status and ECE status (Supplementary Table S1). Specificity and sensitivity of HPV16 DNA positivity in the total patient population to predict loco-regional tumour control were 93% and 38%, the corresponding values for overexpression of p16 were 86% and 41%. In oropharyn- geal cancer specificity and sensitivity of HPV16 DNA positivity were 91% and 53%, for p16 overexpression 73% and 56% were obtained (1 out of 11 loco-regional recurrences occurred within the HPV16 DNA positive group, 3 out of 11 were tested positive for p16). Stratified for tumour site, univariate analyses in oropha- ryngeal cancer showed that HPV16 DNA (HR 0.09; *p* = 0.02; [Fig. 1](#_bookmark89)B, Supplementary Table S1) but not p16 overexpression or p53 positivity have a significant impact on loco-regional tumour control, whereas p53 showed a significant impact on loco-regional tumour control in oral cavity cancer (HR 3.61; *p* < 0.05; Supple- mentary Table S1).

[Table 4](#_bookmark91) summarises the results of the multivariate analyses,

including the significant parameters of the univariate analyses plus ECE status, which had shown a significant impact on the secondary endpoints (Supplementary Table S1). For the total patient popula- tion, positive HPV16 DNA status was significantly associated with a high chance of loco-regional tumour control (HR 0.20; *p* = 0.04). Oral cavity cancer showed significantly poorer loco-regional tumour control than oropharyngeal cancers (HR 2.30; *p* = 0.04). Multivariate analysis stratified for tumour site showed that positive HPV16 DNA status correlated with loco-regional tumour control in patients with oropharyngeal carcinoma (HR 0.09; *p* = 0.03) but not in the oral cavity carcinoma group ([Table 4](#_bookmark91)). As HPV16 DNA and p16 are strongly correlated, a second multivariate Cox model assessing p16 overexpression was performed. The results of this Cox model showed that the HPV status was a borderline independent prognos- tic marker for loco-regional tumour control in the total patient pop- ulation (HR 0.36; *p* = 0.07; Supplementary Table S2).

Multivariate evaluation of the secondary endpoints in the total population revealed a significant association of HPV16 DNA positivity with overall survival (HR 0.36; *p* < 0.01) but not with

Table 1

Patient characteristics and treatment parameter.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Patient characteristic |  |  |  |  | *n* % | |
| Number of patients |  |  |  |  | 221 |  |
| Sex | Male | 180 | 81.4 |
|  | Female | 41 | 18.6 |
| UICC stage | II | 8 | 3.6 |
|  | III | 33 | 18.6 |
|  | IV | 180 | 81.4 |
| Tumour localisation | Oral cavity | 60 | 27.1 |
|  | Oropharynx | 126 | 57.0 |
|  | Hypopharynx | 35 | 15.8 |
| R status[⇑](#_bookmark85) | Negative  Positive | 125  94 | 56.6  42.5 |
| ECE status | Negative | 102 | 46.2 |
|  | Positive | 119 | 53.8 |
| Treatment parameter | | Median | Percentiles  10% | 25% | 75% | 90% | Range |
| Applied cisplatin-dose (mg/m2 body surface area) RT dose (Gy)  Boost volume Per fraction  Adjuvant volume Per fraction  Time between last surgery and first radiotherapy (weeks) Overall treatment time of PORT-C (days)  Follow-up time (months) | | 200 | 100 | 200 | 200 | 240 | 100–300 |
| 64.0 | 60.0 | 63.9 | 66.0 | 66.0 | 57.2–68.4 |
| 2.0 | 1.8 | 1.8 | 2.0 | 2.0 | 1.8–2.1 |
| 50.4 | 50.0 | 50.0 | 55.9 | 60.0 | 46.8–66.0 |
| 2.0 | 1.8 | 2.0 | 2.0 | 2.1 | 1.8–2.2 |
| 6.0 | 4.1 | 5.0 | 7.5 | 9.6 | 1.0–23.0 |
| 44.0 | 41.0 | 43.0 | 46.5 | 50.0 | 31.0–57.0 |
| 47.3 | 11.1 | 30.7 | 61.2 | 71.7 | 2.5–98.6 |

\* Two patients were not evaluable.

Table 2

Number of patients per treatment centre and tumour localisation.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Treatment | centre | *n* | Oral | cavity | Oropharynx | Hypopharynx |
| Dresden | 42 | | 21 | 14 | | 7 |
| Frankfurt | 27 | | 12 | 14 | | 1 |
| Tübingen | 33 | | 9 | 19 | | 5 |
| Freiburg | 30 | | 5 | 20 | | 5 |
| Essen | 32 | | 2 | 22 | | 9 |
| Berlin | 24 | | 9 | 11 | | 4 |
| Munich[a](#_bookmark86) | 17 | | 0 | 16 | | 1 |
| Heidelberg | 15 | | 2 | 10 | | 3 |
| Total | 221 | | 60 | 126 | | 35 |

a Partner site Munich consists of Technische Universität and Ludwig- Maximilians-Universität.

Table 3

Number of tumours with positive biomarkers per tumour localisation.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Biomarker | Overall | Oral cavity | Oropharynx | Hypopharynx |
| HPV16 DNA | 72 (33.5%) | 7 (12.1%) | 59 (48.0%) | 5 (14.7%) |
| HPV16/18 DNA[a](#_bookmark88) | 1 | – | 1 | – |
| HPV18 DNA | 1 | 1 | – | – |
| HPV33 DNA | 1 | – | 1 | – |
| p16 | 79 (36.9%) | 11 (18.3%) | 65 (53.7%) | 3 (9.1%) |
| p53 | 85 (38.5%) | 32 (53.3%) | 40 (31.7%) | 13 (37.1%) |

a Patient was included in HPV16 DNA positive group.

distant metastases. ECE status showed significant association with distant metastases (HR 2.55; *p* < 0.01) and borderline significance with overall survival ([Table 4](#_bookmark91) and Supplementary Table S1). Overexpression of p16 showed a significant association with distant metastases (HR 0.31; *p* = 0.02) and on overall survival (HR 0.44; *p* = 0.01) (Supplementary Table S2).

Discussion

While several previous studies provided strong evidence that the HPV status is a significant prognostic marker of loco-regional

tumour control and/or survival in patients treated with primary radiotherapy or radiochemotherapy for locally advanced HNSCC [[14,16,18,19]](#_bookmark96), the impact of HPV status on outcome of postopera- tive radio(chemo)therapy is less well investigated. The results of the present multicentre retrospective study of the DKTK-ROG show that HPV16 DNA positivity is a significant prognosticator of loco- regional tumour control and survival of patients treated with cis- platin-based postoperative radiochemotherapy after surgical resection of locally advanced HNSCC. The effect appears to be robust over all treatment centres and is driven by the results in oropharyngeal cancers. Our findings are in line with a study by Snietura et al. who investigated the influence of HPV infection on the clinical outcome in a posthoc analysis of a randomised clinical trial of two different schedules of PORT without chemotherapy in 279 HNSCC patients. HPV analysis was conducted in tumours of 131 patients. From the 66 patients with oral cavity or oropharyn- geal carcinoma, 9 were found to be positive for HPV16 DNA and were locally controlled after 5 years, whereas the loco-regional tumour control rate in the whole HPV DNA negative group was only 58% [[24]](#_bookmark98). Taken together, HPV16 DNA appears to be a poten- tially promising biomarker for stratification and individualised prescription of postoperative radiotherapy. HPV-positivity seems to be sufficient to define a patient cohort that is highly unlikely to develop loco-regional recurrences after PORT-C, which is in con- trast to primary radiochemotherapy where more stratification parameters are necessary [[25]](#_bookmark98). This difference between the two treatment approaches may be caused by the fact that such addi- tional factors, most obviously tumour volume, play a lesser role when the tumour is resected. Other patient-related risk factors like smoking status could not be evaluated in our dataset but might be relevant as well. We are currently performing similar analyses in a patient cohort that has been treated by the same centres and within the same period of time with primary radiochemotherapy to further evaluate such differential prognosticators using a multi- dimensional statistical approach including radiobiological esti- mates e.g., on tumour cell number. For the group of HPV- negative patients, the situation is largely different. Here, HPV can-

###### All patients

**100**

HPV16 DNA +

HPV16 DNA -

###### Pts. with oropharyngeal carcinoma

**100**

HPV16 DNA + HPV16 DNA -

**80 80**

Loco-regional control

Loco-regional control

**60 60**

**0**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| p<0.01  **0**  **0 12** | | | **24** | **36** | **48** | **60** | p=  **0** | 0.02  **12** | **24** | **36** | **48** | **60** |
| **Patients at risk** Months after begin of treatment Months after begin of treatment | | | | | | | | | | | | |
| HPV16 DNA + | 72 | 69 | 65 | 58 | 43 | 24 | 60 | 60 | 57 | 51 | 38 | 21 |
| HPV16 DNA - | 143 | 116 | 102 | 86 | 60 | 36 | 63 | 56 | 49 | 42 | 33 | 20 |

Fig. 1. Kaplan–Meier estimates of loco-regional tumour control. (A) Patients with HPV16 DNA positive HNSCC had significantly higher loco-regional tumour control compared to HPV16 DNA negative tumours. (B) In subgroup analysis, patients with HPV16 DNA positive oropharyngeal tumours showed significantly higher loco-regional tumour control rates than those with HPV16 DNA negative tumours confined to the oropharynx.

Treatment centre

Loco-regional control, HR (95% CI)

HPV16 DNA positive HPV16 DNA negative Events Total Events Total

\* No CI was calculated in case of no event in the HPV16 DNA positive group

²-test of heterogeneity not significant (p>0.2)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Centre 1 | 0.23 | (0.02-1.80) | 1 | 12 | 9 | 30 |
| Centre 2 | 0 | \* | 0 | 7 | 4 | 20 |
| Centre 3 | 0 | \* | 0 | 11 | 3 | 20 |
| Centre 4 | 0 | \* | 0 | 10 | 3 | 20 |
| Centre 5 | 0 | \* | 0 | 9 | 4 | 23 |
| Centre 6 | 0 | \* | 0 | 4 | 2 | 17 |
| Centre 7 | 0.32 | (0.02-5.12) | 1 | 13 | 1 | 4 |
| Centre 8 | 0 | \* | 0 | 6 | 1 | 9 |
| **Overall** | **0.13** | **(0.03-0.54)** | **2** | **72** | **27** | **143** |

0 1 2 3 4 5

HR (95% CI)

Fig. 2. Forest plot demonstrating the impact of HPV16 DNA status on loco-regional tumour control at the different treatment centres and the pooled estimate (univariate analyses; Supplementary Table S1). HPV16 DNA status is a prognostic parameter for loco-regional tumour control at all treatment centres.

Table 4

Multivariate analyses of prognostic factors for loco-regional control, distant metastases and overall survival. HR = hazard ratio; 95% CI = 95 percent confidence interval.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| All sites Oral cavity Oropharynx    HR (95% CI) *p*-value HR (95% CI) *p*-value HR (95% CI) *p*-value | | | | | | | | | |
| *Loco-regional control* |  |  |  |  |  |  |  |  |  |
| HPV16 DNA | 0.20 | (0.04–0.92) | 0.04 | 0.83 | (0.10–6.75) | 0.87 | 0.09 | (0.01–0.74) | 0.03 |
| p53 positivity | 1.81 | (0.81–4.06) | 0.15 | 3.21 | (0.87–11.8) | 0.08 | 0.89 | (0.23–3.50) | 0.87 |
| ECE status | 1.35 | (0.62–2.93) | 0.45 | 3.68 | (0.83–16.4) | 0.09 | 1.09 | (0.29–4.14) | 0.90 |
| Oral cavity[⇑](#_bookmark92) | 2.30 | (1.02–5.16) | 0.04 | – |  | – | – |  | – |
| Hypopharynx[⇑](#_bookmark92) | 0.71 | (0.19–2.58) | 0.60 | – |  | – | – |  | – |
| *Distant metastases* |  |  |  |  |  |  |  |  |  |
| HPV16 DNA | 0.56 | (0.22–1.42) | 0.23 | 0.97 | (0.21–4.38) | 0.97 | 0.40 | (0.11–1.49) | 0.17 |
| p53 positivity | 1.39 | (0.72–2.70) | 0.32 | 0.93 | (0.35–2.45) | 0.88 | 1.46 | (0.42–5.12) | 0.55 |
| ECE status | 2.55 | (1.26–5.15) | <0.01 | 9.10 | (1.21–68.8) | 0.03 | 1.36 | (0.43–4.34) | 0.60 |
| Oral cavity[⇑](#_bookmark92) | 2.37 | (1.11–5.07) | 0.03 | – |  | – | – |  | – |
| Hypopharynx[⇑](#_bookmark92) | 2.73 | (1.15–6.47) | 0.02 | – |  | – | – |  | – |
| *Overall survival* |  |  |  |  |  |  |  |  |  |
| HPV16 DNA | 0.36 | (0.17–0.73) | <0.01 | 0.30 | (0.04–2.23) | 0.24 | 0.36 | (0.15–0.82) | 0.02 |
| p53 positivity | 1.07 | (0.65–1.79) | 0.78 | 1.27 | (0.58–2.79) | 0.55 | 1.03 | (0.46–2.30) | 0.94 |
| ECE status | 1.63 | (0.98–2.70) | 0.06 | 3.60 | (1.24–10.5) | 0.02 | 0.99 | (0.47–2.13) | 1.00 |
| Oral cavity[⇑](#_bookmark92) | 1.73 | (1.00–2.96) | <0.05 | – |  | – | – |  | – |
| Hypopharynx[⇑](#_bookmark92) | 0.66 | (0.30–1.46) | 0.31 | – |  | – | – |  | – |

\* Baseline oropharynx.

not be used as a sole biomarker to predict tumour recurrences, as shown by the low sensitivity of 38% or 41% for HPV16 DNA or p16 positivity. Thus, the HPV-negative group needs further investiga- tions into potential biomarkers to stratify for patients who may need treatment intensification and for patients for whom local recurrences are not to be expected.

Currently a prospective multicentre study of the DKTK-ROG is ongoing to validate the prognostic value of HPV16 DNA positivity on loco-regional tumour control after PORT-C in 240 HNSCC patients. If the results of the present retrospective cohort are con- firmed, an interventional trial to de-escalate PORT-C radiation doses in HPV16 DNA positive, clinically suitable oropharyngeal cancer patients will be initiated. Specificity assessment of HPV16 DNA positivity for loco-regional tumour control from the present investigation suggests that very few if any recurrences should be expected from a moderate decrease of radiation dose in these patients, therefore strict stopping rules for patient safety against the risk of inferior treatment may be applied in such trial, using e.g., a Pocock boundary approach [[26]](#_bookmark98). Further refinement of risk stratification specifically for the HPV-negative group may result from prospective assessment of clinical parameters [[27]](#_bookmark98) in the val- idation trial, and from ongoing efforts to identify further biomark- ers in the current retrospective and in the validation patient cohort.

The low risk of loco-regional recurrence in HPV16 DNA positive oropharyngeal cancers after curatively intended resection and PORT-C suggests that either less tumour stem cells are present at start of PORT-C, that the remaining HPV-positive tumour cells are more radio(chemo)sensitive, or a combination of both. Recently it was reported that HPV-positive oropharyngeal cancers show low expression of stem cell markers such as CD44 and CD98 compared to HPV-negative oropharyngeal cancers [[28]](#_bookmark98). Furthermore, patients with HPV-positive and low CD98 expressing tumours showed bet- ter overall survival and progression-free survival compared to patients with high CD98 expressing HPV-positive tumours. Increased radiosensitivity of HPV-positive tumour cells is sup- ported by a number of investigations. HPV-positive HNSCC cell lines (all positive for HPV DNA, HPV RNA and p16) assessed by col- ony formation assay in vitro showed a higher cellular radiosensi- tivity when compared to HPV-negative cell lines due to compromised DNA repair capacity [[29]](#_bookmark98). Similar observations have been reported by others [[30,31]](#_bookmark98). Further observations using both in vitro and in vivo approaches suggest that overexpressed p16 impairs the recruitment of RAD51 to the DNA damage site in HPV-positive HNSCC by down-regulation of cyclin D1, thereby affecting the cell cycle and homologous recombination-mediated DNA repair response [[32]](#_bookmark98).

There is currently no generally agreed consensus for the assess-

ment of the HPV infection status as a potential biomarker; general methods used for assessment of HPV infection include HPV DNA, HPV RNA, and p16 overexpression [[33–35]](#_bookmark99). The vast majority of HPV-positive HNSCC has been shown to be positive for HPV16 DNA [[21,36]](#_bookmark105), which is in line with the results reported here. HPV16 DNA showed stronger correlations with outcome parame- ters as compared to p16 immunohistochemistry in a cohort of 50 patients with oropharyngeal tumours who received primary radio- chemotherapy [[37]](#_bookmark101). Also in our study HPV16 DNA appears as a stronger prognosticator for loco-regional tumour control compared to p16 expression ([Table 4](#_bookmark91) vs. Supplementary Table S2), however this needs to be validated in a larger cohort.

In the present study HPV16 DNA positivity was a strong inde- pendent prognosticator for loco-regional tumour control in oropha- ryngeal but not in oral cavity tumours. In contrast, increased p53 positivity was observed in oral cavity tumours, which suggests an alternative pathway for tumour development, e.g. life style factors.

The tumour suppressor gene TP53 is known to be involved in carci- nogenesis of HNSCC [[38]](#_bookmark102) and its overexpression is reported in heavy smokers and heavy drinkers [[39,40]](#_bookmark103). Increased positivity has been linked to TP53 gene mutations, which can cause stabilisation and nuclear accumulation of p53 proteins [[41]](#_bookmark104). It has been demon- strated that the HPV oncoprotein E6 inactivates and inhibits p53 [[42,43]](#_bookmark107), which is in line with the fact that the majority of our HPV-positive study cohort was negative for p53.

In our study, extracapsular extension of lymph nodes is a prog- nostic factor for overall survival in patients with oral cavity carci- nomas but not in the total patient population. This seems to be in contrast to the results of the meta-analysis by Bernier et al., show- ing that positive margins and/or extracapsular extension are the most significant prognosticators for poor outcome/overall survival [[9]](#_bookmark94). However this meta-analysis did not stratify between oral cav- ity and oropharyngeal cancer and molecular biomarkers were not investigated as confounding factors in the trials included in the meta-analysis (EORTC and RTOG trials). Furthermore patients included in the EORTC and RTOG trials might reflect a different population relative to the patient cohort analysed in the present study, underlining the necessity of constant marker adaptation for patient stratification. Further efforts to investigate different biomarkers specifically for the HPV-negative group receiving post- operative radiochemotherapy as well as for primary radiochemo- therapy are currently ongoing in the DKTK-ROG using material of the cohort reported here and of the validation trial.

In conclusion, our results of this retrospective explorative mul- ticentre study show that HPV16 DNA seems to be a strong prog- nosticator of loco-regional tumour control after postoperative cisplatin-based radiochemotherapy of locally advanced oropharyn- geal carcinoma and is therefore a promising biomarker for patient stratification. The effect appeared robust over the 8 treatment cen- tres. For patients with HPV16 DNA positive oropharyngeal carci- noma treatment de-intensification may be a valid interventional option for a prospective trial that is currently prepared.

Conflict of interest statement

The authors have nothing to disclose.

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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.201](http://dx.doi.org/10.1016/j.radonc.2014.11.011) [4.11.011](http://dx.doi.org/10.1016/j.radonc.2014.11.011).

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**Letter to the Editor**

###### Page 1 of 3

**HPV and beyond—looking out for biomarkers for distinguishing the good prognosis from the bad prognosis group in locally advanced and clinically high risk HNSCC**

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In their recent editorial Kimple and Harari (1) reviewed the current knowledge of the importance of HPV status of head and neck squamous cell carcinoma (HNSCC) for outcome of radiotherapy. The editorial summarized, among others, a retrospective multicenter study by the German Cancer Consortium Radiation Oncology Group (DKTK-ROG) which demonstrated significant prognostic value of the HPV status in patients with locally advanced HNSCC on the outcome of postoperative radiochemotherapy (PORT-C) (2). The overall aim of the DKTK-ROG head and neck program is to identify and validate biomarkers including biological and clinical parameters as well as imaging data for patient stratification in terms of individualization of radiotherapy by multimodal treatment. The study design includes retrospective explorative analyses in patients who received PORT-C or primary radiochemotherapy (RCT) for biomarker identification. The most promising biomarkers will then be validated in a prospective validation cohort, which is currently recruiting at all eight DKTK-ROG partner sites. Based on these results, interventional studies are under preparation. Biomarker analyses are being performed at all eight DKTK partner sites and are addressing different topics such as HPV status, hypoxia, cancer stem cells, tumor infiltrating lymphocytes, tumor volume, targeted next generation sequencing, transcriptomics and methylome analyses.

With a 2-year overall survival rate of about 83% in the retrospective PORT-C study, the patients treated at the

DKTK-ROG sites seem to have a favorable prognosis compared to the two landmark studies of the RTOG (#9501) (63%) and EORTC (#22931) (75%) (3,4). This difference may be due to differences in staging (in the DKTK-ROG study staging was performed with contrast-enhanced MRI, CT or PET/CT) and the broad availability of intensity modulated radiotherapy (IMRT). However, also formal differences exist between these three studies, with the DKTK-ROG study evaluating contemporary retrospective data from a limited number of tertiary high volume centers, while the randomized RTOG and EORTC trials have prospectively accrued patients in a larger number of centers. This limits the validity of direct comparison of the survival data of these studies. It is also not straightforward to compare the PORT-C data reported by the DKTK- ROG with the outcome of studies testing primary RCT, as the patient cohorts submitted to these different treatment strategies may vary in important parameters, including stage and co-morbidity. Therefore it appears most rationale to compare the relative impact of biological stratification parameters on outcome separately in different trials.

Impact of HPV in non-oropharyngeal cancer

##### In our DKTK-ROG study, the HPV status was analyzed in patients with locally advanced HNSCC of the oral cavity, oropharynx and hypopharynx. However, 35 patients with

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##### hypopharyngeal carcinoma were included in this study with five of them being HPV16 DNA positive. Because of this low number, we did not draw any conclusion of the impact of the HPV status in this tumor subsite. Kimple and Harari pointed out that in a study by Chung *et al*. (5), patients with p16-positive non-oropharyngeal cancer treated with different RCT schedules had a significantly better overall survival than patients with p16-negative tumors. Whether this applies also to patients in Germany and Europe needs to be addressed in larger studies, as today’s HPV infection rate is lower in Germany than in North America.

Impact of HPV in primary RCT ***vs***. PORT-C

##### In our retrospective DKTK-ROG PORT-C cohort, a very high locoregional tumor control rate was achieved in HPV16 DNA positive (oropharyngeal) tumors. If confirmed in our ongoing multicenter validation trial, this observation is of substantial importance for the design of future clinical studies. Local control rates close to 100% should allow to testing the value of dose de-escalation in HPV positive tumors without compromising locoregional control. This is in contrast to primary RCT of macroscopic tumors (e.g., RTOG0129, RTOG 0522, DAHANCA 5, DAHANCA 6,

DAHANCA 7) where long term local control rates of HPV positive oropharyngeal cancers are in the range between 70% and 80%. Further clinical and biological parameters are needed in this situation to safely stratify patients to less or more aggressive treatments. This can for example be demonstrated in the primary RCT DKTK-ROG cohort, which shows that tumor volume, hypoxia and stem cell markers add significantly to the prognostic power of HPV (Lohaus, Linge, Löck, Baumann *et al*., to be submitted).

Beyond HPV infection

##### To further stratify patients with HPV negative tumors undergoing PORT-C, further biomarkers are currently under investigation. Recently, Balermpas *et al*. showed in the retrospective PORT-C cohort of the DKTK-ROG, the prognostic value of tumor-infiltrating CD8 positive lymphocytes on overall survival and local progression- free survival independent of the HPV status (6). In terms of potential treatment modulation, the question remains to be resolved whether HPV positive HNSCC are more radiosensitive *per se* or if the improved response is due to the immune response. Furthermore, targeted next generation sequencing in the retrospective DKTK PORT-C cohort

revealed that loss-of-function alterations in tumor suppressor genes are related to an increased risk of loco- regional recurrence, distant metastases and death, mainly in HPV negative tumors (7). Also hypoxia and expression of cancer stem cell markers, independently of HPV, correlate with outcome of PORT-C (Linge *et al*., submitted for publication). Thus clinical applicable biomarkers beyond HPV are emerging which, if validated in our ongoing prospective trial, may significantly enhance our arsenal for stratification of patients for clinical trials and individualized treatment approaches, offering new opportunities on the avenue towards personalized precision radiation oncology in head and neck cancer.

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Footnote

*Conflicts of Interest:* The authors have no conflicts of interest

##### to declare.

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# Comparison of detection methods for HPV status as a prognostic marker for loco- regional control after radiochemotherapy in patients with HNSCC

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**Autor** M. Stuschke **Autor** P. Balermpas **Autor** C. Rodel **Autor** H. Bunea **Autor** A. L. Grosu **Autor** A. Abdollahi **Autor** J. Debus **Autor** U. Ganswindt **Autor** K. Lauber **Autor** S. Pigorsch **Autor** S. E. Combs **Autor** D. Monnich **Autor** D. Zips

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**Zusammenfassung** OBJECTIVE: To compare six HPV detection methods in pre-treatment FFPE tumour samples from patients with locally advanced head and neck squamous cell carcinoma (HNSCC) who received postoperative (N=175) or primary (N=90) radiochemotherapy. MATERIALS AND METHODS: HPV analyses included detection of (i) HPV16 E6/E7 RNA, (ii) HPV16 DNA (PCR-based arrays, A-PCR),

(iii) HPV DNA (GP5+/GP6+ qPCR, (GP-PCR)), (iv) p16 (immunohistochemistry, p16 IHC), (v) combining p16 IHC and the A-PCR result and (vi) combining p16

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IHC and the GP-PCR result. Differences between HPV positive and negative subgroups were evaluated for the primary endpoint loco-regional control (LRC) using Cox regression. RESULTS: Correlation between the HPV detection methods was high (chi-squared test, p<0.001). While p16 IHC analysis resulted in several false positive classifications, A-PCR, GP-PCR and the combination of p16 IHC and A-PCR or GP-PCR led to results comparable to RNA analysis. In both cohorts, Cox regression analyses revealed significantly prolonged LRC for patients with HPV positive tumours irrespective of the detection method. CONCLUSIONS: The most stringent classification was obtained by detection of HPV16 RNA, or combining p16 IHC with A-PCR or GP-PCR. This approach revealed the lowest rate of recurrence in patients with tumours classified as HPV positive and therefore appears most suited for patient stratification in HPV-based clinical studies.

**Datum** 2018

**URL** <https://www.ncbi.nlm.nih.gov/pubmed/29295747>

**Extra** Type: Journal Article

**Band** 127

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**Publikation** Radiother Oncol

**DOI** [10.1016/j.radonc.2017.12.007](http://doi.org/10.1016/j.radonc.2017.12.007)

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Adult Aged Carcinoma Local/drug therapy/pathology/radiotherapy/\*virology Oncogene Proteins Squamous Cell/drug therapy/pathology/radiotherapy/\*virology Chemoradiotherapy Female Head and Neck Neoplasms/drug therapy/pathology/radiotherapy/\*virology Human papillomavirus 16/genetics

/\*isolation & purification/metabolism Humans Immunohistochemistry Male Middle Aged Neoplasm Recurrence Viral/genetics Real-Time Polymerase Chain Reaction Repressor Proteins/genetics/metabolism Retrospective Studies Squamous Cell Carcinoma of Head and Neck \*dktk-rog \*hnscc \*hpv \*Loco- regional control \*Radiochemotherapy \*p16 Viral/genetics/metabolism Papillomavirus E7 Proteins/genetics/metabolism Papillomavirus Infections/metabolism/\*virology Prognosis RNA

### Notizen:

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Linge, Annett Schotz, Ulrike Lock, Steffen Lohaus, Fabian von Neubeck, Clare Gudziol, Volker Nowak, Alexander Tinhofer, Inge Budach, Volker Sak, Ali Stuschke, Martin Balermpas, Panagiotis Rodel, Claus Bunea, Hatice Grosu, Anca-Ligia Abdollahi, Amir Debus, Jurgen Ganswindt, Ute Lauber, Kirsten Pigorsch, Steffi Combs, Stephanie E Monnich, David Zips, Daniel Baretton, Gustavo B Buchholz, Frank Krause, Mechthild Belka, Claus Baumann, Michael eng Comparative Study Multicenter Study Research Support, Non-U.S. Gov't Ireland Radiother Oncol. 2018 Apr;127(1):27-35. doi: 10.1016/j.radonc.2017.12.007. Epub 2017 Dec 30.

# HPV and beyond-looking out for biomarkers for distinguishing the good prognosis from the bad prognosis group in locally advanced and clinically high risk HNSCC

**Eintragsart** Zeitschriftenartikel

**Autor** F. Lohaus **Autor** A. Linge **Autor** M. Baumann **Datum** 2015

**URL** <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4620098/pdf/atm-03-17-255.pdf>

**Extra** Type: Journal Article

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**Seiten** 255

**Publikation** Ann Transl Med

**DOI** [10.3978/j.issn.2305-5839.2015.09.35](http://doi.org/10.3978/j.issn.2305-5839.2015.09.35)

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Lohaus, Fabian Linge, Annett Baumann, Michael eng China Ann Transl Med. 2015 Oct;3(17):255. doi: 10.3978/j.issn.2305-5839.2015.09.35.

### Anhänge

* Volltext

3 of 13 28.09.23, 16:00

# 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)

**Eintragsart** Zeitschriftenartikel

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**Autor** G. B. Baretton **Autor** H. D. Thames **Autor** A. Dubrovska **Autor** J. Alsner **Autor** J. Overgaard **Autor** M. Krause **Autor** M. Baumann **Autor** R. O. G. Dktk

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**Zusammenfassung** 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). 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 2years 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.

**Datum** 2016

**URL** <https://www.ncbi.nlm.nih.gov/pubmed/27913065>

**Extra** Type: Journal Article

**Band** 121

**Seiten** 364-373

**Publikation** Radiother Oncol

**DOI** [10.1016/j.radonc.2016.11.008](http://doi.org/10.1016/j.radonc.2016.11.008)

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### Tags:

5 of 13 28.09.23, 16:00

Tumor/metabolism Carcinoma Adult Aged Biomarkers Squamous Cell/genetics/pathology/\*therapy

/virology Cell Hypoxia/genetics Chemoradiotherapy Female Gene Expression Profiling/methods Head and Neck Neoplasms/genetics/pathology/\*therapy/virology Humans Hyaluronan Receptors/metabolism Male Middle Aged Neoplastic Stem Cells/\*metabolism Papillomaviridae/\*isolation & purification Prognosis Radiation Tolerance/genetics Retrospective Studies Squamous Cell Carcinoma of Head and Neck Tumor Burden \*Biomarkers for radiotherapy \*Cancer stem cells \*hnscc \*hpv \*Hypoxia \*Primary radiochemotherapy

### Notizen:

Linge, Annett Lohaus, Fabian Lock, Steffen Nowak, Alexander Gudziol, Volker Valentini, Chiara von Neubeck, Clare Jutz, Martin Tinhofer, Inge Budach, Volker Sak, Ali Stuschke, Martin Balermpas, Panagiotis Rodel, Claus Grosu, Anca-Ligia Abdollahi, Amir Debus, Jurgen Ganswindt, Ute Belka, Claus Pigorsch, Steffi Combs, Stephanie E Monnich, David Zips, Daniel Buchholz, Frank Aust, Daniela E Baretton, Gustavo B Thames, Howard D Dubrovska, Anna Alsner, Jan Overgaard, Jens Krause, Mechthild Baumann, Michael eng Multicenter Study Ireland Radiother Oncol. 2016 Dec;121(3):364-373. doi: 10.1016/j.radonc.2016.11.008. Epub 2016 Nov 29.

# HPV16 DNA status is a strong prognosticator of loco-regional control after postoperative radiochemotherapy of locally advanced oropharyngeal carcinoma: results from a multicentre explorative study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG)

**Eintragsart** Zeitschriftenartikel

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**Autor** H. D. Thames **Autor** M. Krause **Autor** M. Baumann **Autor** R. O. G. Dktk

**Zusammenfassung** OBJECTIVE: To investigate the impact of HPV status in patients with locally advanced head and neck squamous cell carcinoma (HNSCC), who received surgery and cisplatin-based postoperative radiochemotherapy. MATERIALS AND METHODS: For 221 patients with locally advanced squamous cell carcinoma of the hypopharynx, oropharynx or oral cavity treated at the 8 partner sites of the German Cancer Consortium, the impact of HPV DNA, p16 overexpression and p53 expression on outcome were retrospectively analysed. The primary endpoint was loco-regional tumour control; secondary endpoints were distant metastases and overall survival. RESULTS: In the total patient population, univariate analyses revealed a significant impact of HPV16 DNA positivity, p16 overexpression, p53 positivity and tumour site on loco-regional tumour control. Multivariate analysis stratified for tumour site showed that positive HPV 16 DNA status correlated with loco-regional tumour control in patients with oropharyngeal carcinoma (p=0.02) but not in the oral cavity carcinoma group. Multivariate evaluation of the secondary endpoints in the total population revealed a significant association of HPV16 DNA positivity with overall survival (p<0.01) but not with distant metastases.

CONCLUSIONS: HPV16 DNA status appears to be a strong prognosticator of loco- regional tumour control after postoperative cisplatin-based radiochemotherapy of locally advanced oropharyngeal carcinoma and is now being explored in a prospective validation trial.

**Datum** 2014

**URL** <https://www.thegreenjournal.com/article/S0167-8140(14)00489-7/pdf>

**Extra** Type: Journal Article

**Band** 113

**Seiten** 317-23

**Publikation** Radiother Oncol

**DOI** [10.1016/j.radonc.2014.11.011](http://doi.org/10.1016/j.radonc.2014.11.011)

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Carcinoma Squamous Cell/\*genetics/\*therapy/virology Chemoradiotherapy/methods Cyclin-Dependent Kinase Inhibitor p16/\*genetics DNA/genetics Female Head and Neck Neoplasms/\*genetics/\*therapy

/virology Humans Male Oropharyngeal Neoplasms/\*genetics/\*therapy/virology Papillomaviridae/genetics Postoperative Period Prognosis Prospective Studies Retrospective Studies Squamous Cell Carcinoma of Head and Neck Survival Analysis Treatment Outcome Dktk-rog Hnscc Hpv Postoperative radiochemotherapy p16 p53

### Notizen:

Lohaus, Fabian Linge, Annett Tinhofer, Inge Budach, Volker Gkika, Eleni Stuschke, Martin Balermpas, Panagiotis Rodel, Claus Avlar, Melanie Grosu, Anca-Ligia Abdollahi, Amir Debus, Jurgen Bayer, Christine Belka, Claus Pigorsch, Steffi Combs, Stephanie E Monnich, David Zips, Daniel von Neubeck, Clare Baretton, Gustavo B Lock, Steffen Thames, Howard D Krause, Mechthild Baumann, Michael eng Multicenter Study Research Support, Non-U.S. Gov't Ireland Radiother Oncol. 2014 Dec;113(3):317-23. doi: 10.1016/j.radonc.2014.11.011. Epub 2014 Dec 2.

### Anhänge

* Volltext

# Independent validation of the prognostic value of cancer stem cell marker expression and hypoxia-induced gene expression for patients with locally advanced HNSCC after postoperative radiotherapy

**Eintragsart** Zeitschriftenartikel

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**Zusammenfassung** Objective: To validate the impact of HPV status, cancer stem cell (CSC) marker expression and tumour hypoxia status in patients with locally advanced head and neck squamous cell carcinoma (HNSCC), who received postoperative radiotherapy. The results of the exploration cohort have previously been reported by the German Cancer Consortium Radiation Oncology Group (DKTK-ROG; Lohaus et al., 2014;

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Linge et al., 2016). Materials and methods: For 152 patients with locally advanced HNSCC the impact of HPV16 DNA status, CSC marker expression and hypoxia- associated gene signatures on outcome of postoperative radiotherapy were retrospectively analysed. Out of them, 40 patients received postoperative radiochemotherapy. Cox models presented in a previous study were validated using the concordance index as a performance measure. The primary endpoint of this study was loco-regional control. Results were compared to those previously reported by DKTK-ROG. Results: Loco-regional control, freedom from distant metastases and overall survival were inferior to the previously reported cohort. Despite of this, the prognostic value of the combination of HPV infection status, CSC marker expression (SLC3A2) and tumour hypoxia status could be validated in univariate analyses using an independent validation cohort. For multivariate models, the concordance index was between 0.58 and 0.69 in validation, indicating a good prognostic performance of the models. The inclusion of CD44 and the 15-gene hypoxia signature moderately improved the performance compared to a baseline model without CSC markers or hypoxia classifiers. Conclusions: The HPV status, CSC marker expression of CD44 and SLC3A2 as well as hypoxia status are potential prognostic biomarkers for patients with locally advanced HNSCC treated by postoperative radiotherapy.

**Datum** 2016

**URL** <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5893482/pdf/main.pdf>

**Extra** Type: Journal Article

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### Notizen:

Linge, Annett Lock, Steffen Krenn, Constanze Appold, Steffen Lohaus, Fabian Nowak, Alexander Gudziol, Volker Baretton, Gustavo B Buchholz, Frank Baumann, Michael Krause, Mechthild eng Ireland Clin Transl Radiat Oncol. 2016 Dec 22;1:19-26. doi: 10.1016/j.ctro.2016.10.002. eCollection 2016 Dec.

### Anhänge

* Volltext

9 of 13 28.09.23, 16:00

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

**Eintragsart** Zeitschriftenartikel

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**Autor** A. Bandurska-Luque

**Autor** I. Platzek **Autor** C. von Neubeck **Autor** S. Appold **Autor** A. Nowak **Autor** V. Gudziol **Autor** F. Buchholz **Autor** G. B. Baretton **Autor** M. Baumann **Autor** S. Lock

**Autor** M. Krause

**Zusammenfassung** 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 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.

10 of 13 28.09.23, 16:00

**Datum** 2019

**URL** <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6449705/pdf/main.pdf>

**Extra** Type: Journal Article

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Biomarker Cancer stem cells Hnscc Hpv Hypoxia Primary radiochemotherapy

### Notizen:

Linge, Annett Schmidt, Stefan Lohaus, Fabian Krenn, Constanze Bandurska-Luque, Anna Platzek, Ivan von Neubeck, Clare Appold, Steffen Nowak, Alexander Gudziol, Volker Buchholz, Frank Baretton, Gustavo B Baumann, Michael Lock, Steffen Krause, Mechthild eng Ireland Clin Transl Radiat Oncol. 2019 Mar 18;16:40-47. doi: 10.1016/j.ctro.2019.03.002. eCollection 2019 May.

### Anhänge

* Volltext

# Low Cancer Stem Cell Marker Expression and Low Hypoxia Identify Good Prognosis Subgroups in HPV(-) HNSCC after Postoperative Radiochemotherapy: A Multicenter Study of the DKTK-ROG

**Eintragsart** Zeitschriftenartikel

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**Autor** C. von Neubeck

**Autor** M. Jutz **Autor** A. Abdollahi **Autor** J. Debus **Autor** I. Tinhofer

11 of 13 28.09.23, 16:00

**Autor** V. Budach **Autor** A. Sak **Autor** M. Stuschke **Autor** P. Balermpas **Autor** C. Rodel **Autor** M. Avlar **Autor** A. L. Grosu **Autor** C. Bayer **Autor** C. Belka **Autor** S. Pigorsch **Autor** S. E. Combs **Autor** S. Welz **Autor** D. Zips **Autor** F. Buchholz **Autor** D. E. Aust

**Autor** G. B. Baretton **Autor** H. D. Thames **Autor** A. Dubrovska **Autor** J. Alsner **Autor** J. Overgaard **Autor** M. Baumann **Autor** M. Krause **Autor** R. O. G. Dktk

**Zusammenfassung** PURPOSE: To investigate the impact of hypoxia-induced gene expression and cancer stem cell (CSC) marker expression on outcome of postoperative cisplatin- based radiochemotherapy (PORT-C) in patients with locally advanced head and neck squamous cell carcinoma (HNSCC). EXPERIMENTAL DESIGN: Expression of the CSC markers CD44, MET, and SLC3A2, and hypoxia gene signatures were analyzed in the resected primary tumors using RT-PCR and nanoString technology in a multicenter retrospective cohort of 195 patients. CD44 protein expression was further analyzed in tissue microarrays. Primary endpoint was locoregional tumor control. RESULTS: Univariate analysis showed that hypoxia-induced gene expression was significantly associated with a high risk of locoregional recurrence using the 15-gene signature (P = 0.010) or the 26-gene signature (P = 0.002). In multivariate analyses, in patients with HPV16 DNA-negative but not with HPV16 DNA-positive tumors the effect of hypoxia-induced genes on locoregional control was apparent (15-gene signature: HR 4.54, P = 0.006; 26-gene signature: HR 10.27, P = 0.024). Furthermore, MET, SLC3A2, CD44, and CD44 protein showed an association with locoregional tumor control in multivariate analyses (MET: HR 3.71, P = 0.016; SLC3A2: HR 8.54, P = 0.037; CD44: HR 3.36, P = 0.054; CD44

protein n/a because of no event in the CD44-negative group) in the HPV16 DNA- negative subgroup. CONCLUSIONS: We have shown for the first time that high hypoxia-induced gene expression and high CSC marker expression levels correlate with tumor recurrence after PORT-C in patients with HPV16 DNA-negative

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HNSCC. After validation in a currently ongoing prospective trial, these parameters may help to further stratify patients for individualized treatment de-escalation or intensification strategies. Clin Cancer Res; 22(11); 2639-49. (c)2016 AACR.

**Datum** 2016

**URL** <https://www.ncbi.nlm.nih.gov/pubmed/26755529>

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**Seiten** 2639-49

**Publikation** Clin Cancer Res

**DOI** [10.1158/1078-0432.CCR-15-1990](http://doi.org/10.1158/1078-0432.CCR-15-1990)

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Tumor/metabolism Carcinoma Antineoplastic Agents/therapeutic use Biomarkers Heavy Chain/metabolism Human papillomavirus 16/genetics Humans Hyaluronan Receptors/metabolism Kaplan- Meier Estimate Mouth Neoplasms/\*metabolism/mortality/pathology/therapy Multivariate Analysis Neoplastic Stem Cells/\*metabolism Papillomavirus Infections/diagnosis Prognosis Prospective Studies Transcriptome Treatment Outcome Squamous Cell/\*metabolism/mortality/pathology/therapy Cell Hypoxia Chemoradiotherapy Cisplatin/therapeutic use Fusion Regulatory Protein 1

### Notizen:

Linge, Annett Lock, Steffen Gudziol, Volker Nowak, Alexander Lohaus, Fabian von Neubeck, Clare Jutz, Martin Abdollahi, Amir Debus, Jurgen Tinhofer, Inge Budach, Volker Sak, Ali Stuschke, Martin Balermpas, Panagiotis Rodel, Claus Avlar, Melanie Grosu, Anca-Ligia Bayer, Christine Belka, Claus Pigorsch, Steffi Combs, Stephanie E Welz, Stefan Zips, Daniel Buchholz, Frank Aust, Daniela E Baretton, Gustavo B Thames, Howard D Dubrovska, Anna Alsner, Jan Overgaard, Jens Baumann, Michael Krause, Mechthild eng Multicenter Study Research Support, Non-U.S. Gov't Clin Cancer Res. 2016 Jun 1;22(11):2639-49. doi: 10.1158/1078-0432.CCR-15-1990. Epub 2016 Jan 11.

### Anhänge

* Volltext

13 of 13 28.09.23, 16:00