Clinical Cancer Research

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

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Abstract

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 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. ©2016 AACR.*

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Translational Relevance

Locally advanced, HPV-positive head and neck squamous cell carcinomas (HNSCC) are responding very well to postoperative radiochemotherapy with virtually no recurrences detected in our recent study. To define patient groups for treatment individualization, it is important to establish further markers that could help to identify patients with a favorable prognosis but also with a very high risk of tumor recurrence within the HPV-negative group. In resected tumor specimens obtained prior to radiochemotherapy, we show for the first time that HPV16 DNA–negative, hypoxic tumors are significantly associated with an increased expression of cancer stem cell (CSC) markers and poor locoregional tumor control. In contrast, HPV16 DNA–negative HNSCC with low hypoxia and low CSC marker expression have a favorable locoregional tumor control rate. After validation in prospective trials, these markers may be used for further patient stratification for treatment intensification or de-escalation strategies.

Introduction

Although treatment efficacy has been improved for patients with locally advanced head and neck squamous cell carcinomas (HNSCC), the overall 5-year survival rate is still about 50% (1, 2). Resected locally advanced HNSCC with high-risk features are routinely treated with postoperative radiochemotherapy (PORT-C) after three randomized independent trials have shown an improved locoregional control and overall survival compared with postoperative radiotherapy alone (3–5). However, tumors respond very heterogeneously to this treatment and biomarkers are needed to identify patient groups who require de-escalated or intensified treatment schedules.

Recently, we have shown in a systematic multicentric retrospective evaluation that the human papillomavirus type 16 deoxyribonucleic acid (HPV16 DNA) status is a strong prognosticator for locoregional control after PORT-C in HNSCC, especially in patients with locally advanced oropharyngeal squamous cell carcinomas (6). HPV-positive tumors are more radio(chemo) sensitive than HPV-negative tumors (7, 8) and locoregional control after PORT-C was achieved in our analysis in virtually all patients with HPV16 DNA–positive tumors (6). In contrast, in patients with HPV16 DNA–negative tumors locoregional control rates of approximately 80% were reached, implying that the HPV16 DNA infection status is not suitable as a sole biomarker for the prediction of locoregional tumor recurrences in these patients. Additional biomarkers are needed for the stratification of HPV16 DNA–negative patients into two groups: those, who are very likely to develop tumor recurrence and may profit from treatment intensification and into those, who are not expected to develop tumor recurrences.

Tumor hypoxia has been shown to be negatively associated with radiotherapy treatment response (9, 10). Nordsmark and colleagues showed that pretreatment tumor hypoxia in HNSCC with partial oxygen pressure (pO_2) values of less than 2.5 mmHg is associated with significantly lower locoregional tumor control rates compared with patients with higher tumor oxygenation values (11). In a multi-institutional study on 397 patients with locally advanced HNSCC, Nordsmark and colleagues found that pretreatment tumor oxygenation is a highly significant prognostic factor for survival after primary radiotherapy applied alone or combined with chemotherapy, surgery, or radiation sensitizers (12). In recent years, hypoxia detection using [(18F)]-fluoroazomycin arabinoside (FAZA) PET/CT and [(18F)]-fluoromisonidazole (FMISO) PET/CT imaging have been found to have a prognostic potential in HNSCC (13, 14).

The molecular response of the tumor to hypoxia can also be analyzed on the transcriptional level. Several gene classifiers have been developed as a measure of tumor hypoxia in biopsies routinely taken for diagnosis (15–18). Toustrup and colleagues developed a 15-gene classifier for patients with HNSCC who received primary radiotherapy (15). This gene classifier is based on studies using oxygen electrode measurements in xenograft models and in an independent training set of 58 patients with HNSCC. It is therefore associated with the oxygen levels in tumors and has been shown to correlate with the benefit from hypoxic cell sensitization during radiotherapy. Another highly prognostic hypoxia metagene signature was developed by Buffa and colleagues for patients with head and neck, breast, and lung cancers (18). The 26-gene classifier by Eustace and colleagues, that is based on this metagene signature (18), has been shown to predict the benefit from hypoxia-modifying treatment in laryngeal cancer (16). However, as the gene classifiers are based on the measurement of hypoxia-induced gene expression and not on hypoxia itself, the classification by the gene signature does not necessarily correlate with hypoxia assessed by PET (13, 19).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-15-1990

To date, the potential of hypoxia gene signatures to discriminate pretreatment tumor specimen regarding their hypoxia-induced gene expression levels has not been tested in the postoperative setting (15, 16, 18). However, there is some evidence from an experimental study of our laboratory, which showed that pretreatment hypoxia impacts local tumor control after radiotherapy also when radiotherapy was applied under homogenous anoxic conditions (20). This would suggest that hypoxia impacts the outcome of radiotherapy not only by a direct biochemical or radiobiologic effect on cellular radioresistance, but that also other biologic mechanisms are involved. Hypoxia, as an external factor, might also contribute to increased radioresistance of cancer stem cells (CSC). According to the CSC hypothesis, CSCs are the only cells, which are capable of self-renewal and of tumor recurrence after treatment (21). Therefore, the number of CSCs is an important determinant for the radiation dose needed to achieve local tumor control (reviewed in ref. 22) and this may also differ between tumors of the same histopathologic type (23). In addition, CSC-related radiobiologic mechanisms such as repopulation during treatment and recovery from radiation-induced damage between the single fractions have been shown to increase tumor resistance against radiotherapy (24–26).

HPV16 DNA positivity may be used in future prospective clinical trials as a stratification marker for a very good patient outcome after PORT-C in high-risk HNSCC and thereby as a basis for radiotherapy de-escalation strategies. An important question is whether further markers could help to identify patients with a favorable prognosis within the HPV16 DNA–negative group, which would therefore also be candidates for treatment de-escalation strategies. At the same time, additional markers could help to detect a subgroup within the patients with HPV-negative tumors with a very high risk of recurrence, who would need to be included into treatment intensification trials. To further stratify patients with HPV16 DNA–negative HNSCC, we assessed the effects of pretreatment tumor hypoxia and the expression of potential CSC markers (*CD44* and CD44 protein, *MET* and *SLC3A2*) on the primary endpoint locoregional tumor control in the multicenter retrospective patient cohort of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG).

Materials and Methods

Patients

The patient eligibility criteria have been described previously (6). Briefly, patients with histologically proven squamous cell carcinoma arising from the oral cavity, oropharynx, or hypopharynx who were treated between 2004 and 2012 in eight DKTK partner sites were included in this study. They all received curatively intended cisplatin-based PORT-C according to standard protocols with a minimum follow-up of 24 months. All patients had a high risk for locoregional tumor recurrence with a tumor stage pT4 and/or >3 positive lymph nodes and/or positive microscopic resection margins and/or extracapsular spread. In total, 221 patients were included in this study, with a median follow-up of 47.3 months. The patient characteristics are shown in Supplementary Table S1A.

Processing of FFPE material

From all DKTK partner sites, formalin-fixed paraffin-embedded (FFPE) material of primary tumor specimens (removed by surgery) were collected centrally and processed under standardized procedures at the DKTK partner site Dresden. All FFPE tissue specimens were subjected to hematoxylin and eosin staining in order to confirm the presence of squamous cell carcinoma.

DNA extraction and PCR array-based analysis of HPV status

Genomic DNA was extracted from 5-μm FFPE tissue sections using the QIAamp DNA FFPE tissue kit (Qiagen GmbH) according to the manufacturer's instructions and stored at −20°C until required. HPV DNA analyses including genotyping were performed using the LCD-Array HPV 3.5 kit (CHIPRON GmbH) according to the manufacturer's instructions. Briefly, PCR was performed using the Primer Mix A (My 11/09) and B ("125") provided with the LCD-Array HPV 3.5 kit and the HotStarTaq Plus Master Mix (Qiagen GmbH). Hybridization mix including 5 μL of each amplified PCR product A and B were added to each field of the LCD-Array. After staining and washing, the hybridization spots were scanned and analyzed 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.

RNA extraction

Total RNA was extracted from 5-μm FFPE tissue sections (about 400 mm^2 tissue area per sample) centrally at the DKTK partner site Dresden using the fully automated Tissue Preparation System (TPS; Siemens Healthcare Diagnostics; for *in vivo* diagnostic use) according to the manufacturer's instructions. RNA quantity and purity were estimated using the Qubit fluorometer (Life Technologies GmbH), and 25 samples were omitted due to limited tissue material or poor RNA quality. Control RNA (positive control) and sections of tissue-free paraffin blocks (negative control) were included in each RNA extraction set. Their RNA was analyzed in parallel for quality control purposes. For RNA extractions and subsequent analyses by real-time PCR (RT-PCR) and nanoString technology, FFPE samples from all partner sites were randomized. The technical assistants and scientists involved in processing and analyses of the biomaterial were blinded to the clinical data.

nanoString analyses

Gene expression analyses were performed using a custom designed code set and nanoString Elements reagents (nanoString Technologies) including the genes of two hypoxia gene signatures (Supplementary Table S1B) as well as the potential stem cell markers *MET*, *SLC3A2*, and *CD44*. As the *CD44* probe design was found to be incorrect, this marker had to be omitted from the analysis. *CD44* expression was therefore analyzed by RT-PCR (see: cDNA generation and RT-PCR). Total RNA as well as reporter and capture probes specific to the genes of interest were mixed and incubated at 62°C for 22 hours. Samples were then kept at 4°C for a maximum of 18 hours and subjected to the nCounter system and processed as described elsewhere (27). Raw counts were logarithmized and then normalized to the mean of the internal level of reference genes *ACTR3*, *NDFIP1*, *RPL37A*, *B2M*, *GNB2L1*, *RPL11*, *POLR2A* or to the reference genes of the corresponding hypoxia gene signatures (Supplementary Table S1B), respectively (15, 16).

cDNA generation and RT-PCR

RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies). Because of low RNA yield, each cDNA was preamplified for genes of interest using the pooled TaqMan gene expression assays according to the manufacturer's protocol (10 cycles). TaqMan gene expression assays were used for both hypoxia classifier and reference genes (15, 16) as well as for *CD44*. RT-PCR was performed on an ABI StepOne Plus Real-Time PCR System (Life Technologies). For every patient, all RT-PCR reactions were performed on a single PCR plate. Gene expression values were logarithmized and then normalized to the mean of the reference genes *ACTR3*, *NDFIP1*, *RPL37A*, *B2M*, *GNB2L1*, *RPL11*, or the reference genes of each hypoxia gene classifier.

Immunohistochemical analysis of CD44

For immunohistochemical analysis, tissue microarrays (TMA) of primary HNSCC specimen were generated. They consisted of up to 3 cores (1-mm diameter each) per tumor representing the tumor heterogeneity. Cores of HNSCC of 195 patients were analyzable for CD44 protein expression. Immunohistochemical staining was performed as described previously (6), with minor modifications. After antigen retrieval (pH 6; Dako) for 35 minutes at 630 W, sections were incubated with the monoclonal mouse anti-human CD44 antibody (dilution 1:500; clone DF1485; Dako) for 30 minutes at room temperature. Negative control slides were incubated with corresponding mouse IgG1 antibody control (Dako). Blinded samples were evaluated semiquantitatively by two independent observers (A. Linge and C. von Neubeck) with an interobserver variability of <5%. CD44 staining intensity was scored (0, +, ++, +++) and tumors with a minimum of one positive core (+, ++, +++) were considered as positive.

Clinical endpoints and statistical analysis

The primary endpoint was locoregional tumor control (LRC). As secondary endpoints, freedom from distant metastases (DM) and overall survival (OS) were evaluated. All endpoints were calculated from the first day of radiotherapy to the date of event or censoring.

The corresponding survival curves were estimated by the Kaplan–Meier method. The impact of potential prognostic variables on the endpoints was evaluated using the Cox regression model in which the respective gene expressions were included as binarized parameters. Parameters found to be significant in univariate analysis were included in a multivariate Cox model. Statistical analyses were performed for all patients and for the subgroup of patients with HPV16 DNA–negative tumors. In the subgroup of patients with HPV16 DNA–positive tumors only two recurrences occurred. Therefore, it was not possible to detect any significant differences in LRC for this subgroup. To obtain the optimal cutoffs for the binarization of the CSC marker expression, for every CSC marker, each possible cutoff leading to a different patient stratification was considered. For each of these cutoffs, univariate Cox regressions with the binarized CSC marker were performed for the subgroup of HPV16 DNA–negative tumors using 10.000 bootstrap samples of the patient cohort stratified for tumor localization. Afterwards, the fraction of significant results (power) was calculated for each cutoff, leading to the optimal cutoff, which has the largest power. For the CSC markers *CD44*, *MET*, and *SLC3A2* this results in the cut-off values 0.2 (power 73%), −4.135 (power 77%), and −3.135 (power 66%), respectively. For stratification with respect to hypoxia-induced gene expression, tumors were assigned to a less hypoxic or a more hypoxic class, according to low or high expression levels of the corresponding hypoxia gene signature, using two-class *k*-means clustering based on the Euclidian distance. To compare the survival curves of patients stratified by HPV16 DNA status, CSC marker expression and hypoxia status, log-rank tests were employed. To assess correlations between continuous variables the Pearson correlation coefficient was used. Between binary parameters the mean square contingency coefficient (phi coefficient) was employed. For all analyses, two-sided tests were performed and P values <0.05 were considered statistically significant. All analyses were performed by the SPSS 21 software (IBM Corporation) except for the bootstrapping procedure, which was performed by STATA 11 (StataCorp LP).

Results

Hypoxia gene expression

In this study, tumor hypoxia was assessed using the 15-gene signature developed by Toustrup and colleagues (15) and the 26-gene signature by Eustace and colleagues (16) in parallel. Using nanoString analyses, univariate analysis showed that hypoxic tumors are significantly associated with a high risk of locoregional tumor recurrence after PORT-C using the 15-gene signature (HR, 3.55; $P = 0.010$) and the 26-gene signature (HR, 9.37; $P = 0.002$; Fig. 1; Supplementary Table S2). The 26-gene signature was also significant for the secondary endpoints DM (HR, 2.15; $P = 0.046$) and OS (HR, 2.48; $P = 0.002$). For the subgroup of patients with HPV16 DNA –negative tumors, the effect of hypoxia was also significant for both locoregional control and overall survival (15-gene signature: LRC, HR 4.66, $P = 0.005$; OS, HR 1.88, $P = 0.031$; 26-gene signature: LRC, HR 11.3, $P = 0.017$; OS, HR 2.05, $P = 0.040$; Fig. 1; Supplementary Table S2), whereas no significance has been revealed for patients with HPV-positive tumors (not shown).

The results of the multivariate analyses of the total patient population are summarized in Supplementary Table S3. The multivariate analyses also included extracapsular extension (ECE) status (significant for secondary endpoints) and tumor localization, because these variables were significant prognostic factors in our previous analysis (6). Both hypoxia gene classifiers revealed a significant association of tumors classified as "hypoxic" with poor locoregional control (15-gene classifier: HR 3.73, $P = 0.008$; 26-gene classifier: HR 6.00, $P = 0.017$; Supplementary Table S3).

In patients with HPV16 DNA–negative tumors, the effect of hypoxia on locoregional control was apparent in multivariate analysis (15-gene classifier: HR 4.54, $P = 0.006$; 26-gene classifier: HR 10.27, $P = 0.024$; Table 1). Comparing the two hypoxia classifiers, the 26-gene signature was stronger correlated with HPV status ($R = -0.271$, $P < 0.001$) and therefore may have less power regarding patient stratification compared with the 15-gene signature ($R = 0.007$, $P = 0.92$). As nanoString analysis is a relatively new technique for analyzing gene expression levels, hypoxia gene expression levels of both classifiers were validated by RT-PCR and revealed similar results (not shown). The results of both techniques were highly correlated as expected (Supplementary Table S4).

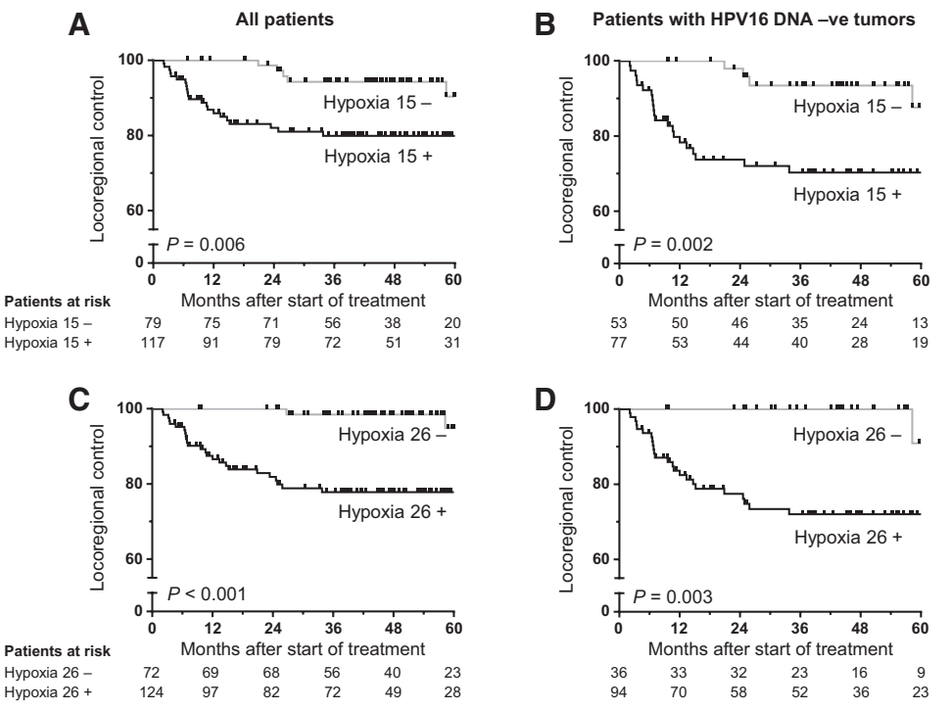

Figure 1.
(A–D) Kaplan-Meier estimates of locoregional tumor control of (A and C) all patients or (B and D) patients with HPV16 DNA–negative HNSCC. Patients with tumors expressing low hypoxia gene levels showed significantly better locoregional tumor control rates compared to patients with highly hypoxic tumors.

CSC marker expression

The potential CSC markers *MET* and *SLC3A2* were included in the nanoString gene panel. *CD44* was analyzed using RT-PCR and its protein expression was assessed by IHC. In univariate analysis, *MET*, *SLC3A2*, *CD44*, and CD44 protein were significantly associated with the primary endpoint locoregional tumor control (*MET*: HR 5.19, $P = 0.001$; *SLC3A2*: HR 6.54, $P = 0.002$; *CD44* (RT-PCR): HR 3.56, $P = 0.01$; CD44 (IHC): HR 9.09, $P = 0.03$; Fig. 2, Supplementary Table S5). In contrast to *CD44* and CD44 protein, *MET* and *SLC3A2* were also significantly associated with increased distant metastases and decreased overall survival (Supplementary Table S5). Similar effects for locoregional control were observed in the subgroup of HPV16 DNA–negative tumors for *MET*, *SLC3A2*, *CD44*, and CD44 protein (Supplementary Table S5; Fig. 2). As the CSC markers are correlated (Supplementary Table S6A), four Cox models, each including one CSC marker, were performed for multivariate analyses in the total patient cohort (Supplementary Table S6B). In this multivariate analysis, *MET* and *SLC3A2* showed a strong association with locoregional control (*MET*: HR 3.61, $P = 0.011$; *SLC3A2*: HR 3.61, $P = 0.045$) and with distant metastases (*MET*: HR 2.78, $P = 0.01$; *SLC3A2*: HR 3.75, $P = 0.006$). Both *CD44* and CD44 protein did not reveal a significant association with any of the three endpoints in the total patient cohort. However, in patients with HPV16 DNA–negative tumors, *MET*, *SLC3A2*, *CD44*, and CD44 protein showed an association with the primary endpoint locoregional tumor control (*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; Table 2).

In addition, patients were stratified by the smoking status during therapy. Except for the potential CSC marker *MET* ($R = 0.21$, $P = 0.004$) there were no significant correlations between smoking and CSC marker expression or hypoxia. As in univariate analysis smoking had no impact on the clinical endpoints, this factor was not considered for multivariate analyses.

Table 1. Multivariate analyses of hypoxia gene signatures and additional prognostic factors for patients with HPV16 DNA–negative tumors only

	Locoregional control		Distant metastases		Overall survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
15-gene signature	4.54 (1.56–13.2)	**0.006**	1.42 (0.67–3.01)	0.356	1.80 (1.01–3.21)	**0.047**
ECE	1.18 (0.52–2.69)	0.694	2.77 (1.22–6.28)	**0.015**	1.81 (1.02–3.20)	**0.042**
Oral cavity[a]	1.81 (0.76–4.27)	0.178	1.63 (0.71–3.74)	0.251	1.54 (0.86–2.75)	0.144
Hypopharynx[a]	0.69 (0.19–2.54)	0.581	2.05 (0.78–5.38)	0.146	0.63 (0.26–1.55)	0.317
26-gene signature	10.27 (1.36–77.2)	**0.024**	1.53 (0.63–3.69)	0.347	1.84 (0.90–3.77)	0.095
ECE	1.39 (0.62–3.15)	0.428	2.83 (1.25–6.39)	**0.013**	1.93 (1.09–3.42)	**0.025**
Oral cavity[a]	1.51 (0.65–3.52)	0.337	1.59 (0.69–3.66)	0.278	1.46 (0.81–2.62)	0.209
Hypopharynx[a]	0.95 (0.26–3.49)	0.944	2.17 (0.82–5.75)	0.118	0.71 (0.29–1.76)	0.462

NOTE: *P* values <0.05 were considered statistically significant.
Abbreviation: ECE, extracapsular extension.
[a]Baseline oropharynx.

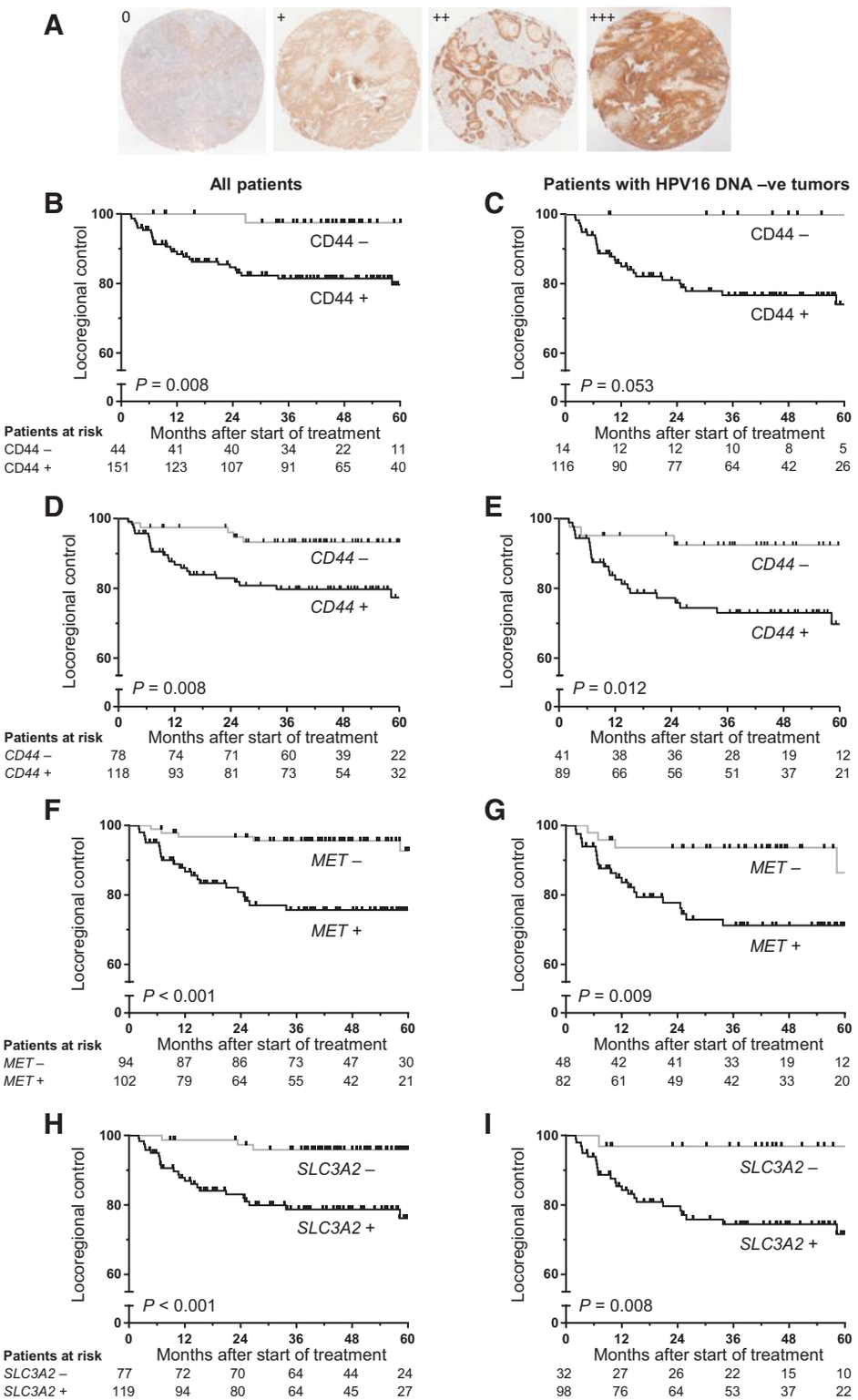

Figure 2.

A, immunohistochemical staining of CD44 showing different staining intensities. All staining intensities (+, ++, +++) were considered as positive staining. B–I, Kaplan–Meier estimates of locoregional tumor control of CD44 protein (assessed by IHC) and *CD44*, *MET*, and *SLC3A2* gene expression of (B, D, F, H) all patients or (C, E, G, I) patients with HPV16 DNA–negative HNSCC. Patients with little or no CSC marker expression showed better locoregional tumor control compared with patients with stronger CSC marker expression.

Table 2. Multivariate analyses of CSC markers and additional prognostic factors for patients with HPV16 DNA–negative tumors only

	Locoregional control		Distant metastases		Overall survival	
	HR (95% CI)	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
MET	3.71 (1.28–10.8)	**0.016**	2.83 (1.16–6.92)	**0.022**	2.52 (1.32–4.81)	**0.005**
ECE	1.30 (0.58–2.94)	0.524	2.83 (1.25–6.42)	**0.013**	1.96 (1.10–3.49)	**0.022**
Oral cavity[a]	1.81 (0.78–4.20)	0.169	1.65 (0.73–3.77)	0.232	1.52 (0.86–2.71)	0.152
Hypopharynx[a]	0.70 (0.19–2.57)	0.594	2.06 (0.78–5.41)	0.144	0.61 (0.25–1.51)	0.285
SLC3A2	8.54 (1.14–63.9)	**0.037**	4.61 (1.35–15.8)	**0.015**	1.44 (0.72–2.88)	0.297
ECE	1.43 (0.64–3.23)	0.386	3.23 (1.41–7.39)	**0.005**	1.93 (1.08–3.42)	**0.025**
Oral cavity[a]	1.60 (0.69–3.74)	0.274	1.43 (0.63–3.27)	0.397	1.55 (0.87–2.78)	0.141
Hypopharynx[a]	0.86 (0.24–3.17)	0.823	2.66 (1.00–7.10)	0.051	0.69 (0.28–1.70)	0.419
CD44	3.36 (0.98–11.5)	0.054	1.62 (0.65–4.02)	0.300	1.11 (0.58–2.11)	0.757
ECE	1.41 (0.64–3.10)	0.398	2.84 (1.25–6.48)	**0.013**	1.87 (1.05–3.31)	**0.033**
Oral cavity[a]	1.71 (0.74–3.94)	0.211	1.57 (0.68–3.64)	0.295	1.57 (0.86–2.86)	0.140
Hypopharynx[a]	0.81 (0.22–2.97)	0.751	1.79 (0.64–5.02)	0.267	0.52 (0.20–1.36)	0.181
CD44	—	**	2.43 (0.57–10.4)	0.231	1.30 (0.51–3.31)	0.584
ECE	—	—	3.09 (1.40–6.82)	**0.005**	1.86 (1.07–3.24)	**0.029**
Oral cavity[a]	—	—	1.63 (0.72–3.70)	0.246	1.55 (0.87–2.76)	0.136
Hypopharynx[a]	—	—	2.84 (1.14–7.04)	**0.025**	0.65 (0.28–1.51)	0.315

NOTE: *P* values <0.05 were considered statistically significant.

Abbreviation: ECE, extracapsular extension.

[a]Baseline oropharynx.

**, as there were no events in the CD44 protein negative group, the Cox model did not converge.

Association of tumor hypoxia and cancer stem cells

To assess whether the hypoxic microenvironment is contributing to the radio(chemo)resistance associated with CSC marker expression, the correlation between hypoxia-induced gene expression and CSCs was analyzed. Interestingly, hypoxic tumors showed a strong positive association with CSC marker expression, whereas tumors with low hypoxia showed much more heterogeneous CSC marker expression in the total patient cohort (Table 3). Similar correlation coefficients between hypoxia and CSC marker expression were also obtained within the subgroups of patients with HPV16 DNA–positive or negative tumors (not shown).

The 15-gene but not the 26-gene signature was able to further stratify patients with HPV16 DNA–negative/high CSC marker–expressing tumors into a group with significantly lower locoregional tumor control rate and a more favorable group. In univariate analysis, this was true for the CSC markers *SLC3A2* ($P = 0.03$, Fig. 3A) and *MET* ($P = 0.046$, Fig. 3B), but not for *CD44* or CD44 protein. Multivariate analyses including HPV16 DNA, hypoxia, and CSC markers showed a significant independent correlation of *MET* and the 15-gene signature with locoregional tumor control, while the other CSC markers *CD44*, CD44 protein and *SLC3A2* were not significant (Supplementary Table S7). The Cox model including hypoxia and *MET* fitted the data significantly better than the model with hypoxia alone ($P = 0.026$, likelihood-ratio test of nested models). Combining patients with HPV-negative tumors, which simultaneously are hypoxic and have a high CSC marker expression, gives a patient subgroup with the highest risk of recurrence (Fig. 3C and D).

Association of tumor hypoxia and CSCs with HPV16 DNA status

To assess the impact of the HPV16 DNA status on tumor hypoxia and CSC marker expression, their correlation was analyzed. A significant correlation of the HPV16 DNA status with tumor hypoxia was found for the 26-gene signature ($R = -0.271$, $P < 0.001$) but not for the 15-gene signature ($R = 0.007$, $P = 0.918$; Supplementary Table S8A). Specifically, in the group of HPV16 DNA–negative HNSCC, the majority of the tumors were found to be hypoxic, whereas for HPV16 DNA–positive tumors similar numbers of more hypoxic and less hypoxic tumors were seen.

The expression of the potential CSC markers CD44, *SLC3A2*, and *MET* was also significantly correlated with HPV16 DNA status (CD44: $R = -0.399$, $P < 0.001$; *MET*: $R = -0.319$, $P < 0.001$; *SLC3A2*: $R = -0.430$, $P < 0.001$; Supplementary Table S8B).

Discussion

Previously, we and others have shown that the HPV infection status is a strong prognosticator for locoregional control in patients with locally advanced HNSCC who received postoperative radio(chemo)therapy (6, 28, 29) with an improved locoregional control and radiosensitivity of HPV16 DNA positive in comparison with HPV16 DNA–negative tumors. In our previous multicenter evaluation, we found virtually no recurrences in the HPV16 DNA–positive group among high-risk HNSCC patients who received PORT-C (6). Patients in this highly advantageous group may therefore be candidates for potential radiotherapy de-escalation trials. However, for further stratification of patients with HPV-negative HNSCC, additional biomarkers are required to predict locoregional control to identify additional patients who could be assigned to the very good prognosis group and to define a patient group with very unfavorable outcome that could be a candidate for treatment intensification strategies.

The oxygenation status of the tumors is one of the known biomarkers for outcome of primary radiotherapy in HNSCC,

Table 3. Correlation of CSC marker expression and tumor hypoxia for all patients

	15-gene signature		26-gene signature	
	Hypoxia		Hypoxia	
	Low	High	Low	High
CD44				
Low	21	16	23	14
High	48	96	42	102
	$R = 0.194$	$P = 0.009$	$R = 0.277$	$P < 0.001$
CD44				
Low	39	37	49	27
High	38	74	19	93
	$R = 0.174$	$P = 0.017$	$R = 0.485$	$P < 0.001$
MET				
Low	52	42	59	35
High	27	75	13	89
	$R = 0.294$	$P < 0.001$	$R = 0.518$	$P < 0.001$
SLC3A2				
Low	44	33	55	22
High	35	84	17	102
	$R = 0.276$	$P < 0.001$	$R = 0.579$	$P < 0.001$

that is, of macroscopic tumors, which have not undergone surgical removal. Here, we aimed to determine the relevance of the hypoxia status using the hypoxia gene signatures by Toustrup and colleagues (15) and Eustace and colleagues (16) in the tumor specimen obtained by surgical resection before PORT-C. We found that the pretreatment hypoxia status was prognostic in patients with PORT-C. Patients with high hypoxia levels in the surgical specimen obtained prior to PORT-C had a poor locoregional tumor control compared with patients with low hypoxic tumors. This effect mainly attributes to the subgroup of HPV-negative HNSCC. Taking into account that hypoxia was evaluated within the surgical specimen and radiochemotherapy was applied to the potential residual tumor cells after surgery, the finding of a significant association of hypoxia within the resected primary tumor with outcome of PORT-C at a first glance is surprising. As it is unlikely that the low number of residual tumor cells after surgery also differs in hypoxia, this finding suggests that hypoxia impacts outcome of radiotherapy not only by a direct biochemical or radiobiologic effect on cellular radioresistance of the tumor but also by other radiobiologic mechanisms. This would be in line with previous experimental data from our laboratory, showing that pretreatment hypoxia impacts local tumor control after radiotherapy also when radiotherapy was applied under homogeneous anoxic conditions (20). As recent studies suggested that tumor hypoxia also favors stemness and invasive growth as an external factor (reviewed in refs. 30–33), we explored the role of potential CSC markers in our cohort. CSCs are known to play a major role in radioresistance (reviewed in ref. 34), and a highly hypoxic tumor microenvironment contributes to an increased clonogenic potential (20, 35).

CD44 is a widely explored CSC marker in HNSCC (30, 36, 37). A study by de Jong and colleagues showed that *CD44* and CD44 protein levels significantly predict local recurrence after radiotherapy in patients with early-stage laryngeal cancers (38). Here, we showed that patients with surgically resected tumors with no detectable CD44 protein expression have increased locoregional tumor control rates compared with those with CD44 protein–expressing tumors.

Recently, CD98 has been established experimentally as a putative CSC marker (39). In a study on 711 patients with oropharyngeal squamous cell carcinoma, HPV-positive tumors have been shown to express less CSC markers such as CD44 and CD98, whereas CD44 and CD98 positivity was associated

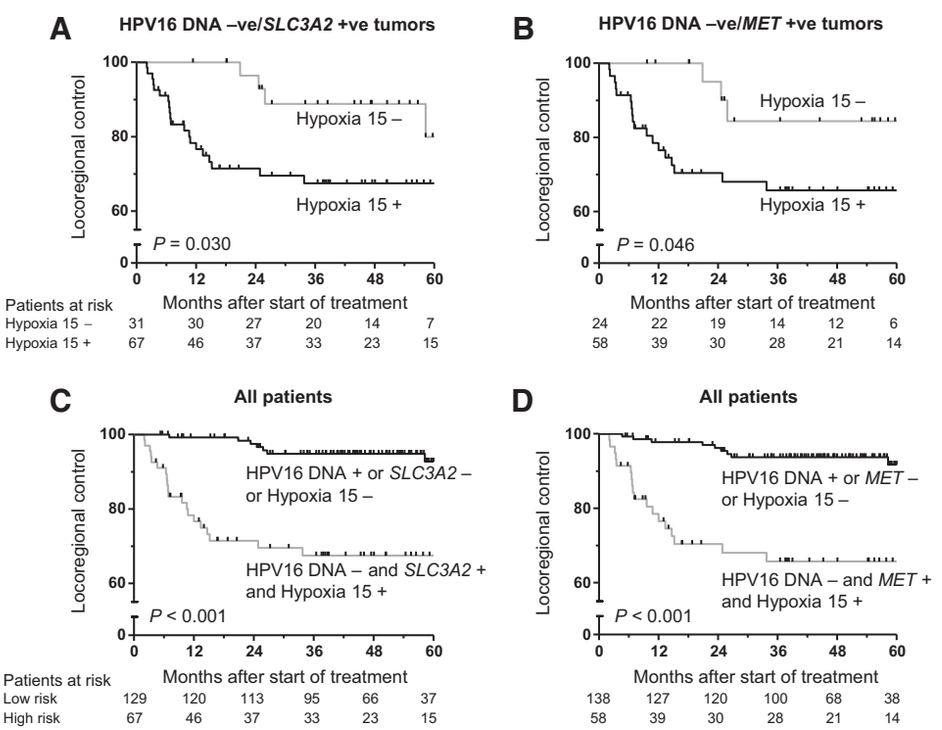

Figure 3.
Kaplan–Meier estimates of locoregional tumor control of patients with (A and B) HPV16 DNA–negative HNSCC and high expression levels of *SLC3A2* (A) or *MET* (B). Patients with low hypoxic tumors have better locoregional control rates compared with patients with highly hypoxic tumors. C and D, patients with high risk of tumor recurrence (HPV16 DNA–negative, CSC marker positive, high hypoxia) show significantly poor locoregional control rates than the patients with HPV16 DNA positive, low CSC marker expressing and low hypoxic tumors.

with significantly lower progression-free and OS (40). In our patient cohort, we found that patients with high expression of *SLC3A2*, which is encoding for one of the CD98 heterodimers, show a poor locoregional tumor control and an increased risk for distant metastases also after PORT-C.

The MET pathway has been shown to promote self-renewal and tumorigenicity in HNSCC stem-like cells (41) and pharmacologic selective inhibition of MET has been shown to lead to elimination of CSCs (42). Furthermore, MET was found to be associated with poor prognosis in patients with locally advanced p16-negative HNSCC who were treated with primary radiochemotherapy (43). In the current study, we have shown that *MET* overexpressing HPV16 DNA–negative HNSCC are associated with poor locoregional tumor control and increased distant metastases after PORT-C. Interestingly, Pennacchietti and colleagues showed that hypoxia activates the transcription of the MET proto-oncogene, that MET overexpression is associated with hypoxic areas of tumors and that MET inhibition prevents hypoxia-induced cell growth (44). Our data, showing a positive correlation of *MET* with tumor hypoxia, support this association of both factors. Still, the relatively low number of low hypoxic tumors among the HPV16 DNA–negative/*MET* positive group showed a significantly higher locoregional control rate compared with the highly hypoxic *MET*–positive tumors. In addition, our data indicate that, in patients with HPV16 DNA–negative tumors, *SLC3A2* and *MET* may be suitable markers to determine a group of patients who have a very poor prognosis and therefore may profit from treatment intensification.

While the hypoxia profiles used in this study were prognostic for locoregional tumor control, they were not indicative for an increased risk of distant metastases (Table 1). This appears counterintuitive in light of experimental and clinical data showing that hypoxia may drive distant metastases (31, 45–47). However, to our best knowledge, this so far has not been investigated in HNSCC in the postoperative setting. Some of the genes in the hypoxia profiles utilized in our study might also be associated with other biologic phenomenons, including stemness of cancer cells. The coefficients of correlation between the different CSC markers and hypoxia profiles (Table 3) indicate that, although a significant correlation exists, both parameters to a large extent are expressed independent of each other. In contrast to the hypoxia profiles, CSC marker expression correlated in our study not only with locoregional control but also with distant metastases. Expression of CSC markers has been suggested as a potential surrogate of CSC density, that is, of the number of cells at risk to metastasize per given volume of tumor tissue (30, 34, 36, 46, 48, 49). Further mechanistic investigations into the relationship of hypoxia, stemness, and metastatic risk in HNSCC, in correlation with clinical data, appears therefore to be an interesting avenue for better stratification of patients for systemic therapies and for discovery of novel targets. An important particularity of our study is that biomarkers have been investigated in a cohort of patients treated postoperatively by radiochemotherapy, with overall higher locoregional control rates compared with studies evaluating primary radiochemotherapy in HPV-negative or -positive HNSCC (50). Currently, it is unknown whether the predictive potential of biomarkers differs for the different clinical risk groups receiving postoperative or primary radiochemotherapy. To approach this question, the latter risk group of patients is currently evaluated using the same biomarkers studied here by the DKTK-ROG.

Taken together, this is the first systematic multicentric analysis showing an association of high levels of tumor hypoxia and CSC marker expression within the surgically removed primary tumor with impaired locoregional tumor control after PORT-C in HNSCC. In HPV16 DNA–negative HNSCC, negativity for these parameters may help to identify a subgroup of patients with locoregional control rates as high as observed in HPV16 DNA–positive tumors. In addition, hypoxia and CSC marker positive tumors seem to constitute a subgroup of patients that is undertreated with current standard PORT-C. After validation in a currently ongoing prospective study, these parameters may help to further stratify patients for individualized treatment de-escalation or intensification strategies.

Disclosure of Potential Conflicts of Interest

I. Tinhofer reports receiving commercial research grants from Merck Serono and Pfizer and is a consultant/advisory board member for Merck Serono. C. Belka reports receiving a commercial research grant and speakers bureau honoraria from Merck Darmstadt. J. Alsner and J. Overgaard are listed as a co-inventors on a provisional patent application on a method for determining clinically relevant hypoxia in cancer that is owned by Aarhus University, Aarhus, Denmark, and the part concerning prediction of benefit from Nimorazole is licensed to Azanta Denmark A/S, Hellerup, Denmark. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors gratefully acknowledge the excellent technical assistance by Mrs. Sigrid Balschukat and Mrs. Liane Stolz-Kieslich. The authors also thank all pathologists, head and neck surgeons, and maxillofacial surgeons at the eight treatment centers who provided tumor material and data for this study.

Grant Support

The study was financed by a Joint Funding Grant within the German Cancer Consortium (DKTK) awarded to the DKTK-ROG (principle

investigator: Michael Baumann). The DKTK is funded as one of the National German Health Centers by the Federal German Ministry of Education and Research.

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Received August 24, 2015; revised November 18, 2015; accepted December 11, 2015; published OnlineFirst January 11, 2016.

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