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ORIGINAL ARTICLE

WILEY

HIF-1a expression and differential effects on survival in patients with oral cavity, larynx, and oropharynx squamous cell carcinomas

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Abstract

Background: Hypoxia is a negative prognostic factor in head and neck squamous cell carcinomas. Under hypoxia, the hypoxia-inducible factor (HIF)-1a transcription factor is overexpressed. We investigated whether there were site differences in HIF-1a expression and its effect on patient outcomes per subsite.

Design/Method: A total of 941 patients with HNSCC in the squamous cell carcinoma of the oropharynx (OPSCC, n = 302), oral cavity (OSCC, n = 391), or larynx (LSCC, n = 248) were included. Expression of HIF-1a in tissue samples was investigated using immunohistochemistry. Overall survival (OS), disease-free survival (DFS), and locoregional control (LRC) were analyzed.

Results: HIF-1a expression was higher in OSCC than in LSCC and OPSCC. High HIF-1a expression led to worse prognosis in OPSCC (OS $P = .029$, DFS $P = .085$) and LSCC (OS $P = .041$, DFS $P = .011$) and better in OSCC (OS $P = .055$, DFS $P = .012$). There was no association between HIF-1a and LRC.

Conclusions: High HIF-1a expression is related to poor outcome in OPSCC and LSCC and better outcome in OSCC.

KEYWORDS

head and neck neoplasms, hypoxia, oropharyngeal neoplasms, survival, tumor microenvironment

1 | INTRODUCTION

Hypoxia is a state of a reduced tissue oxygen tension and is often observed within the tumor microenvironment. Processes in a tumor that cause hypoxic cells to proliferate in these circumstances are also the basis for a more aggressive phenotype and a higher likelihood of

metastasis.^{1,2} The hypoxia-inducible factor 1 (HIF-1) pathway has an important role in cellular survival under hypoxia. In head and neck squamous cell carcinoma (HNSCC), high expression of HIF-1alpha (HIF-1a) has been shown to lead to worse prognosis.³

HNSCC comprises carcinomas arising in mucosal lining of the upper aero-digestive tract. Biologically, HNSCCs were considered a homogenous group of tumors arising in different anatomic regions, or sites, within the

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upper aero-digestive tract. Pathogenesis of HNSCC is generally attributed to accumulation of mutations in oncogenes and tumor-suppressor genes because of DNA damage that can be caused by smoking and drinking.⁴ However, HPV-related oropharyngeal tumors have been identified as a distinct subgroup within HNSCC. In these tumors, HPV onco-proteins (especially E6 and E7) cause changes in signaling pathways that lead to carcinogenesis, rather than accumulation of mutations. Clinically, HPV-positive oropharyngeal tumors show better response to radiotherapy treatment in contrast to HPV-negative oropharyngeal tumors.⁵ Interestingly, survival benefits for HPV-related tumors were not identified in non-oropharyngeal sites.^{6,7} These findings highlight the genetic and biological heterogeneity of HNSCC.

These biological differences between HNSCCs of different sites warrant further investigation into HIF-1 α expression and its effect on clinical outcome. The aim of the present study was to compare both the amount of HIF-1 α expression and the effect of HIF-1 α over-expression on clinical outcome between sites. We studied the effect of HIF-1 α expression in four well-documented patient cohorts, all treated in our center, that encompass the three major sites of HNSCC: the oral cavity, oropharynx, and larynx. Because all patient tissues were stained using an identical staining protocol, we were able to compare the amount and effect of HIF-1 α expression across different sites.

2 | MATERIALS AND METHODS

2.1 | Patient cohort

Patients diagnosed with a first primary oral, oropharyngeal, or laryngeal carcinoma and treated with curative intent at the University Medical Center Utrecht in the Netherlands were included in this retrospective study. All data and tissue samples were handled according to the General Data Protection Regulation (GDPR).

2.1.1 | Oral cancer cohorts

Two previously described patient cohorts were analyzed in the present study.^{8,9} All 391 patients had been diagnosed with an OSCC between 1996 and 2010. OSCC patients treated with primary (chemo)radiotherapy (CRT) were not included in this study, because generally these patients had (functionally) irresectable disease, or either did not wish or were not fit to undergo extensive surgery.

2.1.2 | Oropharyngeal cancer cohort

The oropharyngeal cancer (OPSCC) cohort has also been previously described.¹⁰ A total of 274 patients were included who were diagnosed with an OPSCC between 1997 and 2011 and had tumor tissue available in the pathology archives for tissue microarray (TMA) construction. In addition, 28 patients with an OPSCC included in the study by Van Hooff et al were also included.⁸

2.1.3 | Laryngeal cancer cohort

The laryngeal cancer (LSCC) cohort consisted of all consecutive patients diagnosed with a laryngeal squamous cell carcinoma between January 2007 and June 2014. Exclusion criteria were (a) no tissue available at the University Medical Center Utrecht, (b) insufficient material for TMA construction, and (c) receiving systemic therapy for another, non HNSCC, malignancy within less than 5 years before diagnosis. Given that this was a retrospective cohort study, there were no means to collect additional tissue in case of insufficient material. A total of 248 patients were included.

2.1.4 | Treatment strategy

For all patients, a treatment proposition was offered based on the clinical TNM-staging (seventh edition), the most recent guidelines during the time of treatment and after discussion in the weekly head and neck oncology multidisciplinary team (MDT) meeting. Adjuvant treatment was advised based on the criteria in Appendix S1. In our center, this team consists of at least an otolaryngologist, maxillofacial surgeon, radiation oncologist, medical oncologist, radiologist, nuclear physician, and pathologist, all specialized in treatment of HNSCC. The final treatment was ultimately decided by the patient in concurrence with his or her treating physicians.

2.2 | Definitions

HPV-status was determined using the algorithm described by Smeets et al:¹¹ a p16 staining was performed, followed by an HPV-PCR if positive. HPV-positivity outside the oropharyngeal site was rare and was only deemed clinically relevant in patients with oropharyngeal cancer. HPV-status was therefore categorized as “positive,” “negative,” or “non-oropharyngeal site”.^{8,12} Patients treated with radiotherapy and cisplatin or carboplatin, as well as patients treated with radiotherapy

and cetuximab, were analyzed as a single treatment group (chemo-radiotherapy). All tumors were staged according to the UICC TNM seventh edition.

2.3 | Immunohistochemistry

All tissues were leftover materials following routine clinical care. All clinical patient data were analyzed anonymously. Therefore, obtaining informed consent or ethical board approval was not necessary according to the Dutch best practice guidelines at the time of the study (www.federa.org).

Immunohistochemistry was performed on TMAs, which is widely accepted to investigate protein expression in large cohorts.^{13,14} In brief, hematoxylin and eosin-stained sections from resection specimens or biopsies from primary tumors were reviewed by a dedicated head and neck pathologist (SMW), who marked areas of tumor tissue. From the formalin-fixed and paraffin-embedded (FFPE) tissue blocks, three representative 0.6 mm cylinders were transferred to a recipient block using the TMA-Grandmaster (3D Histech, Budapest, Hungary). In general, the tissues were taken from resection specimens for OSCC. As LSCC and OPSCC patients were mostly treated with radiotherapy, tissues were mostly derived from biopsies.

Staining was performed by deparaffination and rehydration, followed by blocking of endogenous peroxidase activity using a buffer consisting of 3% H₂O₂ solution in PBS. Antigen retrieval was performed by boiling the slides in a pH 9.0 EDTA buffer for 20 minutes. The Novolink kit (Leica Biosystems, Rijswijk, the Netherlands) was used according to the manufacturer's instructions for staining. Incubation with the primary antibody (Mouse-anti-HIF-1a, BD biosciences, cat# 610959, lot 4 073 775, diluted 1:50 in PBS-BSA) was performed overnight at four degrees. During every staining procedure, the same positive control tissue sample (renal cell carcinoma) was stained and the staining pattern was compared to ensure similarity of the staining. The same tissue was used as a negative control, by incubation with PBS-BSA instead of the primary antibody.

Scoring was performed visually by an experienced head and neck pathologist (SMW) and ENT-resident (JES) in consensus, blinded to the patient IDs and clinical outcome data. Only nuclear staining of HIF-1a was regarded as biologically relevant and was scored as a percentage expression of tumor cells. A score of 15% or more was considered positive in accordance with our previous study.¹⁵ Cores were regarded as missing if more than half of the core was lost during processing or if a core contained less than 25% tumor tissue.

2.4 | Sub-analysis of HIF-1a expression in biopsies vs resection specimens

A subgroup of 180 OSCC patients had tissue from both the tumor center and the tumor periphery available for analysis (paired samples). These tissues were used to assess whether an observed difference between OSCC and LSCC or OPSCC is caused by a different tissue origin (biopsy vs resection specimen) or due to an actual difference between sites. HIF-1a staining was performed in tumor center and tumor periphery tissue. These samples were tested for concordance using a McNemar test.

2.5 | Data analysis

All analyzes were performed using SPSS (IBM, version 25.0). For analysis of immunohistochemical staining, a mean was calculated of the available cores. Baseline data were compared using chi-square analysis for categorical data, student's *t*-tests for normally distributed data (assessed using Kolmogorov-Smirnov test), and the independent-samples median test for non-normally distributed data. Analysis of variables related to HIF-1a positivity was investigated using logistic regression.

2.6 | Missing data handling

Missing values for all investigated variables were handled using multiple imputation with 15 datasets. This method of handling missing data has shown increased accuracy compared to performing only a complete case analysis.^{16,17} Please find Table S1 for an overview of missing data and further details on imputation.

2.7 | Survival analyzes

For survival analyzes, Kaplan-Meier analysis (including Log-rank tests), univariate and multivariate Cox-regression were used. Regression analyzes were performed in each of the 15 multiple imputed datasets. The presented results were pooled automatically in SPSS, unless stated otherwise. Variables that were significant on univariate Cox-regression were included in the multivariate analysis. HIF-1a expression was included in all multivariate analyzes based on clinical relevance. Multivariate analysis was performed per site. We analyzed effect modification by introducing interaction variables into the analyzes when appropriate. If the interaction variable had a significant relation to the outcome, this factor was considered an effect modifier and split

analyses were performed for each category of the effect modifier.

The balance between the degrees of freedom and the survivors and deceased was no lower than 10 patients per degree of freedom (in the OSCC cohort 391 patients with 45% deceased and thus a maximum of 17° of freedom, in OPSCC: 302 patients with 45% survivors and thus a maximum of 13° of freedom, in LSCC: 248 patients with 33% deceased and thus a maximum of 8° of freedom). A step-wise backward method was used to establish the Cox-regression model of the survival outcomes, by excluding variables with a *P*-value of more than 0.157 (Akaike's Information Criterion).¹⁸

The following outcomes were analyzed:

1. Overall survival (OS). An event was defined as death by any cause (time between date of first positive biopsy and date of death). Patients were censored if they were alive at the end of follow-up (time between date of first positive biopsy and date that the patient was last confirmed alive).
2. Locoregional control (LRC). An event was defined as the occurrence of local and/or regional disease after therapy (time between date of first positive biopsy and date of histological, radiological and/or clinically evident disease). Patients were censored if they did not have a locoregional recurrence at the last follow-up visit, or at the time of death.
3. Disease-free survival (DFS). An event was defined as the occurrence of local, regional, or distant disease after therapy (time between date of first positive biopsy and time of histological or clinically evident disease) or death (time between date of first positive biopsy and date of death). Patients were censored if they were alive and free of disease at the end of follow-up (time between date of first positive biopsy and last follow-up that included full ENT-examination)
4. Disease-specific survival data were not available for all patients and could not be analyzed. Instead we performed a separate OS analysis where the duration of follow-up was cut off at 60 months of follow-up. This cut-off point was chosen as we would expect that most disease-related death would occur in the first 5 years after disease onset. Later deaths may also be explained by second primary tumors or non-disease-related causes of death.

3 | RESULTS

3.1 | Clinical characteristics

A total of 941 patients were included in the study (Table 1): 248 patients with a LSCC, 391 patients with an

OSCC, and 302 patients with an OPSCC. Most patients were male and had a history of smoking and alcohol use. For LSCC and OSCC, most tumors were early stage (T1 or T2). In contrast, more than half of patients with an OPSCC were seen with an advanced stage primary tumor or with neck metastases. For the OPSCC cohort, HPV-status was positive in 15.9%. Most tumors were of moderate differentiation grade. After a median of 66 months of follow-up, approximately half of patients were alive.

3.2 | HIF-1a expression is correlated to site

An example of the HIF-1a staining can be found in Figure 1. HIF-1a expression was significantly different between sites ($P < .001$, independent samples median test). Expression of HIF-1a was significantly higher in patients with an oral cancer (median 39.9% positive cells), compared to oropharyngeal (median 23.9%) and laryngeal cancer (median 25.7%). Positivity for HIF-1a (more than 15% HIF-1a positive cells) was present in 290 (76.9%) OSCC patients, 146 (64.2%) LSCC patients, and 164 (57.1%) OPSCC patients. There was no correlation between HIF1a expression and either T-classification, N-classification, differentiation grade or HPV-status.

3.3 | HIF-1a expression is associated with poor OS in patients with laryngeal and oropharyngeal, but not in oral squamous cell carcinomas

Age, smoking, clinical T-classification and N-classification, site, HPV-status, differentiation grade, and choice of treatment were significantly related to OS (Table 2). When analyzed in the whole cohort, HIF-1a expression was not related to OS (Figure 2). However, there was significant effect modification between tumor site and HIF-1a expression: there was a significant difference in the effect of HIF-1a expression on OS between the different sites. HIF-1a expression was associated with worse OS in LSCC and OPSCC. In contrast, HIF-1a expression was associated with better OS in patients with OSCC, although this difference did not reach statistical significance. In a subgroup analysis of HPV-associated OPSCC, HIF-1a positivity was a significant predictor of worse survival with a hazard ratio (HR) of 5.47 (95% CI: 1.16-25.8).

Treatment strategy was a significant univariate predictor of OS in OSCC with a HR of 0.57 (95% CI 0.42-0.77, $P < .001$) for surgery only vs surgery with postoperative radiotherapy (PORT). It was also a significant predictor in OPSCC with a HR of 0.50 (95% CI 0.23-1.08, $P = .079$) for surgery vs CRT, a HR of 0.54 (95% CI 0.38-0.78, $P = .001$)

TABLE 1 Baseline patient characteristics according to site

Variable	Larynx n = 248	Oral Cavity n = 391	Oropharynx n = 302	Total n = 941
Sex				
Men	202 (81.5)	240 (61.4)	212 (70.2)	654 (69.5)
Women	46 (18.5)	151 (38.6)	90 (29.8)	287 (30.5)
Age	66.0 (9.7)	62.1 (11.8)	59.3 (9.0)	62.3 (10.7)
Smoking				
Active or quit	232 (94.3)	263 (67.6)	270 (90.0)	765 (81.8)
Never	14 (5.7)	126 (32.4)	30 (10.0)	170 (18.2)
Alcohol use				
Active or quit	188 (78.7)	204 (52.6)	257 (86.2)	649 (70.2)
Never	51 (21.3)	184 (47.4)	41 (13.8)	276 (29.8)
Clinical T-classification				
cT1 _{a/b}	108 (43.5)	129 (33.0)	36 (12.0)	273 (29.0)
cT2	63 (25.4)	173 (44.2)	89 (29.6)	325 (34.6)
cT3	49 (19.8)	19 (4.9)	68 (22.6)	136 (14.5)
cT4 _{a/b}	28 (11.3)	70 (17.9)	108 (35.9)	206 (21.9)
Clinical N-classification				
cN0	212 (85.5)	304 (77.7)	106 (35.3)	622 (66.2)
cN1	12 (4.8)	46 (11.8)	48 (16.0)	106 (11.3)
cN2 _{a/b/c}	24 (9.7)	41 (10.5)	132 (44.0)	197 (21.0)
cN3	0 (0)	0 (0)	14 (4.7)	14 (1.5)
TNM-stage				
Stage I	106 (42.7)	119 (30.4)	16 (5.4)	241 (25.7)
Stage II	53 (21.4)	140 (35.8)	36 (12.0)	229 (24.4)
Stage III	55 (17.7)	42 (10.7)	57 (19.1)	143 (15.2)
Stage IVA	45 (18.1)	90 (23.0)	158 (52.8)	293 (31.2)
Stage IVB	0 (0)	0 (0)	32 (10.7)	32 (3.4)
HPV-status				
Positive	NA	NA	46 (15.9)	NA
Differentiation grade				
Well	10 (9.4)	33 (8.6)	4 (1.7)	47 (6.5)
Moderate	76 (71.7)	298 (77.6)	162 (70.1)	536 (74.3)
Poor	20 (18.9)	53 (13.8)	65 (28.1)	138 (19.1)
Primary treatment				
Surgery	61 (24.6)	199 (51.0)	16 (5.3)	276 (29.4)
Surgery+PORT	40 (16.1)	191 (49.0)	96 (31.8)	327 (34.8)
Surgery+POCRT	3 (1.2)	0 (0)	5 (1.7)	8 (0.9)
Radiotherapy	137 (55.2)	0 (0)	74 (24.5)	211 (22.4)
Chemoradiation	7 (2.8)	0 (0)	111 (36.8)	118 (12.6)
HIF-1a expression				
Median % (IQR)	25.7 (46.6)	39.9 (42.5)	23.9 (45.0)	30.1 (45.0)
Positive (> 15%)	147 (64.2)	290 (76.9)	164 (57.1)	601 (67.3)
Negative (≤ 15%)	82 (35.8)	87 (23.1)	123 (42.9)	292 (32.7)
Survival				
Alive (%)	163 (65.7)	215 (55.0)	135 (44.7)	513 (54.5)
Median months of follow-up for surviving patients	60.0	79.0	66.0	66.0

Note: Categorical variables are shown as n (%), normally distributed data are shown as mean (SD), non-normally distributed, continuous data are shown as median (IQR). Some baseline characteristics were not available and were scored as missing, this is further shown in Table S1.

Abbreviations: IQR, interquartile range; NA, not applicable; POCRT, postoperative chemoradiotherapy; PORT, postoperative radiotherapy.

FIGURE 1 Example of HIF-1a staining. Examples of immunohistochemical staining for HIF-1a. A, Laryngeal cancer tissue microarray (TMA) core. One percent of tumor cells showed positive nuclear staining for HIF-1a. B, Oral squamous cell carcinoma TMA core with 10% HIF-1a positivity. C, Squamous cell carcinoma of the oropharynx (OPSCC) TMA core with 50% HIF-1a positivity. D, OPSCC TMA core with 90% HIF-1a positivity [Color figure can be viewed at wileyonlinelibrary.com]

for surgery with PORT vs CRT, HR of 0.89 (95% CI 0.28–2.82, $P = .84$) for surgery with postoperative CRT vs CRT, and HR of 0.58 (95% CI 0.39–0.89, $P = .011$) for primary RT vs CRT. There was no significant difference between treatment strategies in LSCC.

Because of the site difference, multivariate analyses were performed separately for each tumor site (Table 3). In LSCC, HIF-1a expression retained a significant association with reduced OS independent of age, T-classification and N-classification. In OPSCC, HIF-1a expression retained a significant association with reduced OS independent of age, T-classification and N-classification, HPV-status, and treatment modality. In OSCC, age, T-classification and N-classification, and differentiation grade were significantly associated with OS. Also in the multivariate analysis in OSCC, HIF-1a was associated

with better survival, but the difference did not reach statistical significance.

When OS time was cut off at 60 months the results were mostly in concurrence with the overall survival analysis (Table S2). However in this analysis, we found a statistically significant better survival in HIF-1a positive OSCC.

To further investigate the site differences, we investigated several variables for confounding or effect modification in the relation between site and survival. These variables were treatment strategy, smoking history, and alcohol history. None of these variables were significant effect modifiers or confounders in the relation between HIF-1a expression and survival. Site was the only effect modifier. Moreover, treatment strategy was not a significant effect modifier in the relation between HIF-1a expression and survival overall and within each site. This suggests that

TABLE 2 Univariate survival analyzes

Variable			Overall survival		Locoregional control		Disease-free survival	
			HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age	Per year increase	Overall	1.02 (1.02-1.03)	<.001	1.00 (0.99-1.02)	NS	1.03 (1.02-1.04)	<.001
Sex	Women vs men	Overall	0.94 (0.76-1.15)	NS	1.08 (0.78-1.49)	NS	1.00 (0.82-1.22)	NS
Alcohol	Active/quit vs never	Overall	1.17 (0.94-1.45)	NS	0.97 (0.70-1.35)	NS	1.20 (0.98-1.48)	NS
Smoking	Active/quit vs never	Overall	1.41 (1.07-1.85)	.014	0.77 (0.54-1.11)	NS	1.32 (1.03-1.70)	.031
Clinical T-classification	cT3-4 vs cT1-2	Overall	2.53 (2.08-3.04)	<.001	1.65 (1.22-2.24)	.001	2.10 (1.75-2.52)	<.001
Clinical N-classification	cN+ vs cN0	Overall	2.57 (2.13-3.11)	<.001	1.83 (1.35-2.49)	<.001	2.10 (1.75-2.52)	<.001
Site	Laryngeal vs oropharyngeal	Overall	0.58 (0.45-0.76)	<.001	0.76 (0.51-1.14)	NS	0.82 (0.64-1.04)	NS
	Oral cancer vs oropharyngeal		0.68 (0.55-0.84)	<.001	0.73 (0.52-1.03)	NS	0.68 (0.55-0.83)	<.001
HPV-status	Positive vs negative ^a	Overall	0.27 (0.14-0.52)	<.001	0.40 (0.16-1.00)	NS	0.39 (0.22-0.68)	.001
Differentiation grade	Moderate vs well	Overall	1.84 (1.07-3.17)	.028	2.16 (0.81-5.74)	NS	1.68 (0.97-2.91)	NS
	Poor vs well		1.80 (0.97-3.31)	NS	2.54 (0.95-6.82)	NS	1.74 (0.98-3.10)	NS
HIF-1a	Positive vs negative	Overall	1.14 (0.92-1.41)	NS	1.13 (0.81-1.59)	NS	1.04 (0.85-1.27)	NS
		Within larynx ^b		.020		NS		.022
		Within oral cavity ^b	1.86 (1.10-3.14)	NS	1.76 (0.81-3.79)	NS	1.72 (1.08-2.73)	.049
		Within oropharynx ^b	0.77 (0.55-1.09)	.023	0.77 (0.44-1.34)	NS	0.72 (0.51-1.00)	NS
			1.46 (1.05-2.02)		1.41 (0.84-2.37)		1.30 (0.96-1.77)	
Treatment	Surgery + PORT vs surgery	Overall	1.82 (1.42-2.35)	<.001	1.50 (1.00-2.26)	.049	1.42 (1.11-1.81)	.005
	Surgery + POCRT vs surgery			.017		NS		.043
	Primary RT vs surgery		3.01 (1.90-4.77)	.016	2.07 (0.50-8.59)	.023	2.54 (1.03-6.24)	.001
	Primary CRT vs surgery			<.001		.001		<.001
			1.45 (1.07-1.97)		1.69 (1.07-2.64)		1.59 (1.20-2.10)	
			2.94 (2.16-4.00)		2.26 (1.38-3.70)		2.49 (1.85-3.33)	

Abbreviations: CI, confidence interval; CRT, chemoradiotherapy; HR, hazard ratio; NS, not significant ($P > .05$); POCRT, postoperative chemoradiotherapy; PORT, postoperative radiotherapy; RT radiotherapy.

^aOropharyngeal cancer only.

^bWithin analyzes were performed by including the interaction variable HIF-1a*Site, not by a subgroup analysis.

in all treatment strategies, HIF-1a expression was associated with better (OSCC) or worse (LSCC, OPSCC) survival.

3.4 | No association of HIF-1a expression and LRC

T-classification and N-classification as well as treatment modality predicted LRC. HIF-1a expression was not related to LRC. Therefore, no further analyzes were performed.

3.5 | HIF-1a expression is associated with poor DFS in laryngeal and oropharyngeal and improved DFS in oral squamous cell carcinomas

DFS analyzes were mostly concurrent with OS analyzes. The location of recurrence per site is shown in Table S3. In univariate analyzes, age, smoking, clinical T-classification and N-classification, site, HPV-status, and treatment were significant predictors of DFS (Table 2). As with OS,

there was effect modification caused by the site of the primary tumor: the effect of HIF-1a expression on DFS was significantly different among sites. In LSCC and OPSCC, HIF-1a positivity was associated with worse DFS, although this difference did not reach statistical significance in OPSCC. In OSCC, HIF-1a positivity was associated with significantly better survival DFS.

The significant effect of HIF-1a positivity in LSCC and OSCC remained statistically significant in multivariate analyses with age, clinical T-classification and N-classification, grade (OSCC only), and treatment strategy (LSCC only), as seen in Table 4. In multivariate analysis in OPSCC, there was no statistically significant effect of HIF-1a positivity for DFS.

3.6 | Concordance between tumor center and periphery in oral squamous cell carcinomas

There was no significant discordance between samples taken from the tumor center or tumor periphery in the 180 OSCC patients that had paired samples available (McNemar test $P = 1.00$). Moreover, in these patients HIF-1a positivity in the tumor center and tumor periphery were both associated with better OS, although the difference did not reach statistical significance on univariate survival analysis in this smaller subgroup.

4 | DISCUSSION

This study compared the effect of HIF-1a on OS, LRC, and DFS in 941 patients with a squamous cell carcinoma in the three major sites in HNSCC: the oral cavity, oropharynx, and larynx. High HIF-1a expression was observed more often in OSCC as compared to the other two sites. In LSCC and OPSCC, HIF-1a expression was associated with worse OS and DFS, but with better OS and DFS in OSCC. The association between HIF-1a expression and survival was independent of other known prognosticators such as T-classification, N-classification, and HPV-status. These findings underline biological differences between squamous cell tumors in the head and neck region.

HIF-1a is a protein that is continuously synthesized, but also degraded under normoxic conditions. As this degradation process is oxygen-dependent, hypoxia leads to accumulation of HIF-1a, activating transcription of its downstream targets. This activates cellular survival mechanisms that enable cells to survive under hypoxic conditions. In the case of cancer cells, these survival mechanisms also improve resistance to radiotherapy and

some forms of chemotherapy.¹⁹ HIF-stabilization can also occur by oncogenic activation, independent of hypoxia.^{20,21} In most studies within HNSCC, high HIF-1a expression is associated with worse survival in each of the major sites, including OSCC.³

Interestingly, there are several studies that describe an association of high HIF-1a expression with better survival and these findings were all in OSCC.²²⁻²⁴ It is suggested in these studies that the high expression of HIF-1a is caused by oncogenic activation. The transcription of the downstream targets, such as VEGF, then improves vascularity and oxygen availability. This in turn is suggested to increase the efficacy of (post-operative) radiotherapy, improving survival. While this is a viable explanation of how high HIF-1a expression is associated with improved survival, this fails to explain why this phenomenon only occurs in OSCC and not in the other sites, which are more often treated with radiotherapy. However, it does concur with our finding that high HIF-1a expression leads to worse survival in OPSCC and LSCC, but not in OSCC. We also investigated the effect of HIF-1a expression on survival in patients who underwent surgery vs organ preservation therapy independent of site and found no differences between treatment strategies. We therefore conclude that the differences in the relation between HIF-1a expression and survival are to be attributed to the site and not the treatment strategy. It can be hypothesized that in OSCC the oncogenic activation of HIF-1a plays a major role in HIF-1a expression, while in the other sites, hypoxia is the major driver of HIF-1a stabilization, illustrating the heterogeneity between tumors of different sites.

In addition, we found that HIF-1a expression only had a significant effect of the survival outcomes OS and DFS, but not LRC. This suggests that the effect of HIF-1a expression on survival may occur through distant metastasis. It has been described that hypoxia may cause hypoxia-induced proteome changes, causing delayed recurrences or dormant micrometastasis.²⁵

Site differences are immediately observed when performing clinical staging according to the TNM system.²⁶ In the oral cavity, tumor size and depth of invasion are the criteria for staging of oral or oropharyngeal tumors, while in the larynx tumors are considered of a higher stage when invading multiple anatomical subregions, independent of gross tumor size. Treatment strategy, that is, the choice of a primary surgical or (chemo-)radiation approach, also differs per site. However, this difference has mostly to do with functionality and the best chance of eradicating disease while maintaining function.

In the present study, the proportion of patients who had ever smoked or had used alcohol was lower in the OSCC patients than in the LSCC and OPSCC patients.

FIGURE 2 Overall survival by HIF-1a expression. In laryngeal cancer (LSCC) and squamous cell carcinoma of the oropharynx (OPSCC) patients, HIF-1a positive tumors show significantly worse survival. In the OSCC patient cohort, HIF-1a positive tumors appear to have better survival, but this is not statistically significant [Color figure can be viewed at wileyonlinelibrary.com]

We did not find significant effect modification of smoking or drinking status on the relation between HIF-1a and survival. Still, it is possible that this has led to other, unknown differences in tumor biology or etiology that have contributed to the observed differences.

HPV-associated (HPV+) OPSCC is a subgroup that has much better survival than non-HPV associated (HPV-) OPSCC. We found that HIF-1a expression is associated with worse survival even in the subgroup of HPV+ tumors. Still the overall OS and DFS in the whole group of OPSCC patients were relatively low. This can be explained by the relatively low percentage of HPV+ OPSCC in our country, as compared to countries such as the United States.^{27,28} The observed adverse survival effect of HIF1a expression, independent of HPV status is worthy of confirmation in other OPSCC cohorts.

Treatment strategy was a significant predictor of survival in LSCC (DFS), OSCC (OS), and OPSCC (OS, DFS) in univariate analysis. In OSCC, patients treated with

surgery and PORT had worse survival than patients treated with surgery only. In multivariate analysis, controlling for other variables, treatment strategy was no longer related to survival in OSCC. Therefore, we conclude that the univariate survival difference between surgery and surgery+PORT is related to the tumor characteristics that lead to the indication for PORT.

Several points concerning the present study should be taken into account. HIF-1a staining and scoring was performed using the same staining protocol and in a TMA; therefore, tumors from different sites and different tumor sizes could be scored in a similar way and were scored by the same pathologist. It has been shown that TMA analysis with three or more spots from the same tumor yields a representative sample of the tumor and this has been shown to be reliable for high-throughput molecular profiling.^{13,29-32} In addition, specifically for HIF-1a staining, a previous study shows that single-core TMAs have a good concurrence with full sections and that false-

TABLE 3 Final multivariate models for overall survival

Variable		Laryngeal cancer		Oral cancer		Oropharyngeal cancer	
		HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age	Per year increase	1.06 (1.03-1.08)	<.001	1.04 (1.03-1.06)	<.001	1.03 (1.01-1.05)	.005
Smoking	Active/quit vs never	NA	NA	NA	NA	NA	NA
Clinical T-classification	cT3-4 vs cT1-2	2.06 (1.29-3.29)	.003	1.71 (1.23-2.38)	.002	1.80 (1.21-2.69)	.004
Clinical N-classification	cN+ vs cN0	2.96 (1.73-5.04)	<.001	3.03 (2.14-4.29)	<.001	2.18 (1.51-3.16)	<.001
HPV-status	Positive vs negative	NA	NA	NA	NA	0.24 (0.13-0.47)	<.001
Differentiation grade	Moderate vs well Poor vs well	NA	NA	2.01 (0.97-4.13) 2.25 (1.01-5.01)	NS .047	NA	NA
HIF-1a	Positive vs negative	1.72 (1.02-2.89)	.041	0.71 (0.50-1.01)	NS	1.46 (1.04-2.05)	.029
Treatment <i>Larynx</i>		NA	NA				
Treatment <i>Oral Cavity</i>				NA	NA		
Treatment ^a <i>Oropharynx</i>	Surgery vs CRT					0.92 (0.40-2.10)	NS
	Surgery + PORT vs CRT					0.58 (0.36-0.93)	.024
	Surgery + POCRT vs CRT					0.82 (0.24-2.79)	NS
	Radiotherapy alone vs CRT					1.10 (0.67-1.79)	NS

Note: NA: HPV was only included in oropharyngeal cancer analyzes, other variables are reported NA if they were removed from the model by backward selection using Akaike's information criterion ($P > .157$) for removal.

Abbreviations: CI, confidence interval; CRT, chemoradiotherapy; HR, hazard ratio; NS, not significant ($P > .05$); POCRT, postoperative chemoradiotherapy; PORT, postoperative radiotherapy.

^aTreatment: the largest category was chosen as reference category for each site.

TABLE 4 Final multivariate models for disease-free survival

Variable		Laryngeal cancer		Oral cancer		Oropharyngeal cancer	
		HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age	Per year increase	1.03 (1.01-1.06)	.004	1.04 (1.03-1.06)	<.001	1.02 (1-1.04)	.018
Smoking	Active/quit vs never	NA	NA	NA	NA	NA	NA
Clinical T-classification	cT3-4 vs cT1-2	2.18 (1.32-3.62)	.002	1.46 (1.05-2.02)	.023	1.71 (1.18-2.48)	.005
Clinical N-classification	cN+ vs cN0	3.22 (1.87-5.55)	<.001	2.63 (1.88-3.68)	<.001	1.83 (1.29-2.6)	.001
HPV-status	Positive vs negative	NA	NA	NA	NA	0.35 (0.20-0.63)	<.001
Differentiation grade	Moderate vs well Poor vs well	NA	NA	2.29 (1.11-4.71) 2.17 (0.97-4.83)	.024 NS		
HIF-1a	Positive vs negative	1.89 (1.16-3.07)	.011	0.65 (0.46-0.91)	.012	1.32 (0.96-1.82)	.085
Treatment ^a <i>Larynx</i>	Surgery vs RT	0.64 (0.37-1.13)	NS				
	Surgery + PORT vs RT	0.32 (0.16-0.62)	.001				
	Surgery + POCRT vs RT	0.43 (0.10-1.97)	NS				
	CRT vs RT	0.57 (0.16-2.03)	NS				
Treatment <i>Oral Cavity</i>				NA	NA		
Treatment ^a <i>Oropharynx</i>	Surgery vs CRT					0.84 (0.37-0.91)	NS
	Surgery + PORT vs CRT					0.55 (0.37-0.81)	.002
	Surgery + POCRT vs CRT					0.80 (0.25-2.55)	NS
	RT alone vs CRT					0.93 (0.59-1.48)	NS

Note: NA: HPV was only included in oropharyngeal cancer analyzes, other variables are reported NA if they were removed from the model by backward selection using Akaike's information criterion ($P > .157$) for removal.

Abbreviations: CI, confidence interval; CRT, chemoradiotherapy; HR, hazard ratio; NS not significant ($P > .05$), POCRT, postoperative chemoradiotherapy; PORT, postoperative radiotherapy; RT, radiotherapy.

^aTreatment: the largest category was chosen as reference category for each site.

positivity is rare.³³ We believe that by using triplicate cores concurrence will be comparable or better. The use of TMAs makes the present study one of the largest single-center

studies on the effect of HIF-1a expression on survival. All patients were treated by the same team of physicians and treatment decisions made in the same MDT.

In addition, it could be argued that the site difference between OSCC and LSCC and OPSCC could be explained because of the tissue origin; OPSCC and LSCC tissues were mostly derived from biopsies, while in most OSCC patients the analyzed tissues originated from resection specimens. To investigate this, we performed sensitivity analyses in a subgroup of OSCC patients that had tissue taken from the tumor center and the tumor periphery (which could be considered a location where a biopsy might be taken). Because there was no significant discordance in HIF-1 α positivity between these samples and because the observed effect was similar in these samples (improved survival in HIF-1 α positive OSCC patients), we conclude that the observed outcome is not due to a difference in tissue origin, that is, biopsy or resection specimen.

This study was retrospective by design and patients were included in different cohorts. Some patients had undergone biopsy in other hospitals, before being referred to our tertiary hospital. The tissues of these patients were not always available for analysis. However, apart from this we included all consecutive patients who were treated in our hospital. Because no patients had been included or excluded based on either the outcome (survival) or the determinant (HIF-1 α) expression, we do not believe that substantial bias was introduced. Moreover, the year of treatment differed between cohorts. Unfortunately because the data had been anonymized, it could not be investigated whether the year of treatment has had a direct effect on survival. However, we do not find it likely that the year of treatment confounds the relation between site, HIF-1 α expression and survival.

In conclusion, we found that HIF-1 α expression is significantly different across sites within HNSCC. Moreover, HIF-1 α overexpression had a significantly different effect on patient outcomes between sites. Tumor hypoxia is a target for therapy: hypoxia radiosensitizers, such as nimorazole in conjunction with primary radiotherapy or ARCON therapy, are available to increase susceptibility to radiotherapy.^{34,35} Also, drugs that directly inhibit HIF-1 α are currently being investigated as anticancer treatment.³⁶ If HIF-1 α is considered to be a marker of hypoxia in OPSCC and LSCC, particularly patients with tumors of these sites will benefit from a hypoxia radiosensitizer. In OSCC, high expression of HIF-1 α may be caused by oncogenic activation, which improves vascularity and oxygen availability. These tumors will less likely benefit from these therapy modalities. The present study highlights biological differences between HNSCC sites and warrants further investigation into these differences, rather than treating HNSCC as a single entity.

DATA AVAILABILITY STATEMENT

Data available on request from the authors

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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