HPV非関連進行咽頭癌におけるHIF-1α発現と予後

Prognostic significance of hypoxia-inducible factor-1α expression in advanced pharyngeal cancer without human papillomavirus infection

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Abstract

Objective

To clarify the association between hypoxia-inducible factor-1α (HIF-1α)/glucose transporter type 1 (Glut-1) expression and survival outcome in advanced pharyngeal cancer without human papillomavirus (HPV) infection.

Methods

Twenty-five oropharyngeal and 55 hypopharyngeal cancer patients without HPV infection were enrolled. All subjects had stage III–IV lesions and underwent concurrent chemoradiotherapy or surgery. HIF-1α and Glut-1 expression was investigated in primary lesions by immunohistochemistry.

Results

There were 41 and 39 cases with low and high HIF-1α expression, and 28 and 52 cases with low and high Glut-1 expression, respectively. There was no significant correlation between HIF-1α and Glut-1 expression. In univariate analysis, nodal metastasis, clinical stage, and high HIF-1α expression, but not Glut-1 expression, predicted significantly worse prognosis. In multivariate analysis, HIF-1α overexpression was significantly correlated with poor overall survival, disease-specific survival, and recurrence-free survival.

Conclusion

High HIF-1α expression was an independent risk factor for poor prognosis advanced HPV-unrelated pharyngeal cancer.

Key words: hypoxia-inducible factor 1, glucose transporter type 1, prognosis, pharyngeal neoplasms, papillomavirus infections

Introduction

The head and neck regions are closely associated with quality of life and social activity, so their functional preservation is important for the treatment of head and neck cancer (HNC) as well as for fair prognosis. For organ preservation, concurrent chemoradiotherapy (CCRT) has been successfully introduced for the treatment of HNC.[1, 2] However, patients sometimes have severe early and late adverse toxic reactions such as mucositis, disturbance of salivary secretion, dysphagia, laryngeal necrosis, and mandibular osteomyelitis.[3-5] These adverse events decrease the treatment completion rate and increase the mortality rate. Salvage surgery is used to control tumors after CCRT failure. However, surgical treatment after CCRT can cause a number of complications, for example, local infection and suture breakage, because of scarring and decreased local blood flow.[6-9] Thus, biomarkers for predicting the effects of CCRT have been examined.

Since cancer cells proliferate chaotically, angiogenesis in a tumor cannot maintain cancer growth and the vascular network, leading to hypoxia. Cancer cells in a heterogeneously hypoxic environment acquire an adaptive capacity to the hypoxic environment through changes in their signaling system, in which a key molecule is hypoxia-inducible factor-1 (HIF-1).[10-13] HIF-1 is a transcription factor composed of two subunits, HIF-1α and HIF-1β. HIF-1β is constitutively expressed not only in cancer cells but also in normal cells, and HIF-1α levels are extensively regulated by the concentration of oxygen.[12] Under normal oxygen conditions, HIF-1α is hydroxylated by a prolyl hydroxylase, and is degraded through the ubiquitin pathway via binding to the von Hippel-Lindau tumor suppressor.[10] However, under low oxygen conditions, HIF-1α is stabilized without being degraded and moves into the nucleus, where it subsequently

binds to HIF-1β to function as a transcription factor[14] that promotes the expression of many hypoxic adaptation-related factors, including glucose transport proteins such as glucose transporter type 1 (Glut-1). Because these adaptations to a hypoxic environment influence treatment resistance,[11, 14-17] the expression levels of HIF-1α and/or Glut-1 could be used to predict the therapeutic effect, recurrence, and prognosis in advanced HNC.[15, 18, 19]

HIF-1α is overexpressed in various types of cancer including HNC.[20, 21] In HNC, HIF-1α overexpression has been investigated extensively in patients with oral squamous cell carcinoma.[18, 22, 23] Although HIF-1α overexpression has been linked with poor prognosis in oral squamous cell carcinoma,[18, 22] the findings of these reports have not been conclusive.[24] By contrast, there are a relatively small number of reports examining the relationship between HIF-1α expression and disease prognosis in oropharyngeal or hypopharyngeal squamous cell carcinoma (OPSCC or HPSCC, respectively). HIF-1α is overexpressed in the vast majority of patients with OPSCC, and its degree of expression has predictive and prognostic significance in patients undergoing radiation therapy[25]. However, one report demonstrated that human papillomavirus (HPV) status, and not HIF-1α expression, was a predictor of survival outcome in patients with OPSCC.[26]

Patients with advanced hypopharyngeal and HPV-unrelated oropharyngeal carcinoma have a poor prognosis among patients with HNC. Thus, the aim of this study was to clarify the association between HIF-1α/Glut-1 expression and survival outcome in advanced pharyngeal cancer patients without HPV infection.

Materials and methods

Patients

The patients were diagnosed with OPSCC or HPSCC by pathologic examination of biopsy samples and were treated by surgery or CCRT with curative intent at the Department of Otorhinolaryngology, Head and Neck Surgery, University of the Ryukyus, between 2006 and 2017. Clinical tumor staging was performed according to the Union for International Cancer Control TNM Classification (7th edition, 2009). All subjects had clinical stage III or IV disease. Since HPV-related OPSCC has a fair survival rate, patients with HPV-related OPSCC/HPSCC were excluded. The minimum follow-up period was set to 6 months after completion of treatment. Finally, this study enrolled 80 treatment-naive patients without distant metastasis. HPV status was determined by PCR analysis of HPV DNA and immunohistochemistry of p16, as reported previously.[27] No subjects in the present study had HPV DNA or p16 overexpression in the primary lesion.[28]

To determine clinical stage and to detect concomitant multiple primary cancers, the patients underwent physical and endoscopic examinations of the upper gastrointestinal tract, ultrasonic inspection of the neck, and computed tomography (CT) and ^{18}F-fluorodeoxyglucose-positron emission tomography-CT (PET-CT) imaging. Patient evaluation and the decision-making processes were conducted by head and neck surgeons and radiation oncologists before treatment was initiated.

Treatment protocol

Patients with T3/T4 HPSCC were usually treated with 1 cycle of induction chemotherapy for organ preservation and prevention of distant metastasis. The basic regimen of induction chemotherapy was 1 or 2 cycles of a combination of 5-fluorouracil

(600 mg/m^2 on days 1–5), nedaplatin (60 mg/m^2 on day 2), and docetaxel (60 mg/m^2 on day 2). The therapeutic response was evaluated using the four categories of the Response Evaluation Criteria in Solid Tumors (RECIST version 1.1) guidelines: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). The initial CT or magnetic resonance imaging scans were used as reference images. The response to induction chemotherapy was classified into the response (CR or PR) and no-response (SD or PD) groups. Those cases for whom a PR or CR to induction chemotherapy was achieved underwent CCRT as organ preservation treatment, and cases with SD or PD were recommended to undergo total pharyngo-laryngectomy. As a general rule, we performed postoperative RT (60 Gy) with a triweekly infusion of 80 mg/m^2 cisplatin for 3 times within 6 weeks after surgery if the patients had the following pathological high-risk factors for recurrence: lymph node metastasis with extracapsular extension and/or a positive/close surgical margin (tumor located < 5 mm from the surgical margin). HPSCC patients with CCRT underwent definitive RT (total 50.4 Gy, 1.8 Gy per day, or 50 Gy, 2 Gy per day, 5 times per week) that was administered to the primary site and whole neck including the bilateral neck lymph nodes. The primary site and metastatic lymph nodes were subsequently treated with boost doses of a further 16.2 or 20 Gy in 9 or 10 fractions, respectively. Thus, the cumulative dose to the gross primary tumor and metastatic neck lymph nodes was 66.6 Gy or 70 Gy (once daily fraction).

In patients with OPSCC, the primary treatment was CCRT. The patients received platinum-based chemotherapy (nedaplatin and 5-fluorouracil, given twice with a 4-week interval) combined with 66.6 Gy RT. The radiological response of the primary lesion was determined at 39.6 Gy irradiation in all patients by CT, according to the revised

RECIST guidelines (version 1.1). If the primary lesion showed a PR, CCRT was continued as per the protocol. When the primary tumor failed to show a PR regardless of the neck lymph node response, the patients underwent curative surgery for the primary lesion combined with neck dissection. Patients with N2 and N3 lesions underwent neck dissection at 2–3 months after CCRT.[29]

Immunohistochemical study of HIF-1α and Glut-1 expression

For HIF-1α and Glut-1 immunohistochemistry, 4-μm-thick sections from paraffin-embedded block samples were deparaffinized in xylene and hydrated in a graded series of alcohol. Epitope retrieval was achieved by heating at 100 ℃ for 10 min in 1 mM EDTA buffer (pH 8.0) for HIF-1α immunohistochemistry or in 10 mM citrate buffer (pH 6.0) for Glut-1 immunohistochemistry. Endogenous peroxidase activity was quenched by incubating the sections in 0.3% H_2O_2 in methanol for 20 min at room temperature. A SAB-PO Kit (Nichirei Bioscience, Inc., Tokyo, Japan) was used to detect immunoreactivity to HIF-1α and Glut-1 according to the manufacturer's protocol. After blocking non-specific reactions by incubation in 10% goat serum, the sections were incubated with primary antibodies for 1 h at room temperature. A mouse monoclonal anti-HIF-1α antibody (H1α67-immunoprecipitation assay grade; Abcam, Tokyo, Japan) was diluted with Protein Block Serum-Free (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) at 1:100 for HIF-1α immunostaining, and a rabbit monoclonal anti-Glut-1 antibody (ab115730; Abcam, Tokyo, Japan) was diluted with Protein Block Serum-Free at 1:250 for Glut-1 immunostaining. Subsequently, a biotin-labeled secondary antibody and peroxidase-labeled streptavidin were applied. Immunolabeling was visualized by incubation in 3-3′-diaminobenzidine, and stained slides were

counterstained with hematoxylin.

Positive HIF-1α expression was defined as having a stained nucleus and cytoplasm (Fig. 1A). Positive Glut-1 expression was defined as having a stained cell membrane of tumor cells as observed for erythrocytes in the same field (Fig. 1B). Sample scoring was performed by semiquantitative microscopic analysis, considering the positive rates of cancer cells and signal intensity in 3 fields of view under 400× magnification. Considering the percentage of HIF-1α immuno-positive tumor cells, a score of 1 was given when <10% of cells were positive; 2 when ≥10% and <30% of cells were positive; 3 when ≥30% and <70% of cells were positive; and 4 when ≥70% of cells were positive. Signal intensity was scored as negative (0), weak (1), moderate (2), and strong (3). The sum of the two scores was used to categorize HIF-1α expression as negative to weak (≤2; hereafter, low expression) and moderate to strong (>3; hereafter, high expression). A representative case is shown in Fig. 1A. Considering the percentage of Glut-1 immuno-positive tumor cells, a score of 1 was given when <10% of cells were positive; 2 when ≥10% and <70% of cells were positive; 3 when ≥70% and ≤90% of cells were positive; and 4 when >90% of cells were positive. Signal intensity was scored as negative (0), weak (1), moderate (2), and strong (3). The sum of the two scores was used to categorize Glut-1 expression as negative to weak (≤3; hereafter, low expression) and moderate to strong (≥4; hereafter, high expression). A representative case is shown in Fig. 1B. These analyses were performed by S.A., T.I., and N.H., who were blinded to the patients' clinical information.

Survival estimation

The clinicopathologic parameters and treatment outcome of each patient were

recorded at scheduled intervals during the observation period. The status of each patient, including information about recurrence and metastasis, was recorded at least every 4–6 weeks for the first year, every 2–3 months from 2–5 years, and thereafter every 6 months.

Overall survival (OS), disease-specific survival (DSS), and recurrence-free survival (RFS) were investigated as prognostic indicators. Survival curves were estimated according to the Kaplan-Meier method, and survival distributions were compared using the log-rank test. Final prognosis was judged in February 2017. OS was defined as the time from the start of treatment to death from any cause (both related and unrelated to OPSCC or HPSCC) or to February 2017. DSS was defined as the time from the start of treatment to death related to OPSCC or HPSCC or to February 2017. DSS denotes the probability of remaining free of disease after primary treatment. RFS was defined as the time from the start of treatment to locoregional or distant metastasis or to February 2017. All tests were two-sided, and p-values < 0.05 were considered statistically significant. The multivariate prognostic significance of tumor variables on OS, DSS, and RFS was assessed using Cox proportional hazards analysis to identify prognostic parameters. Analyses were performed using the SPSS statistical package (SPSS for Windows version 25.0; SPSS, Inc., Chicago, IL, USA). The significance level was set at $p < 0.05$.

This study was approved by the Institutional Review Board of the University of the Ryukyus and carried out in accordance with the 1975 Declaration of Helsinki, as revised in 2008. Informed consent was obtained from all patients before enrollment. Since the present investigation was a prognostic biomarker study of HIF-1α and Glut-1, we followed the REMARK guideline checklist.

Results and analysis

Patient characteristics (Table I)

The subjects consisted of 69 men (86%) and 11 women (14%) with a median age of 66 (range, 39–82) years. The median follow-up period, excluding those patients who died during this time, was 76 (range, 7–132) months. All patients were followed for at least 24 months except for 1 patient who was lost to follow-up at 7 months. Of the 80 patients, there were 25 (31%) with OPSCC and 55 (69%) with HPSCC. Clinical stage IV was observed in 66 patients (82%). According to T classification, there were 6, 28, 25, and 21 patients with T1, T2, T3, and T4, respectively. According to N classification, there were 6, 16, 52, and 6 patients with N0, N1, N2, and N3, respectively.

Of the 80 patients, 30 (38%) underwent surgery and 50 (62%) received CCRT as a primary treatment. Of the 30 patients in the surgery group, 24 had hypopharyngeal carcinoma.

Of the 55 HPSCC patients, 34 had T3/T4 lesions. Of these 34 patients, 27 (79.4%) received induction chemotherapy and the remaining 7 did not undergo induction chemotherapy due to renal dysfunction (4 patients), previous history of irradiation to the neck (1 patient), and refusal of induction chemotherapy (2 patients).

Immunohistochemical examinations

Overall, HIF-1α expression tended to be weaker than Glut-1 expression (Fig. 1A and B). For HIF-1α immunohistochemistry, 41 (51.3%) of 80 samples demonstrated 0–30% positive cell counts and 59 (73.8%) showed negative to weak staining (Table II). Strong HIF-1α expression was observed in only 5 of 80 subjects (6.3%). On the other

hand, 71 of 80 patients (88.8%) showed weak to strong Glut-1 expression in >70% of primary cancer cells, and 70 (87.5%) showed moderate to strong staining (Table II).

High HIF-1α expression was observed in 39 cases (49%) and high Glut-1 expression was found in 52 cases (65%). However, there was no correlation between HIF-1α and Glut-1 expression (Table III, $p = 0.087$).

Survival estimation

OS, DSS, and RFS in relation to the clinical features and immunoexpression of HIF-1α and Glut-1 are shown in Tables I and IV, respectively. N classification and clinical stage classification showed a significant difference in univariate analysis of OS, DSS, and RFS. However, the other clinical features including T classification and primary sites did not reach significance (Table I). For immunohistochemical analysis, patients with high HIF-1α expression had significantly worse OS, DSS, and RFS than those with low HIF-1α expression (Table IV and Fig. 2). However, Glut-1 expression had no significant impact on survival (Table IV and Fig. 3). In multivariate analysis, there was a significant difference in OS, DSS, and RFS between patients with low and high HIF-1α expression (Table V). N category and clinical stage classification did not reach significance.

There were no significant differences in clinical characteristics between low and high HIF-1α expression (Table VI). However, the high HIF-1α expression group contained a relatively large number of patients with OPSCC ($p = 0.066$). Figure 4 shows the difference in OS between low and high HIF-1α expression in the OPSCC and HPSCC patients. HPSCC patients with high HIF-1α expression had worse OS than those with low HIF-1α expression (Fig. 4B, $p = 0.026$). OPSCC patients also showed

the same tendency for OS, despite the small number of samples (Fig. 4A, $p = 0.114$).

Discussion

In this study, high HIF-1α expression was found to be an independent risk factor for poor prognosis in patients with advanced OPSCC or HPSCC. This is the first report of HIF-1α expression focusing on advanced pharyngeal cancer without HPV infection.

A meta-analysis of 1474 oral cancers demonstrated that HIF-1α was associated with tumor size, clinical stage, and lymph node metastasis, and high HIF-1α expression was an indicator for worse survival outcome.[18] In subgroup analysis, this phenomenon was observed exclusively in Asian patients. According to another systematic review of HIF expression in HNC, HIF-1α overexpression was also significantly associated with poor prognosis in Asian patients, but not in European patients.[30] Regarding tumor location in HNC, oral carcinoma, nasopharyngeal carcinoma, and oropharyngeal carcinoma, but not laryngeal carcinoma, an association was seen between HIF-1α overexpression and worse OS. A previous report on the association between HIF-1α overexpression and the survival rate in HPSCC demonstrated no clear relationship between HIF-1α and locoregional control and DSS.[31] In the present study, all patients were Japanese (i.e., Asians) with HPSCC or OPSCC and a clear association was seen between HIF-1α expression and OS, DSS, and RFS. Although only a small number of reports have examined HIF-1 expression in patients with OPSCC or HPSCC, the findings of the present study are in line with those of previous reports. Given that racial differences in HIF-1 expression have been observed between Asian and European countries,[18, 30] further studies in different ethnic groups are needed to confirm our

observations.

Because this study focused on advanced pharyngeal cancer, clinical T, N, and stage classifications were not found to be significant prognostic factors in multivariate analysis. Oropharyngeal carcinoma[32] and HNC[33] with radiotherapy showed worse local control and survival rate when high HIF-1α expression was observed.[32] On the other hand, HIF-1α expression in surgically treated patients with head and neck squamous cell carcinoma (31 oral cavity, 23 oropharynx, 16 larynx, and 9 hypopharynx) was associated with improved disease-free survival and OS[21], while the results of the present study were similar to those of previous reports.[32, 33] Elevated HIF-1α protein levels have been shown to be associated with increased hypoxic radiation resistance in FaDu human pharyngeal carcinoma cells *in vitro*.[34] The discrepancies between these contradictory results may reflect different treatment modalities, and HIF-1α-overexpressing HNC might be treated by surgery, and not by radiation-based therapy. Further study is needed to clarify an appropriate treatment modality for HIF-1α-overexpressing HNC.

Glut-1 is a cell membrane transport protein that determines glucose uptake and is abnormally expressed in HNC.[19] Although Glut-1 shows only weak expression in normal mucosal lesions, it is strongly expressed in dysplasia and squamous cell carcinoma.[35] Glut-1 expression is considered to be a prognostic marker in HNC.[36, 37, 38] However, in the present study, Glut-1 expression did not demonstrate a clear correlation with disease prognosis. We found that Glut-1 expression was much stronger than HIF-1α expression (Table II). Since PET-CT studies have indicated that glucose uptake is highly increased in HNC,[19, 39, 40] the immunohistochemical approach used in the present study might not detect differences in glucose uptake ability in advanced pharyngeal carcinoma. In addition, although a significant correlation between Glut-1 and HIF-1α

expression in HNC has been reported,[40] we found no such association. Other methods, such as metabolic tumor volume in PET-CT, might be more appropriate than Glut-1 immunohistochemistry for evaluating glucose metabolism.

In conclusion, high HIF-1α expression, but not high Glut-1 expression, was an independent risk factor for poor prognosis in advanced HPV-unrelated OPSCC and HPSCC patients who underwent CCRT as a primary treatment. Racial differences in the association of HIF-1α expression with survival outcome between Asian and European countries and treatment modality in cases with high HIF-1α expression should be examined to facilitate the design of better treatment protocols.

Figure legends

Figure 1. Representative cases with HIF-1α (A) and Glut-1 (B) immunohistochemistry.
A: Hypopharyngeal carcinoma case. Strong HIF-1α expression was observed in nuclei.
B: Oropharyngeal carcinoma case. There was strong and diffuse Glut-1 expression in cell membranes. Scale bar, 50 μm. IHC, immunohistochemistry.

Figure 2. Kaplan-Meier curves of overall survival (A), disease-specific survival (B), and recurrence-free survival (C) in 80 pharyngeal carcinoma patients without human papillomavirus infection.
A: Overall survival in relation to HIF-1α expression. Patients with high HIF-1α expression had worse overall survival than those with low HIF-1α expression (p = 0.033).

B: Disease-specific survival in relation to HIF-1α expression. Patients with high HIF-1α expression had worse disease-specific survival than those with low HIF-1α expression ($p = 0.033$).

C: Recurrence-free survival in relation to HIF-1α expression. Patients with high HIF-1α expression had worse recurrence-free survival than those with low HIF-1α expression ($p = 0.015$).

low ex., low expression; high ex., high expression.

Figure 3. Kaplan-Meier curves of overall survival (A), disease-specific survival (B), and recurrence-free survival (C) in 80 pharyngeal carcinoma patients without human papillomavirus infection.

A: Overall survival in relation to Glut-1 expression. There was no significant difference in overall survival between patients with high and low Glut-1 expression.

B: Disease-specific survival in relation to Glut-1 expression. There was no significant difference in disease-specific survival between patients with high and low Glut-1 expression.

C: Recurrence-free survival in relation to Glut-1 expression. There was no significant difference in recurrence-free survival between patients with high and low Glut-1 expression.

low ex., low expression; high ex., high expression.

Figure 4. Kaplan-Meier curves of overall survival in patients with oropharyngeal carcinoma (A) and hypopharyngeal carcinoma (B).

A: Oropharyngeal cancer patients with high HIF-1α expression tended to have worse overall survival than those with low HIF-1α expression, but the difference did not reach significance ($p = 0.114$).

B: Hypopharyngeal cancer patients with high HIF-1α expression had worse overall survival than those with low HIF-1α expression ($p = 0.026$).

Figure 1

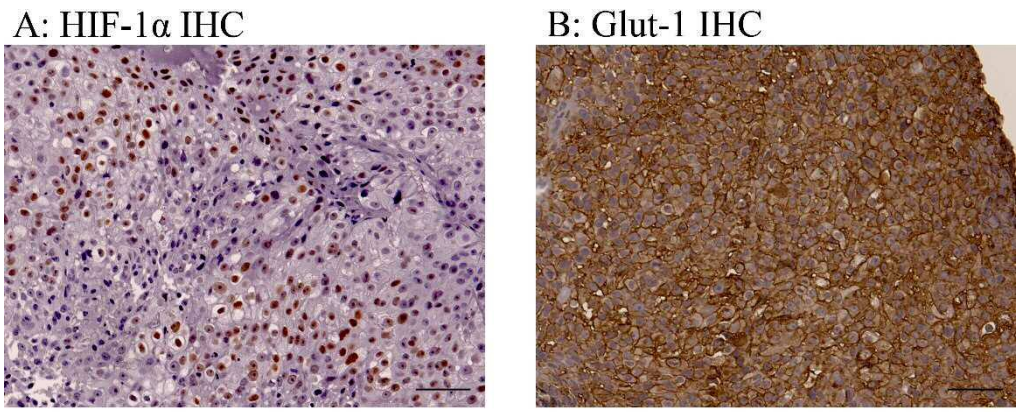


Figure 2

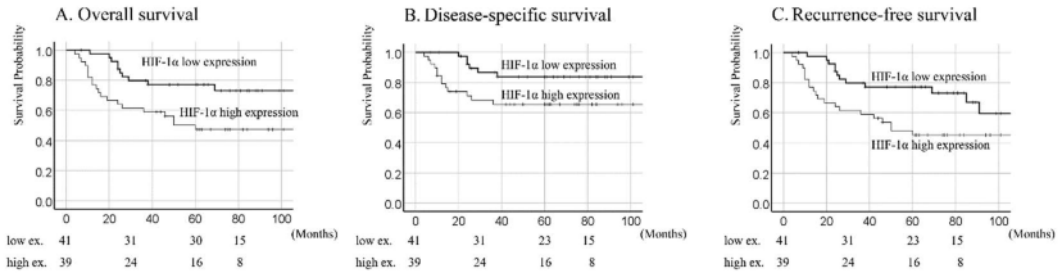


Figure 3

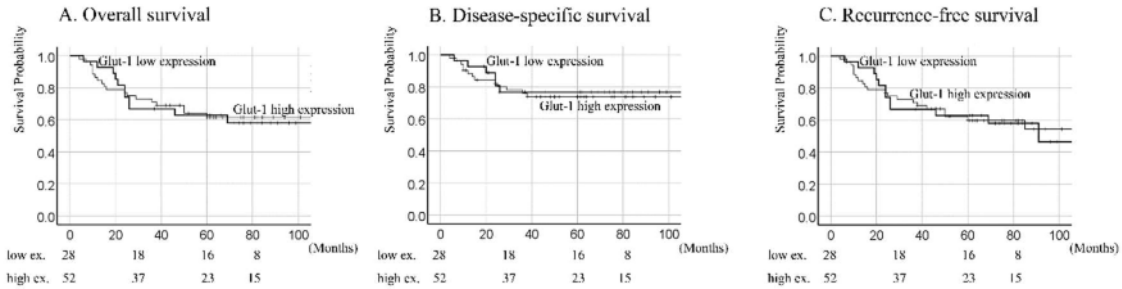
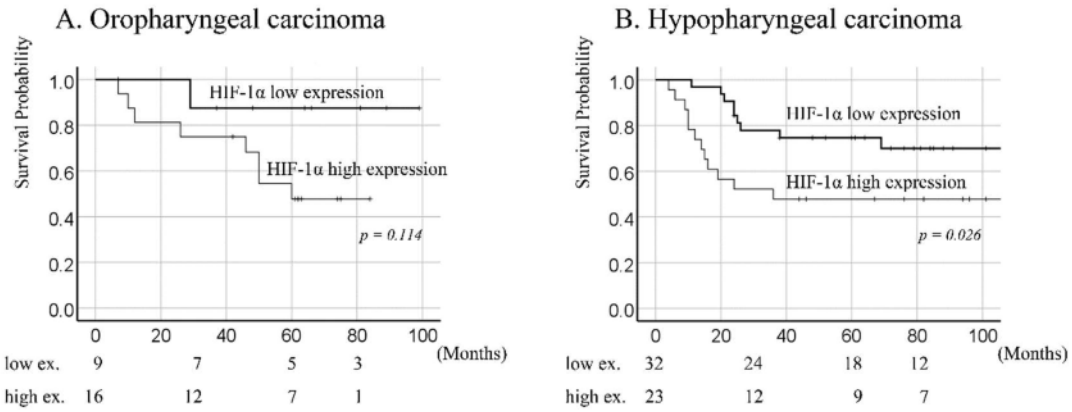


Figure 4


A. Oropharyngeal carcinoma
Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
HIF-1α low expression
HIF-1α high expression
p = 0.114
0 20 40 60 80 100
(Months)
low ex. 9 7 5 3
high ex. 16 12 7 1
B. Hypopharyngeal carcinoma
Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
HIF-1α low expression
HIF-1α high expression
p = 0.026
0 20 40 60 80 100
(Months)
low ex. 32 24 18 12
high ex. 23 12 9 7

Acknowledgements

The study was supported by and conducted in cooperation with the Ryukyu Society for the Promotion of Oto-Rhino-Laryngology. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Financial Support

This work was supported by JSPS KAKENHI (M.S., grant number JP17K11390).

Conflict of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guidelines on human experimentation (Institutional Review Board of the University of the Ryukyus) and with the Helsinki Declaration of 1975, as revised in 2008.

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Summary

- HIF-1α is overexpressed in various types of cancer including HNC.
- Only a few reports have examined the relationship between HIF-1α expression and disease prognosis in pharyngeal squamous cell carcinoma.
- No study has reported the association between HIF-1α and prognosis in advanced pharyngeal cancer without HPV infection.
- High HIF-1α expression, but not high Glut-1 expression, was an independent risk factor for poor prognosis in patients with advanced HPV-unrelated OPSCC and HPSCC.

Tables

Table I. Clinical features and survival estimation

Factor	N (%)	5-year OS	Univariate analysis *p*-value	5-year DSS	Univariate analysis *p*-value	5-year RFS	Univariate analysis *p*-value
Age							
<65 years	35 (44)	52.8	0.262	64.2	0.086	52.8	0.556
≥65 years	45 (56)	69.6		83.4		67.3	
Sex							
Male	69 (86)	62.7	0.770	71.9	0.221	61.1	0.616
Female	11 (14)	60.6		90.9		60.6	
Primary site							
Oropharynx	25 (31)	59.7	0.795	79.3	0.561	56.4	0.938
Hypopharynx	55 (69)	63.4		72.6		63.4	
T							
T1, T2 (T1, 6; T2, 28)	34 (43)	56.1	0.463	72.2	0.550	53.6	0.314
T3, T4 (T3, 25; T4, 21)	46 (57)	66.7		76.5		66.7	
N							
N0–N1 (N0, 6; N1, 16)	22 (28)	90.9	0.003	95.2	0.012	85.9	0.011
N2–N3 (N2, 52; N3, 6)	58 (72)	51.9		66.5		51.9	
Stage							
III	14 (18)	100.0	0.004	100.0	0.023	91.7	0.026
IV	66 (82)	54.6		69.0		54.6	
Tumor differentiation							
Well/moderate	63 (79)	68.4	0.072	78.4	0.235	66.8	0.121
Poor	17 (21)	40.3		60.1		40.3	
Neoadjuvant chemotherapy							
No	45 (56)	61.3	0.713	74.6	0.991	59.3	0.882
Yes	35 (44)	59.2		75.2		62.5	
Primary treatment							
CCRT/CCRT wih/without OP	50 (62)	60.1	0.967	73.4	0.720	58.3	0.709
CCRT	36						
CCRT to Operation	4						
CCRT to PND	10						
Operation (30)	30 (38)	65.2		77.3		65.2	
Multiple primary cancers							
No	58 (72)	59.8	0.832	72.4	0.516	58.2	0.528
Yes	22 (28)	68.2		81.3		68.2	
Brinkman index							
<800	48 (60)	64.5	0.491	80.1	0.183	62.6	0.544
≥800	32 (40)	59.2		66.7		59.2	
Sake index							
<40	38 (48)	63.3	0.832	77.7	0.581	63.3	0.711
≥40	42 (52)	61.4		72.2		58.9	

CCRT, concurrent chemoradiation therapy; DSS, disease-specific survival; OP, operation; OS, overall survival; PND, planned neck dissection; RFS, recurrence-free survival.

Table II. Immunohistochemical findings

HIF-1α expression				**Glut-1**			
Positive cell count (%)	No. of subjects	Staining degree	No. of subjects	Positive cell count (%)	No. of subjects	Staining degree	No. of subjects
0–30	41	negative	41	0–10	0	negative	0
30–70	21	weak	18	10–70	9	weak	10
70	18	moderate	13	70–90	16	moderate	38
		strong	5	≥90	55	strong	32

Table III. Correlation between HIF-1α and Glut-1 expression

	Glut-1 expression	
HIF-1α expression	Low	High
Low	18	23
High	10	29

Table IV. Immunoexpression and survival estimation

Factor	N (%)	5-year OS	Univariate analysis			5-year DSS	Univariate analysis			5-year RFS	Univariate analysis		
			p -value	risk ratio	*95% CI*		*p* -value	risk ratio	*95% CI*		*p* -value	risk ratio	*95% CI*
HIF-1α expression													
	Low 41 (51)	83.8	0.033	0.476	0.256-0.884	83.8	0.033	0.439	0.185-1.040	77.1	0.015	0.544	0.311-0.949
	High 39 (49)	65.5				65.5				45.2			
Glut-1 expression													
	Low 28 (35)	76.7	0.729	1.075	0.600-1.927	76.7	0.765	0.857	0.366-2.008	60.0	0.959	1.061	0.618-1.822
	High 52 (65)	73.9				73.9				61.2			

CI, confidence interval; DSS, disease-specific survival; OS, overall survival; RFS, recurrence-free survival.

Table V. Multivariate analysis of survival data

Factor	N (%)	5-year OS multivariate analysis			5-year DSS multivariate analysis			5-year RFS multivariate analysis		
		p -value	*hazard ratio*	*95% CI*	*p* -value	*hazard ratio*	*95% CI*	*p* -value	*hazard ratio*	*95% CI*
N		0.221	0.407	0.097-1.714	0.267	0.319	0.043-2.394	0.22	0.407	0.097-1.709
N0–N1 (N0, 6; N1, 16)	22 (28)									
N2–N3 (N2, 52; N3, 6)	58 (72)									
Stage		0.965	0.000	0.000-7.0776e+239	0.973	0.000	0.000-3.124e+304	0.468	0.483	0.068-3.448
III	14 (18)									
IV	66 (82)									
HIF-1α expression		0.01	0.367	0.171-0.787	0.037	0.357	0.137–0.942	0.012	0.402	0.196-0.822
Low	41 (51)									
High	39 (49)									

CI, confidence interval; DSS, disease-specific survival; OS, overall survival; RFS, recurrence-free survival.

Table VI. Clinical characteristics of HIF-1α-positive cases

	Low HIF-1α expression	High HIF-1α expression	*p* -value
Age			
<66 years	18	17	0.978
≥66 years	23	22	
Sex			
Male	36	33	0.679
Female	5	6	
Primary Site			
Oropharynx	9	16	0.066
Hypopharynx	32	23	
T			
T1, T2 (T1, 6; T2, 28)	17	17	0.848
T3, T4 (T3, 25; T4, 21)	24	22	
N			
N0–N1 (N0, 6; N1, 16)	12	10	0.716
N2–N3 (N2, 52; N3, 6)	29	29	
Stage			
III	8	6	0.627
IV	33	33	
Tumor differentiation			
Well/Moderate	32	31	0.875
Poor	9	8	
Neoadjuvant Chemotherapy			
No	23	22	0.978
Yes	18	17	
Primary treatment			
CCRT/CCRT with/without OP (50)	19	20	
CCRT	9	16	
CCRT to Operation	3	1	
CCRT to PND	7	3	
Operation (30)	15	15	
Multiple primary cancers			
No	30	28	0.89
Yes	11	11	
Brinkman index			
<800	26	22	0.523
≥800	15	17	
Sake index			
<40	23	15	0.114
≥40	18	24	

CCRT, concurrent chemoradiation therapy; OP, operation; PND, planned neck

dissection.