

Expression of hypoxic signaling markers in head and neck squamous cell carcinoma and its clinical significance

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Received: 28 August 2013 / Accepted: 12 February 2014 / Published online: 14 March 2014
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Abstract Hypoxia is a consistent finding in fast-growing tumors; it contributes to tumor progression and therapeutic responses. We explored the expression of hypoxia-associated biomarkers in head and neck squamous cell carcinoma (HNSCC) to assess their relationship with clinical factors in HNSCC. In total, 90 patients with HNSCC were enrolled. Expression of HIF-1 α , HSP70, HSP90, VEGF, IGF-1R, and P16 was investigated by immunohistochemistry. Their correlations with clinical factors, including location of primary sites, T stage, N stage, M stage, HPV status, primary treatment success/failure, recurrences, disease-free survival (DFS), and overall survival, were analyzed. HIF-1 α , HPS70, HPS90, VEGF, and IGF-1R were positive in 33 of 89 (37.1 %), 62 of 87 (71.3 %), 83 of 89 (93.3 %), 41

of 87 (47.1 %), and 50 of 56 (89.3 %) cases, respectively. Expression levels of some of these markers were correlated. High HIF-1 α or HSP 70 correlated with poor DFS, and expression of HSP70 correlated with LN metastasis. HPV-related carcinomas showed high HSP 70 and IGF-1R expression. Hypoxia-associated proteins were highly expressed and associated with aggressive clinical features in HNSCC. Expression of HIF-1 α or HSP70 can be considered poor prognostic indicator in HNSCC. Our results suggest that hypoxic signaling is activated in HNSCC, especially in HPV-related tumors.

Keywords Hypoxia-inducible factor 1 · Alpha subunit · HSP70 heat-shock proteins · HSP90 heat-shock proteins · Vascular endothelial growth factor A · Receptor · IGF Type 1 · Tumor markers · Biological

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Introduction

Head and neck cancer is the sixth most common type of cancer worldwide, and the most common subtype is squamous cell carcinoma. Almost 36,000 new cases of head and neck squamous cell carcinoma (HNSCC) were diagnosed in the US in 2010 [1]. Most patients were found to have advanced-stage disease at the initial presentation; thus, the 5-year survival rate is <50 %, which has not improved over the past 20 years [2]. To date, little is known about the pathogenesis of or prognostic indicators in HNSCC [3].

Hypoxia is not only a typical phenomenon encountered in rapidly growing cancers, it is also associated with aggressive phenotypes and treatment failure [4]. Hypoxia is also one of the most important environmental factors that induce cancer metastasis [5]. In response to hypoxia, cancer cells activate transcription of “hypoxia-inducible

Table 1 Clinical profile of all 90 patients with head and neck squamous cell carcinoma

	Sex (M:F)	Age (mean, range)	Stage (T1:T2:T3:T4)	LN status (N0:N1:N2-3)	AJCC Stage (I:II:III:IV)	Treatment (Sg:CCRT:RT)
Nasopharynx (<i>n</i> = 6)	3:3	58.7 (34–71)	1:0:1:4	4:1:1	1:0:1:4	1:2:2
Oral cavity (<i>n</i> = 16)	12:4	58.4 (30–78)	6:8:1:1	5:2:9	3:3:2:8	7:6:3
Oropharynx (<i>n</i> = 32)	30:2	57.6 (38–84)	15:7:4:6	6:4:22	2:5:2:23	2:18:3
Hypopharynx (<i>n</i> = 7)	7:0	67.4 (57–84)	2:3:0:2	2:3:2	2:1:1:3	1:5:1
Larynx (<i>n</i> = 29)	29:0	62.9 (40–84)	12:3:7:7	22:4:3	12:3:6:8	9:6:11
Overall (<i>n</i> = 90)	81:9	60.3 (30–84)	36:21:13:20	39:14:37	20:12:12:46	20:37:20

Sg Surgery, CCRT Chemoradiotherapy, RT Radiotherapy

factor-1 α ” (HIF-1 α), heat-shock proteins (HSPs), and angiogenesis-related molecules, such as vascular endothelial growth factor (VEGF) [4, 6, 7]. HIF-1 α is a key transcription factor regulating various responses to hypoxic stimuli [4]. It induces activation of many genes involved in tumor growth and survival, metastasis, invasion, chemoresistance, and angiogenesis [6]. HIF-1 α is protected from degradation by the HSP90 or HSP90-HSP70 complex [8, 9]. These HSPs are involved in many pathways apart from the stabilization of HIF-1 α , such as inhibition of apoptosis [10] and prolonged survival and proliferation of cancer cells [11, 12]. Eventually, elevated levels of HIF-1 α or HSP70 are known to be an indicator of poor clinical outcome [13]. VEGF is a predominant pro-angiogenic factor with specific mitogenicity for endothelial cells, which is crucial for both the growth of tumors and progression with metastasis [14, 15]. Insulin-like growth factor 1 receptor (IGF-1R) is linked to a HIF-1-dependent signaling pathway, and activation of IGF-1R leads to cell proliferation, HIF-1 α expression, and secretion of VEGF in HNSCC [16].

The need for additional therapeutic options, based on molecular profiling of tumors, has been growing for several decades. However, targeted therapy of HNSCC is not common because of a lack of understanding of the molecular pathogenesis and heterogeneity of HNSCC in terms of, for example, anatomical location and etiology [17, 18].

The aim of this study was to examine the role of hypoxia in the pathogenesis of HNSCC and prognosis. We investigated the expression of hypoxia-related proteins in HNSCC and correlated them with various clinicopathological parameters, including human papilloma virus (HPV) status.

Materials and methods

Patients and study design

This study was approved by the Institutional Review Board of Seoul National University Boramae Hospital (IRB No. 06-2012-82).

In total, 90 patients diagnosed with HNSCC at Boramae Medical Center between January 2008 and March 2012 were enrolled. Clinical information including demographic data, clinical stage, treatment methods, treatment response, recurrence, and follow-up data were obtained from electronic medical records. Demographic data and clinical characteristics are summarized in Table 1. The median age of the patients was 60.2 (range 30–84) years and 90.0 % were males (Fig. 1). Tumor stage ranged from I to IV, according to the American Joint Committee on Cancer staging scheme [19]. Induction chemoradiotherapy or Concurrent chemoradiotherapy (CCRT) was used for unresectable cases. In cases with resection margin insufficiency, lymph node metastasis with extracapsular extension, or involvement of adjacent structures (pT3 or T4), CCRT was considered for adjuvant therapy after surgery. During CCRT, cisplatin at 35 mg/m² per week was given to all patients during radiotherapy; the treatment was performed seven or eight times. Radiotherapy was performed 5 days per week and, in total, involved 7,020 cGy in 39 fractions for the gross tumor volume, 5,940 cGy in 33 fractions for the high-risk clinical target volume, and 5,040 cGy in 25–28 fractions for the low-risk clinical target volume.

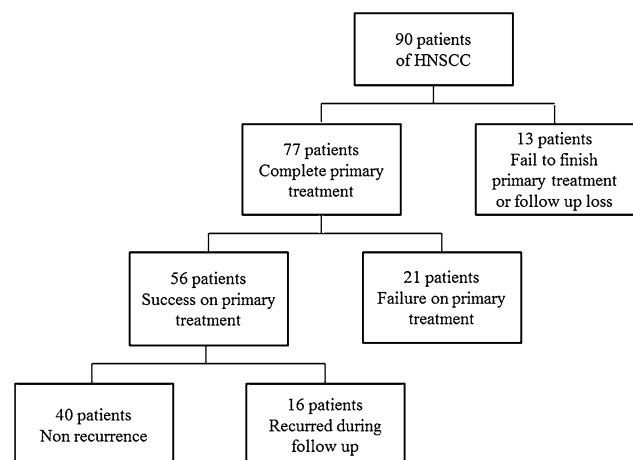


Fig. 1 The enrolled patients of head and neck squamous cell carcinoma

Treatment outcome analysis

Tumor responses were assessed at 3 months after the completion of treatment. Evaluation of tumor response consisted of a clinical examination, nasopharyngolaryngoscopy, and computed tomography (CT), and magnetic resonance imaging (MRI) of the primary sites and the neck. Positron emission tomography (PET) was also performed to evaluate distant metastasis. Recurrence was defined as regional metastasis if found within neck and as distant metastasis if it was found the rest of the body. Data regarding patient demographics, response to treatment, disease-free survival (DFS), and overall survival (OS) were obtained by medical record review. Tumor responses were assessed radiologically at baseline and after every two treatment cycles. Designations of complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) were based on the standardized response definitions established by the World Health Organization [20]. Failure of treatment was defined as the state of PR, SD, or PD on completion of the definite primary treatment. The response rate was defined as the total number of CR patients divided by the total number of evaluable patients. OS was calculated as the time between the beginning of chemotherapy and death or was censored at last follow-up.

Pathological review and construction of tissue microarray blocks

A cancer specimen was taken before the start of primary treatment, and all cases were reviewed by an expert pathologist. Tissue microarray blocks (TMA) were constructed by transferring a 0.2-cm core from the most representative area on the mother block to a new paraffin block containing 40–50 cases of HNSCC. Triplicates of each TMA were used for immunohistochemistry (IHC), as described below.

IHC and interpretation

Immunohistochemistry was performed according to a standard protocol using a Benchmark XT automated immunostainer (Ventana, Tuscon, AZ) on formalin-fixed, paraffin wax-embedded (FFPE) tissue sections. Briefly, all tissue sections underwent heat-induced epitope retrieval in citrate buffer, pH 6.0 (Dako, Carpinteria, CA). Endogenous peroxidase was blocked with a 3 % H₂O₂ solution for 10 min. To avoid nonspecific binding of primary antibodies, a serum-free blocking solution (Dako) was applied for 30 min at room temperature. The antibodies tested were HIF-1 α (Abcam, Cambridge, MA), HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA), HSP90 (Novus Biologicals, Littleton, CO), VEGF (R&D Systems, Minneapolis, MN), IGF1R (Ventana) and P16 (Dako). Reactivity was detected using the Ultra-View

detection kit (Ventana). Appropriate external or internal control slides were run with case slides at the same time; only the process of incubation with primary antibody was omitted in negative control slides. IHC was assessed by a semiquantitative grading method (H-score system); the percentage of positive tumors (0–100) was calculated for each specimen and multiplied by the grade of intensity (0–3, higher number indicates strong expression) [21]. The percentage and grade of intensity were independently interpreted by two pathologists (JEK and JYC) and later reached an agreement in discordant cases. The median H score value was chosen as the cutoff point for separating low and high levels of HIF-1 α , HSP70, HSP90, VEGF, and IGF-1R expression. Tumors with an H score exceeding 0, 30, 120, 0, and 40 were deemed to be in the high expression of HIF-1 α , HSP70, HSP90, VEGF, and IGF-1RR, respectively, groups (Fig. 2).

Detection of HPV

Human papilloma virus was detected using in situ hybridization (ISH) for HPV and HPV genotyping by DNA chip analysis. ISH was performed using the Automated Staining System (Ventana XT) according to the manufacturer's instructions. Inform HPV III probe sets (Ventana), which can identify 13 types of high-risk HPV and 12 low-risk HPVs were used for ISH. Microarray system-based HPV genotyping was carried out from the FFPE tissues, and included 43 types of HPV probes (Goodgene, Seoul, Korea), as described previously [22]. IHC for P16, a sensitive surrogate marker of oncogenic HPV was also performed with the method described above [23].

Statistical analysis

The baseline characteristics of the HNSCC patient groups were compared using Fisher's exact test for discrete variables and the Mann–Whitney *U*-test for continuous variables (Table 1). For statistical analyses, the Pearson χ^2 test was used to compare the anatomical location of the primary tumor, Fischer's exact test for age groups (≤ 60 vs. >60), Fischer's exact test for patient gender, linear by linear association for T-stage and for N stage (from 0 to 2; one N3 case was included in the N2 group), Fischer's exact test for the presence of distant metastasis and the result of primary treatment or recurrence, and the Pearson χ^2 test for treatment modalities (surgery only, radiotherapy only, chemoradiotherapy, surgery with radiotherapy and surgery with chemoradiotherapy) were used (Table 2). Pearson's correlation coefficient was calculated to measure correlations with each marker (Table 3). DFS rates and OS rates were estimated using the Kaplan–Meier method (Table 4). Prognostic values were evaluated using a Cox model, stratified according to center and adjusted for significant prognostic factors for survival (Table 5).

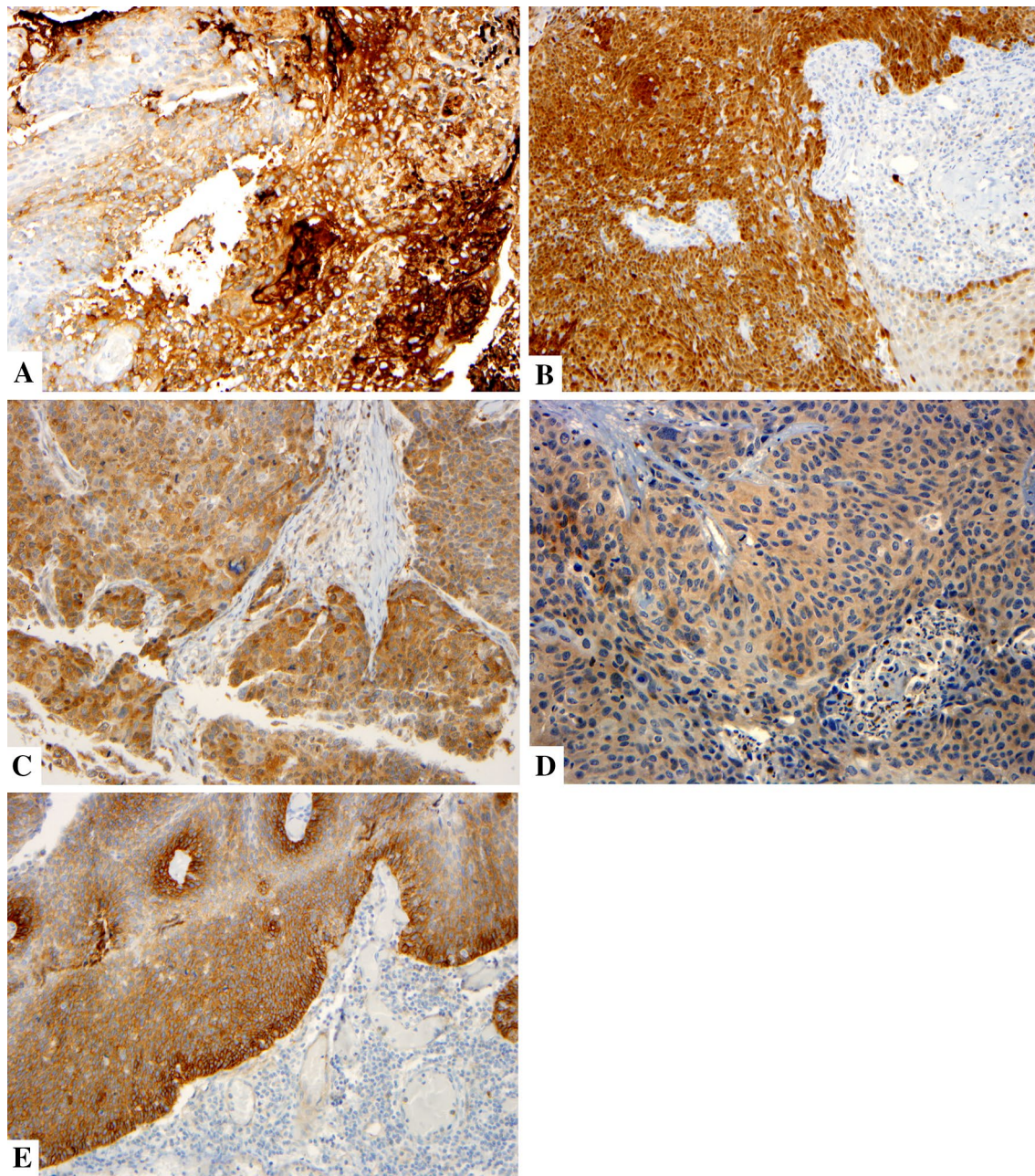


Fig. 2 Representative immunostaining results in head and neck squamous cell carcinoma (all $\times 200$). High expression of HIF-1 α is found in the cytoplasm of tumor cells (a). Expression of HSP70 (b) and

HSP90 (c) is seen in the nuclei and cytoplasm. VEGF is positive in the cytoplasm of tumor cells with moderate intensity (d). IGF-1R is found positive along the cytoplasmic membrane (e)

Results

Patients' characteristics

Table 1 summarizes the general characteristics of the patients. The most common sites were the oropharynx ($n = 32$) and larynx ($n = 29$), followed by the oral cavity ($n = 16$), hypopharynx ($n = 7$), and nasopharynx ($n = 6$). In 90 patients, 20 (22.2 %) were of stage I, 12 (13.3 %) were

of stage II, 12 (13.3 %) were of stage III, and 46 (51.1 %) were of stage IV HNSCC. Lymph node metastasis was found in 51 (56.7 %) cases, and three patients had distant metastases at the time of diagnosis.

Of the 90 patients, 77 (85.6 %) finished their primary treatment completely. As the primary treatment for HNSCC, 20 patients underwent surgery, 25 patients received CCRT with cisplatin, 5 patients were treated with radiotherapy alone, 15 patients were treated with surgery

Table 2 Comparison between expression of HPV, P16, HIF-1 α , HSP70, HSP90, VEGF IGF-1R, and clinical parameters of head and neck squamous cell carcinoma

	HPV	P16	HIF-1 α	HSP70	HSP90	VEGF	IGF-1R
Location	0.038*	0.020*	0.251	0.001*	0.983	0.100	0.669
Age	1.000	0.038*	0.273	0.519	0.385	0.830	0.430
Sex	0.089	1.000	0.475	1.000	0.308	1.000	0.032*
T stage	0.481	0.960	0.704	0.777	0.574	0.919	0.128
N stage	0.007*	0.005*	0.517	0.000*	0.887	0.498	0.829
M stage	0.435	1.000	0.572	0.359	0.124	0.127	0.495
Grade	0.057	0.042*	0.275	0.001*	0.436	0.583	0.827
PTR	0.152	0.736	0.284	0.067	0.204	1.000	1.000
Recurrence	0.224	0.279	1.000	1.000	1.000	1.000	0.719
OTR	0.024*	0.028*	0.088	0.021*	0.746	0.820	0.062

Pearson χ^2 test for location (nasopharynx, oral cavity, oropharynx, hypopharynx, and larynx); Fischer’s exact test for Age group (≤ 60 or >60); Fischer’s exact test for sex (male or female); Linear by linear association for T-stage from 1 to 4; Linear by linear association for N stage from 0 to 2 (1 case of N3 included in N2 group); Fischer’s exact test for M stage 0 and 1; Linear-by-linear association for grade from I to IV; Fischer’s exact test for primary treatments result (success or failure); Fischer’s exact test for recurrence results (yes or no); Pearson χ^2 test for treatment modalities (surgery only, radiotherapy only, chemoradiotherapy, surgery with radiotherapy and surgery with chemoradiotherapy)

PTR Primary treatment response, OTR Overall treatment response

* P value < 0.05

Table 3 Correlations between HPV, P16, HIF-1 α , HSP70, HSP90, VEGF, and IGF-1R in the head and neck squamous cell carcinoma

		HPV	P16	HIF-1 α	HSP70	HSP90	VEGF	IGF-1R
HPV	r	1	0.618**	0.100	0.336*	0.109	0.229	0.452**
	p		0.000	0.456	0.011	0.416	0.090	0.001
P16	r	0.618**	1	0.010	0.320*	0.013	0.238	0.038
	p	0.000		0.935	0.011	0.922	0.060	0.784
HIF-1 α	r	0.100	0.010	1	0.007	0.244*	0.056	0.000
	p	0.456	0.935		0.947	0.022	0.611	0.998
HSP70	r	0.336*	0.320*	0.007	1	0.244*	0.284**	0.104
	p	0.011	0.011	0.947		0.024	0.008	0.453
HSP90	r	0.109	0.013	0.244*	0.244*	1	0.224*	0.261
	p	0.416	0.922	0.022	0.024		0.038	0.052
VEGF	r	0.229	0.238	0.056	0.284**	0.224*	1	0.209
	p	0.090	0.060	0.611	0.008	0.038		0.130
IGF-1R	r	0.452**	0.038	0.000	0.104	0.261	0.209	1
	p	0.001	0.784	0.998	0.453	0.052	0.130	

r pearson’s correlation coefficient, p P-value

* P-value < 0.05 (95 % confidence interval)

** P-value < 0.01 (99 % confidence interval)

followed by radiotherapy, and 12 patients received surgery with CCRT. Primary treatment failed in 21 of 77 patients (27.3 %); 16 of 56 patients (28.6 %) showed local or regional recurrence and 4 (7.1 %) developed lung metastasis (Fig. 1). Of the 90 patients, 11 died during follow-up, with a median follow-up of 29.6 months.

IHC and HPV results

P-values for marker expression and clinical parameters are presented in Table 2. HPV and P16 positivity was seen in 18 of 59 (30.5 %) and 21 of 65 (32.3 %), respectively. As is well known, oropharyngeal tumors showed the highest HPV

and P16 positivity (47.1, 60.1 %) and all six nasopharyngeal tumors were negative for HPV, but there was a low frequency of P16 (16.7 %). HPV and P16 positivity were higher in the advanced N stage group ($P = 0.007$, $P = 0.005$, respectively).

HIF-1 α , HPS70, HPS90, VEGF, and IGF-1R were positive in 33 of 89 (37.1 %), 62 of 87 (71.3 %), 83 of 89 (93.3 %), 41 of 87 (47.1 %), and 50 of 56 (89.3 %) cases, respectively. Expression levels of HIF-1 α , HSP70, HSP90, VEGF, and IGF-1R was divided into high and low groups using the median value of the H-score. High expression for each marker was found in 33 of 89 (37.1 %), 39 of 87 (44.8 %), 35 of 89 (39.3 %), 41 of 87 (47.1 %), and 24 of 56 (42.9 %) cases, respectively.

Table 4 Factors associated with disease free survival (DFS) and overall survival (OS)

	No. of patients	DFS		OS	
		Median DFS (months)	<i>P</i> -value [†]	Mean OS (months)	<i>P</i> -value
Age (years)					
≤60	42	32.4	0.530	49.6	0.881
>60	35	36.6		64.1	
Sex					
Male	70	37.7	0.472	61.2	0.506
Female	7	14.0		32.0	
Organ					
Nphx	5	21.6	0.077*	35.2	0.014**
OC	16	27.4		41.7	
Ophx	23	30.5		42.4	
Hphx	7	12.9		31.3	
Lx	26	46.8		68.7	
T stage					
T1	31	43.8	0.018**	58.2	0.237
T2	18	20.0		44.1	
T3	8	59.2		61.9	
T4	20	22.2		39.8	
N stage					
N0	35	46.9	0.000**	62.2	0.008**
N1	13	24.3		60.8	
N2,3	29	23.5		37.2	
M stage					
M0	64	43.6	0.000**	64.4	0.051*
M1	14	5.5		38.7	
HPV					
Positive	14	26.2	0.234	34.1	0.003**
Negative	35	36.5		64.2	
P16					
Positive	15	28.8	0.767	39.1	0.137
Negative	39	32.4		61.0	
HIF-1α					
High	25	26.4	0.097*	57.7	0.237
Low	51	38.2		55.3	
HSP70					
High	32	26.9	0.043**	54.3	0.451
Low	42	40.7		61.8	
HSP90					
High	37	40.1	0.685	57.8	0.832
Low	39	32.7		61.8	
VEGF					
High	35	34.8	0.866	58.9	0.885
Low	39	35.1		58.7	
IGF-1R					
High	19	33.4	0.710	48.9	0.112
Low	28	28.7		53.7	

Nphx nasopharynx, *OC* oral cavity, *Ophx* oropharynx, *HPhx* hypopharynx, *Lx* larynx

* *P*-value <0.10

** *P*-value <0.05

† *P*-value of Breslow (Generalized Wilcoxon) test

Table 5 Multivariate analysis for disease-free survival (DFS) and overall survival (OS)

	Disease-free survival (DFS)		Overall survival (OS)	
	HR (95 % CI)	<i>P</i> -value	HR (95 % CI)	<i>P</i> -value
HPV	0.634 (0.114–3.542)	0.604	8.684 (0.806–93.579)	0.075*
P16	0.834 (0.175–4.078)	0.834	1.864 (0.170–20.398)	0.610
HIF-1 α	0.431 (0.134–1.384)	0.157	0.265 (0.024–2.925)	0.279
HSP70	0.751 (0.280–2.020)	0.571	0.849 (0.095–7.610)	0.884
HSP90	2.754 (0.952–7.966)	0.062*	0.442 (0.065–3.020)	0.405
VEGF	1.193 (0.355–4.015)	0.775	0.224 (0.016–3.253)	0.274
IGF-1R	2.162 (0.727–6.403)	0.166	3.356 (0.496–22.726)	0.215

* *P*-value <0.10

The high expression rate (high expression group number/total number) of HSP70 was higher in oropharynx cancer (70.9 %) and lower in larynx cancer (17.9 %; $P = 0.001$). The high expression rate also increased with advanced N stage ($P < 0.001$). A high expression rate of HSP70 was also seen in the chemoradiotherapy alone and surgery with chemoradiotherapy groups ($P = 0.021$). None of the female subjects expressed high IGF-1R ($P = 0.032$). Otherwise, there was no significant difference between expression of HIF-1 α , HSP90, and VEGF according to the location, T, N, M, stage, success rate of primary treatment, recurrence rate, or primary treatment modality.

Correlations between HPV, P16, HIF-1 α , HSP70, HSP90, VEGF, and IGF-1R

Pearson's correlation coefficient was calculated to evaluate the correlations of the markers. HPV displayed a strong positive correlation with P16 ($r = 0.618$, $P < 0.001$) and a moderate positive correlation with HSP70 ($r = 0.336$, $P = 0.011$) and IGF-1R ($r = 0.452$, $P = 0.001$).

Expression of P16 showed a moderate positive correlation with HSP70 ($r = 0.320$, $P = 0.001$). HIF-1 α revealed a weak positive correlation with HSP90 ($r = 0.244$, $P = 0.022$). HSP70 showed weak positive correlations with HSP90 ($r = 0.244$, $P = 0.024$) and VEGF ($r = 0.284$, $P = 0.008$). HSP90 showed a weak positive correlation with VEGF ($r = 0.224$, $P = 0.038$; Table 3).

DFS and OS according to protein expression

Survival analysis was performed in 77 patients who completed primary treatment and had a sufficient follow-up period. The 2-year OS rate was 88.1 %, the 2-year DFS rate was 56.0 %, and the median follow-up was 29.6 months in our study group. Figure 3 shows the Kaplan–Meier estimates of the probability of DFS.

Univariate analysis revealed that high HSP70 expression was a significant factor for shorter DFS, along with high T-stage, N-stage, and M-stage (all $P < 0.05$; Table 4). Primary sites and expression of HIF-1 α also showed

statistically significant effects on DFS (within the 90 % confidence interval; $P = 0.077$ and $P = 0.097$, respectively) in a univariate analysis. Larynx cancer was associated with the longest DFS, and hypopharynx cancers the shortest. High HIF-1 α expression was associated with a shorter DFS than low HIF-1 α expression. HPV, P16, HSP90, VEGF, and IGF-1R showed no significant association with DFS (Fig. 3; Table 4).

Regarding OS, primary location was associated with patients' OS; larynx tumors showed the longest OS, while hypopharynx cancer showed the shortest ($P = 0.014$). HPV-negative patients displayed longer OS than HPV-positive patients ($P = 0.003$). Expression of P16, HIF-1 α , HSP70, HSP90, VEGF, and IGF-1R had no statistically significant effect on OS (Table 4).

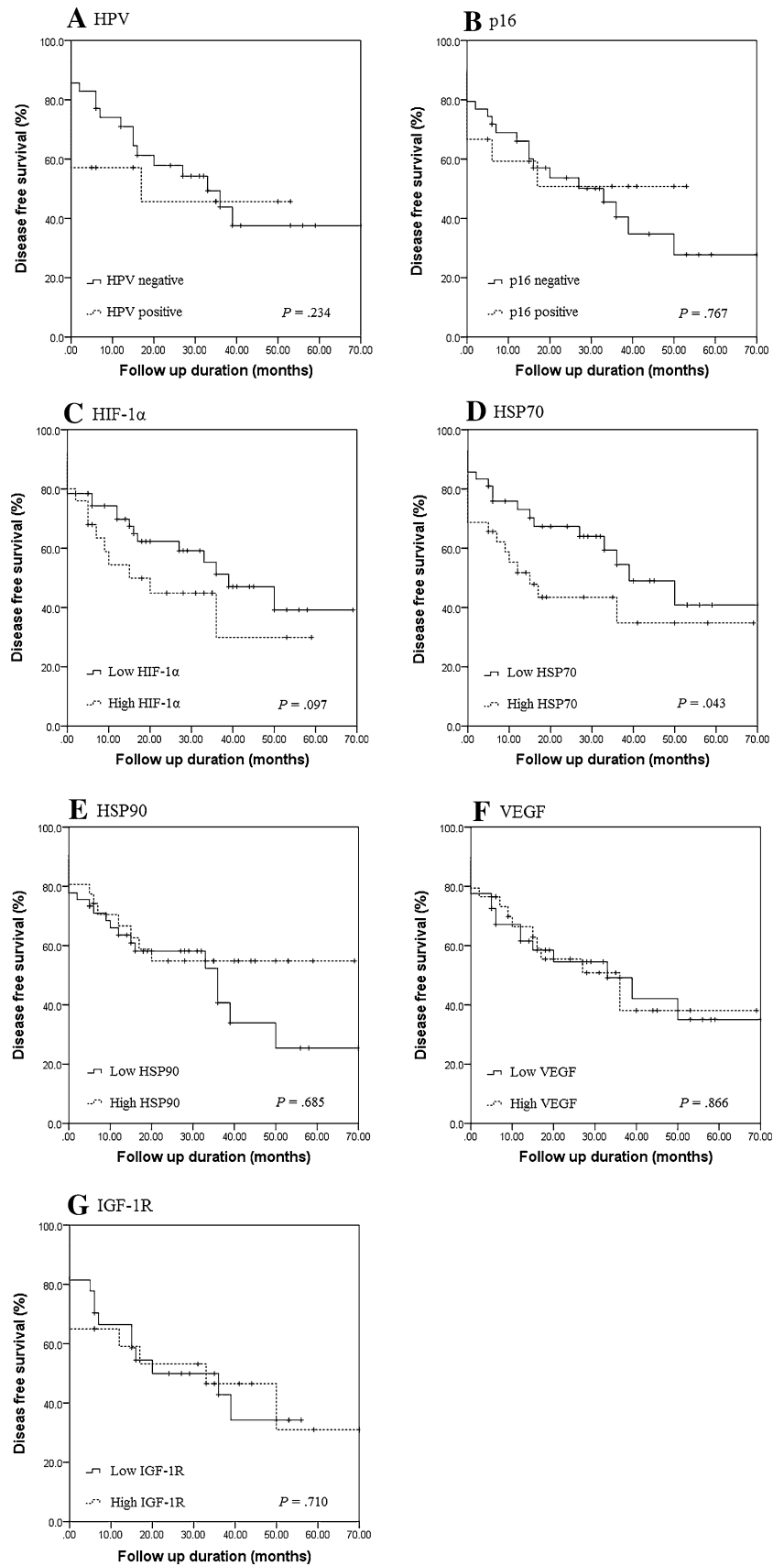
None of the biomarkers examined showed significant associations with DFS or OS in a multivariate analysis (Table 5).

Discussion

Our study revealed that hypoxic signaling markers were highly expressed and associated with adverse clinical outcomes in HNSCC. Of note, HSP70 was frequently overexpressed in oropharyngeal or HPV-positive tumors, and high levels of HSP70 or HIF-1 α predicted a shorter DFS.

A hypoxic microenvironment is established in a rapidly growing tumor lacking sufficient vascularization. In hypoxia, cellular mechanisms to overcome cell death are activated through complex interactions of several molecules. HIF-1 α orchestrates hypoxic signaling with help from HSP90 and HSP70 and also induces transcription of VEGF genes [24, 25]. Although there is some controversy regarding the interaction of HIF-1 α and VEGF [8, 26], these hypoxic signaling molecules exert coordinated effects to overcome cellular stress. In this study, we found positive correlations between the expression of some hypoxic proteins: HSP70 correlated with HSP90 and VEGF, and HSP90 correlated with HIF-1 α and VEGF. Such correlations, along with overall high level of expression of

Fig. 3 Disease-free survival according to protein expression in head and neck squamous cell carcinoma



hypoxic proteins, support the idea that the hypoxic signaling pathway is activated in HNSCC.

Overexpression of HIF-1 α has been found to be related to a poor prognosis in many cancers [27, 28]. In HNSCC, the value of HIF-1 α as a prognostic indicator is unclear because of some inconsistent results, such as in early stage squamous cell carcinoma of the mouth floor [29]. However, most previous studies have shown that expression of HIF-1 α is correlated with frequent treatment failure in HNSCC [26]. Our results are consistent with them; HIF-1 α was associated with poor DFS, with borderline statistical significance. However, we did not find any significant correlation between HIF-1 α expression and clinicopathological parameters, such as T stage, lymph node status, histological grade, or anatomical location. We suspect that activation of HIF-1 α signaling may be a ubiquitous event in carcinogenesis in HNSCC, although higher levels of HIF-1 α are apparently associated with treatment failure.

Prognosis based on HSP70 and/or HSP90 expression has been investigated in many studies [13, 30]; however, the results were inconsistent [31, 32]. In our study, expression of HSP70 correlated with frequent nodal metastasis and was associated with poor DFS. High HSP70 expression was more frequently present in oropharyngeal tumors or HPV-positive cases and correlated with P16. Because HSP70 provokes anti-viral adaptive immune responses in various organs, we suggest that cellular immunity against HPV may also trigger overexpression of HSP70, or vice versa. Recently, enhancing anticancer immunity by molecular engineering of HSP70 and a HPV vaccine has been attempted by several researchers [33, 34]. We found no significant correlation between HSP90 and the various clinicopathological parameters examined. In contrast to HSP70, patients with high HSP90 expression revealed a longer DFS than those with low HSP90 expression, although the difference was not statistically significant. This may be partly explained by a report that expression of HSP90 and HSP70 is regulated in a mutually compensatory manner [35].

There have been several reports that VEGF and IGF-1R are poor prognostic factors in various cancers, including HNSCC [36, 37]. We found frequent expression of VEGF throughout the entire cohort; however, the expression levels were uniformly low and did not correlate with any parameter examined or survival. A strong association between IGF-1R and hypoxic markers, especially with VEGF, has been reported previously [38–40]. In our study, IGF-1R correlated only with HPV positivity. Overexpression of IGF-1R in HPV-related tumors, compared with benign lesions, was reported in one study. They explained that IGF-1R might be high due to the decrease of p53 by means of the promoted proteolysis that is promoted after HPV infection in uterine cervical cancer [41]. Taken together,

our results suggest that hypoxic signaling markers play an important role, especially in HPV-associated tumors.

Conclusions

Hypoxia-associated proteins were highly expressed, and expression of HIF-1 α and/or HSP70 can be considered prognostic indicators in HNSCC. Our results suggest that hypoxic signaling is activated in HNSCC, especially in HPV-related tumors.

Conflict of interest The authors declare that they have no conflict of interest.

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