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Prognostic Significance of Glycolytic Metabolic Change Related to HIF-1α in Oral Squamous Cell Carcinomas

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Background : Growing tumors adapt to a hypoxic environment and increase anaerobic glycolysis. This metabolic switch is related to aggressive behavior. We investigated the relationship between glycolytic metabolism biomarkers associated with hypoxia-inducible factor (HIF)-1α and prognosis. **Methods :** We performed immunohistochemical staining of HIF-1α, pyruvate dehydrogenase kinase (PDK) 1 and lactate dehydrogenase (LDH) 5 in 74 patients with oral squamous cell carcinoma (SCC) who had received curative radical resection. **Results :** High reactivity of HIF-1α, PDK 1 and LDH 5 was observed in 29 (39.2%), 32 (43.2%) and 54 (73.0%) patients, respectively. Expression levels of the three biomarkers were significantly correlated. All three markers were highly expressed in 16 (21.6%) patients. Elevated expression of the three markers was associated with increased invasiveness (p = 0.043) and recurrence (p = 0.017) of tumors. In survival analysis, upregulation of the three markers was additionally associated with shorter disease free survival (DFS, p = 0.001) and overall survival (OS, p = 0.002). High expression of all three markers was a strong independent prognostic factor for DFS (p = 0.030) and OS (p = 0.026). **Conclusions :** Oral SCC with altered glycolytic metabolism exhibits a more invasive and aggressive phenotype. Our results indicate that glycolytic metabolism biomarkers related to HIF-1α may be independent prognostic factors in patients with oral SCC.

Key Words : Carcinoma, squamous cell; HIF1A protein, human; Lactate dehydrogenase 5; Pyruvate dehydrogenase (acetyl-transferring) kinase

The major treatment modalities for squamous cell carcinoma (SCC) in the oropharynx and oral cavity (oral tongue, floor of mouth, buccal, gingival/alveolus, retromolar trigone) at the loco-regional disease stage are surgical resection and radiation therapy, similar to those for other head-and-neck SCCs. However, lymphatics are more well developed in the oral cavity and oropharynx than in other head-and-neck regions; hence lymph node metastasis occurs at earlier stages for tumors in the oral cavity and oropharynx.[1] Complete response is observed in approximately 30-50% of oral cavity and oropharyngeal SCC after treatment, with disease recurrence occurring in the primary area or as neck lymph nodes in 70-90% of cases.[2] Poor prognosis of oral cavity and oropharyngeal cancers may be attributed to the use of the same diagnostic and treatment modalities for these diseases. It is important to note that even similar oral cavity and oropharyngeal SCCs are heterogeneous in terms of tumor biology and molecular biology. However, the prognostic and therapeutic value of biomarkers in the diagnosis and treatment of oral cavity and oropharyngeal cancers remains to be established.

Hypoxia and changes in tumor metabolism are among the decisive stages of carcinogenesis, and mediate important effects in determining the aggressiveness of tumors in several cancers, including head-and-neck SCC.[3,4] During this process, hypoxia-inducible factor (HIF)-1α acts as a key regulator in adaptation to the hypoxic environment and alterations in tumor metabolism.[5] In 1956, Warburg[6] reported increased glucose uptake and glycolysis in tumor cells. Recent research shows that the Warburg

effect is mediated by HIF-1α.[7,8] Under hypoxic conditions, tumor cells produce ATP via anaerobic glycolysis that converts pyruvate to lactate, instead of oxidative phosphorylation of glucose through acetyl-CoA.[7,8] During this process, lactate dehydrogenase A, the enzyme that catalyzes the conversion of pyruvate to lactate, is under the regulation of HIF-1α. Koukourakis *et al*.[9] demonstrated an association of lactate dehydrogenase (LDH) 5 overexpression with poor prognosis in colon cancer. Moreover, tumor LDH and LDH-A mRNA is increased in the head and neck cancer xenograft model.[10] The activity of the pyruvate dehydrogenase complex that converts pyruvate to acetyl-CoA for oxidative phosphorylation is controlled by pyruvate dehydrogenase kinase (PDK) 1 mediated by HIF-1α in head-and-neck-SCCs.[11] Several investigators report that adaptation to hypoxic conditions and changes in tumor metabolism render cancer cells resistant to oxidative stress by reducing reactive oxygen species at the cellular level,[12] consider a process which is associated with resistance to treatment.[13,14]

Head-and-neck SCCs are extremely hypoxic tumors.[15,16] Therefore, in oral cavity and oropharyngeal SCC, hypoxia and consequent changes in glucose metabolism could be major determinants of treatment outcomes. However, the metabolic changes in oral cavity and oropharyngeal SCCs are yet to be elucidated. Here, we investigate the relationships between biomarkers related to glycolytic metabolism (HIF-1α, PDK 1, and LDH 5) and clinicopathologic characteristics, and establish whether these markers can be effectively applied to predict survival of patients with oral cavity and oropharyngeal SCC.

MATERIALS AND METHODS

Patients, specimens and study design

Following approval of the Institutional Review Board, 74 patients diagnosed with oral SCC and treated curatively at Seoul St. Mary's Hospital between 1996 and 2007 were identified through the institutional cancer registry. The criteria for inclusion were as follows: diagnosis of oral SCC, no preoperative therapy, potentially curative radical resection, available follow-up data, and satisfactory tissue preservation. In patients with close (< 1 mm) or positive resection margin or extracapsular spread of positive nodes, postoperative radiotherapy (60 Gy) was administered. Clinicopathologic and follow-up data of the 74 eligible patients were retrospectively reviewed. The sites of primary tumors were recorded as follows: tongue (n = 49, 66.2%), tonsil (n = 13, 17.6%), base of tongue (n = 7, 9.5%), soft palate (n = 3, 4.1%), uvula (n = 1, 1.4%), and floor of mouth (n = 1, 1.4%).

Surgical pathology results were reviewed for size of tumor, depth of invasion, differentiation of primary tumors, surgical margin status, and regional metastasis of the neck dissection specimens. The tumor, node, and metastasis staging categories were determined according to criteria established by the American Joint Committee on Cancer staging system. All patients were followed up with clinical and radiologic examinations.

Tissue preparation and immunohistochemical staining

Immunohistochemistry

Immunohistochemical reactions were performed on paraffin tissue sections using an automated immunohistochemical stainer (Lab Vision Autostainer LV-1, LabVision/Neomarkers, Fremont, CA, USA), according to the manufacturer's protocol. Tissue sections were deparaffinized and quenched with 3% hydrogen peroxide for 10 minutes. Antigen retrieval was performed with 0.01 M citrate buffer (pH 6.0) by heating the sample in a microwave vacuum histoprocessor (RHS-1, Milestone, Bergamo, Italy) at a controlled final temperature of 121℃ for 15 minutes. Primary antibodies were diluted in Dako Antibody Diluent (Dako, Carpinteria, CA, USA) with background-reducing components, and used at the following dilutions: HIF-1α (1 : 800, mouse monoclonal, clone ESEE122, Novus Biologicals, Littleton, CO, USA), LDH isoenzyme V (1 : 800, sheep polyclonal, Abcam, Cambridge, UK), and PDK 1 (1 : 25, rabbit polyclonal, Thermo Fisher Scientific, Fremont, CA, USA). Primary antibodies were incubated at room temperature for 30 minutes, and detection achieved using the Envision plus System (Dako) for HIF-1α and PDK 1, and Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) for LDH isoenzyme V. Immunoreaction was developed with diaminobenzidine (Dako) for 5-10 minutes, and hematoxylin counterstaining applied.

Interpretation of immunohistochemical data

Immunohistochemical data on HIF-1α and LDH 5 were assessed based on nuclear and cytoplasmic staining patterns. The percentages of cancer cells with strong cytoplasmic expression and with nuclear expression were assessed separately in all optical fields. We used a previously established HIF-1α and LDH 5 scoring system to divide samples into groups of low and high reactivity (Table 1).[9] Strong cytoplasmic expression in more than 50% of cancer cells or nuclear expression in more than 10% of

Table 1. HIF-1α and LDH 5 grading system based on the intensity and extent of cytoplasmic and nuclear staining

Scoring system	Score
Complete absence of reactivity	Negative
Weak cytoplasmic reactivity (regardless of the extent)	Low
Strong cytoplasmic reactivity in < 50% of cancer cells	Low
Nuclear expression in sporadic cells (< 10% of cells), with strong cytoplasmic expression in < 50% of cells	Low
Strong cytoplasmic expression in > 50% of cancer cells	High
Nuclear expression in > 10% of cancer cells (regardless of cytoplasmic expression pattern)	High

HIF-1α, hypoxia-inducible factor-1α; LDH 5, lactate dehydrogenase 5.

cancer cells were categorized as bearing 'high reactivity' and other expression patterns were categorized as 'low reactivity.' In case of PDK 1, normal epithelial tonsil cells did not express PDK 1. Strong cytoplasmic expression was noted in muscular cells. In cancer cells, PDK 1 mainly displayed cytoplasmic expression, and the median percentage of cells displaying cytoplasmic staining was used to divide samples into low and high reactivity groups.[11] Assessment was performed by two independent observers.

Statistical analysis

Fisher's exact probability test was applied to evaluate the association between categorical variables. Disease-free survival (DFS) and overall survival (OS) rates were calculated with the Kaplan-Meier method. The log-rank test was used to determine the significance of differences in cumulative survival curves between variables. The Cox proportional hazards model analysis was employed for multivariate analysis using variables that were significant in univariate analysis. Survival rates and odds ratios are presented with their 95% confidence intervals. All statistical comparisons were performed using SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA), with $p < 0.05$ regarded as statistically significant. All tests of statistical significance were two-sided.

RESULTS

Clinicopathologic characteristics of patients

Among the 74 patients, 53 were male and 21 were female, with an overall median age of 60 years (range, 25 to 86 years). Patients were grouped as stage I (25, 33.8%), stage II (17, 23.0%), stage III (8, 10.8%), and stage IV (24, 32.4%). Histopathologic evaluation of lymph node involvement led to the classification of 43 (58.1%) tumors as N0, 7 (9.5%) as N1, and 24 (32.5%) as N2 (Table 2). Relapse was observed in 25 patients and 28 of 74 patients died, with 21 dying of their tumors. Five patients were in the close or positive resection margin category, and 13 (17.6%) received postoperative loco-regional radiotherapy.

Table 2. Patient characteristics

Characteristics	No. of patients (%)
Age (median [range], yr)	60 (25-86)
< 60	33 (44.6)
≥ 60	41 (55.4)
Gender	
Male/Female	53/21 (71.6/28.4)
Primary site	
Oral tongue	56 (75.7)
Oropharynx	18 (24.3)
T stage	
T1/T2	32/35 (43.2/47.3)
T3/T4	5/1 (6.8/1.4)
N stage	
N0/N1/N2	43/7/24 (58.1/9.5/32.5)
Stage	
I/II	25/17 (33.8/23.0)
III/IV	8/24 (10.8/32.4)
Depth of invasion of primary tumor (mm)	
< 10	40 (54.1)
≥ 10	27 (36.5)
Treatment	
Surgery	61 (82.4)
Surgery + radiotherapy	13 (17.6)
Relapse	
Yes	25 (33.8)
No	49 (66.2)

Immunohistochemical staining

Normal mucosa and stroma of tonsil assessed in tissue samples from nononcologic patients were persistently negative for HIF-1α and LDH 5. In the case of HIF-1α, the patterns of expression ranged from negative through weak cytoplasmic staining to strong cytoplasmic reactivity accompanied by a percentage of cells with nuclear reactivity (Fig. 1). According to the previously established HIF-1α scoring system, high reactivity of HIF-1α was recorded in 29 out of 74 patients (39.2%). In

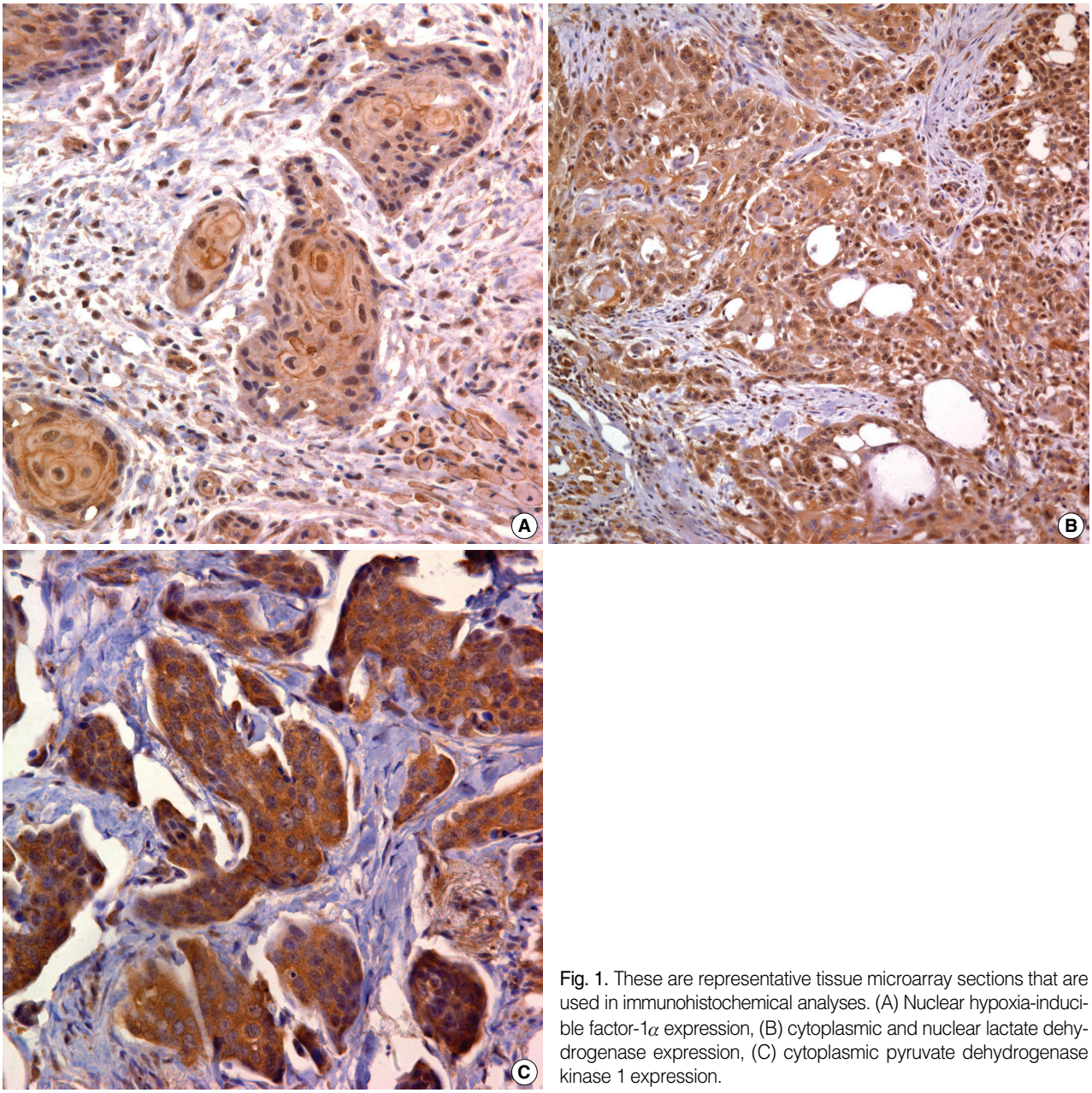

Fig. 1. These are representative tissue microarray sections that are used in immunohistochemical analyses. (A) Nuclear hypoxia-inducible factor-1α expression, (B) cytoplasmic and nuclear lactate dehydrogenase expression, (C) cytoplasmic pyruvate dehydrogenase kinase 1 expression.

terms of LDH 5, the reactivity ranged from limited and weak to extensive and strong cytoplasmic expression with or without a varying percentage of nuclear localization. High reactivity of LDH 5 was recorded in 54 out of 74 patients (73.0%) based on a previously established scoring system (Table 1). In terms of PDK 1, the normal mucosa and stroma of tonsil did not express PDK 1. Staining of PDK 1 was predominantly cytoplasmic. The percentage of cells with strong cytoplasmic expression ranged from 0 to 90% (median, 50%). The median percentage of cells displaying cytoplasmic staining was used to divide samples into low and high reactivity groups.[11] Using 50% as the cut-off value, 32 (43.2%) patients with high PDK 1 activity were identified (Fig. 1).

Association between clinicopathologic characteristics and metabolic markers

The relationships between clinicopathologic characteristics and expression patterns of HIF-1α, PDK 1 and LDH 5 are presented in Table 3. High expression of HIF-1α was significantly

associated with more advanced T stage and invasive tumors (p = 0.029 and p = 0.003, respectively). Elevated PDK 1 expression was significantly correlated with regional lymph node metastasis (p = 0.026), and LDH 5 expression was linked to lymphatic invasion, regional lymph node metastasis, and greater tumor invasiveness (p = 0.028, p = 0.020 and p = 0.015, respectively).

In terms of postoperative recurrence, high expression of HIF-1α and LDH 5 were significantly relevant to tumor recurrence (p = 0.034 and 0.021, respectively).

HIF-1α, PDK 1 and LDH 5 expression levels and their associations

HIF-1α, PDK 1 and LDH 5 expression patterns and associa-

Table 3. Clinical characteristics according to the metabolic markers

	HIF-1α			PDK 1			LDH 5			HIF-1α/PDK 1/LDH 5		
	Low (n = 45)	High (n = 29)	p-value[a]	Low (n = 42)	High (n = 32)	p-value[a]	Low (n = 20)	High (n = 54)	p-value[a]	0-2 high[b] (n = 58)	Triple high[c] (n = 16)	p-value[a]
Age (median, 60 yr)												
< 60	22	11	0.355	21	12	0.284	11	22	0.273	28	5	0.225
≥ 60	23	18		21	20		9	32		30	11	
T stage												
T1	24	8	0.029*	22	10	0.179	11	21	0.214	27	5	0.274
T2-4	21	20		20	21		9	33		31	11	
N stage												
N0	29	14	0.169	29	14	0.026*	16	27	0.020*	36	7	0.189
N1-2	16	15		13	18		4	27		22	9	
Depth of invasion (mm)												
< 10	31	9	0.003*	26	29	0.179	16	24	0.015*	35	5	0.043*
≥ 10	10	17		12	3		4	23		21	10	
Lymphatics invasion												
Absent	26	14	0.423	26	14	0.279	15	25	0.028*	32	8	0.713
Present	19	15		16	18		5	29		26	8	
Perinueral invasion												
Absent	34	23	0.708	36	21	0.140	17	40	0.321	46	11	0.374
Present	11	6		6	11		3	14		12	5	
Nodal ECS												
Absent	31	18	0.779	29	20	0.582	16	33	0.194	39	10	0.750
Present	7	6		6	7		1	12		10	3	
Tumor recurrence												
No recurrent	34	15	0.034*	31	18	0.114	17	36	0.021*	33	6	0.017*
Recurrent	11	14		11	14		3	18		15	10	

*p < 0.05; **p < 0.001.
[a]Fisher's exact test used; [b]Low expression of all markers or high expression of one marker among HIF-1α, PDK 1 or LDH 5 or high expression of two markers among HIF-1α, PDK 1 and LDH 5; [c]High expression of all three markers.
HIF-1α, hypoxia-inducible factor-1α; PDK 1, pyruvate dehydrogenase kinase 1; LDH 5, lactate dehydrogenase 5; ECS, extracapsular spread.

Table 4. Association among HIF-1α, PDK 1 and LDH 5 expression

	HIF-1α			PDK 1		
	Low (%)	High (%)	p-value[a]	Low (%)	High (%)	p-value[a]
LDH 5						
Low (%)	19 (25.7)	1 (1.3)	< 0.001**	17 (23.0)	3 (4.1)	0.003*
High (%)	26 (35.1)	28 (37.8)		25 (33.8)	29 (39.2)	
PDK 1						
Low (%)	30 (40.5)	12 (16.2)	0.032*			
High (%)	15 (20.3)	17 (22.9)				

*p < 0.05; **p < 0.001.
[a]Fisher's exact test used.
HIF-1α, hypoxia-inducible factor-1α; PDK 1, pyruvate dehydrogenase kinase 1; LDH 5, lactate dehydrogenase 5.

tion between markers are evaluated in Table 4. High HIF-1α expression was significantly associated with high LDH 5 and PDK 1 expression ($p < 0.001$ and $p = 0.032$, respectively). Additionally, PDK 1 expression was significantly associated with that of LDH 5 ($p = 0.003$).

Association between clinicopathologic characteristics and co-expression of HIF-1α, PDK 1 and LDH 5

In view of the association between HIF-1α, PDK 1 and LDH 5 levels, we examined the relevance of co-expression of HIF-1α, PDK 1 and LDH 5. Among the 74 specimens, 16 (21.6%) patients co-expressed high levels of HIF- 1α, PDK 1 and LDH 5 (triple high), 17 (22.9%) weakly expressed all three metabolic markers, and 41 (55.4%) displayed elevated expression of one or two of the three markers. High expression of all three metabolic markers was significantly associated with increased invasiveness of tumors ($p = 0.043$) (Table 3).

In terms of postoperative recurrence, co-expression of HIF-1α, PDK 1 and LDH 5 was markedly associated with tumor recurrence ($p = 0.017$). Among 16 patients expressing high levels of all three tumor markers, 10 (62.5%) had tumor recurrence. In contrast, tumor recurrence was seen in 3 (17.6%) of 17 patients expressing low levels of all three markers and 12 (29.2%) of 41 patients expressing high levels of one or two of the three markers (Table 3).

Relationship of HIF-1α, PDK 1 and LDH 5 levels with survival

Among the 74 patients analyzed, 28 died during the follow-up period. The main cause of death was primary tumor recur-

Table 5. Univariate and multivariate analysis for disease-free survival, disease specific survival and overall survival by Cox proportional hazard model

Univariate analysis for disease-free survival and overall survival by Cox proportional hazard model				
	Disease free survival		Overall survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (yr), < 60/≥ 60	0.538 (0.242-1.200)	0.130	0.585 (0.250-1.370)	0.217
T stage (T1/T2-4)	3.144 (1.254-7.887)	0.015*	3.233 (1.191-8.772)	0.021*
N stage (N0/N1-2)	6.633 (2.634-16.701)	< 0.001**	6.561 (2.413-17.836)	< 0.001**
Depth of invasion (mm), < 10/≥ 10	1.013 (1.001-1.024)	0.026*	1.015 (1.004-1.027)	0.008*
Lymphatic invasion, no/yes	4.951 (1.975-12.414)	0.001*	6.547 (2.214-19.357)	0.001*
Perineural invasion, no/yes	2.778 (1.246-6.195)	0.013*	3.681 (1.586-8.546)	0.002*
Nodal ECS , no/yes	1.011 (1.002-1.019)	0.015*	1.012 (1.005-1.021)	0.011*
Resection margin, negative/close (< 1 mm), positive	1.563 (0.461-5.298)	0.473	1.999 (0.581-6.870)	0.272
HIF-1α, low/high	2.384 (1.076-5.284)	0.032*	2.369 (1.033-5.433)	0.042*
PDK 1, low/high	2.131 (1.004-4.524)	0.049*	2.032 (0.887-43652)	0.094
LDH 5, low/high	4.777 (1.425-16.007)	0.011*	3.951 (1.173-13.304)	0.027*
Coexpression of three markers (HIF-1α, PDK 1, and LDH 5)				
0 high[a]	1	0.007*	1	0.008*
1-2 high[b]	3.009 (0.857-10.560)		2.027 (0.554-7.416)	
Triple high[c]	7.302 (1.936-25.545)		5.888 (1.604-21.609)	
Multivariate analysis for disease-free survival and overall survival by Cox proportional hazard model				
	Disease free survival		Overall survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value
N stage (N0/N1-2)	4.716 (1.933-11.507)	0.001*	5.868 (2.306-14.929)	< 0.001**
Coexpression of three markers[d] (HIF-1α, PDK 1, and LDH 5)	2.413 (1.089-5.34)	0.030*	2.552 (1.120-5.814)	0.026*
Perineural invasion, no/yes	2.762 (1.257-6.071)	0.011*		

*p < 0.05; **p < 0.001.

[a]Low expression of all markers; [b]High expression of one marker among HIF-1α, PDK 1 or LDH 5 or high expression of two markers among HIF-1α, PDK 1 and LDH 5; [c]High expression of all three markers; [d]For coexpression of all three markers (HIF-1α, PDK 1 and LDH 5), comparator was 0-2 expression among the three markers.

HR, hazard ratio; 95% CI, 95% confidence interval; ECS, extracapsular spread; HIF-1α, hypoxia-inducible factor-1α; PDK 1, pyruvate dehydrogenase kinase 1; LDH 5, lactate dehydrogenase 5.

rence (n = 21). Median follow-up duration was 28.3 months, and 5-year OS and DFS rates were 58.2% and 55.8%, respectively. Initially, HIF-1α, PDK 1 and LDH 5 expression patterns were analyzed separately. Univariate analysis of HIF-1α expression and Kaplan-Meier survival analysis revealed significantly poorer DFS (p = 0.033), and OS (p = 0.036) for patients displaying high HIF-1α expression, compared to those with low HIF-1α expression (Table 5). In univariate analysis and Kaplan-Meier survival analysis, high LDH 5 expression was associated with significantly poorer DFS (p = 0.005) and OS (p = 0.016) compared to low LDH 5 expression (Table 5), and elevated PDK 1 expression was associated with poorer DFS (p = 0.044) and OS (p = 0.087) compared to low PDK 1 expression (Table 5).

HIF-1α, LDH 5 and PDK 1 expression patterns were assessed as separate variables in multivariate analysis of survival. Other variables included in the model were advanced T stage (T2-4), advanced N stage (N1, 2), more invasive tumor (depth of invasion $\geq$ 10 mm), lymphatic invasion, perineural invasion, and extracapsular extension. In our experiments, advanced N stage was an independent prognostic factor for OS (p < 0.0001), and advanced N stage and perineural invasion were independent prognostic factors for DFS (p < 0.0001 and p = 0.011).

We subsequently evaluated the relevance of the combined expression patterns of HIF-1α, LDH 5, and PDK 1 in survival for each patient due to the significant association of expression of these markers and the connection in their regulation mechanisms. Kaplan-Meier survival analysis revealed that patients in the group displaying high expression of all three markers had significantly poorer DFS and OS, compared with the group expressing low levels of all markers (p = 0.002 and 0.005, respectively) or high levels of 1 or 2 markers (p = 0.027 and 0.012, respectively) or high levels of 0 or 1-2 markers (p = 0.001 and 0.002, respectively) (Fig. 2).

Multivariate analysis with combined expression of HIF-1α, LDH 5, and PDK 1 disclosed that elevated expression of all three markers was associated with significantly poorer DFS (p = 0.030) and OS (p = 0.026). Again, elevated expression of 1 or 2 of the 3 markers was not associated with survival differences. Other factors that were significant in survival outcomes are shown in Table 5.

DISCUSSION

Changes in the glycolytic metabolism are associated with poor prognosis in oral SCC. The correlation between poor prognosis and HIF-1α overexpression in oral SCC has been reported in earlier studies. In our analysis, HIF-1α was highly expressed in 39.1% of patients who displayed significantly decreased DFS (p = 0.033 and p = 0.026, respectively).[13,14] Elevated HIF-1α expression was additionally linked to advanced T stages and increased invasiveness of tumors (p = 0.029 and p = 0.003, respectively). The hypoxic area increases with larger tumor sizes (advanced T stage), and this hypoxia is a strong inducer of HIF-1α. In terms of tumor invasion, depth of invasion in oral SCC is a reliable parameter for predicting regional nodal involvement and survival in oral SCC, especially in patients with N0 stage disease.[17] Cohen *et al*.[18] reported that dysregulation of HIF-1α expression is associated with invasive potential in a head-and-neck SCC cell line. Recently, hypoxia induced epithelial-mesenchymal transi-

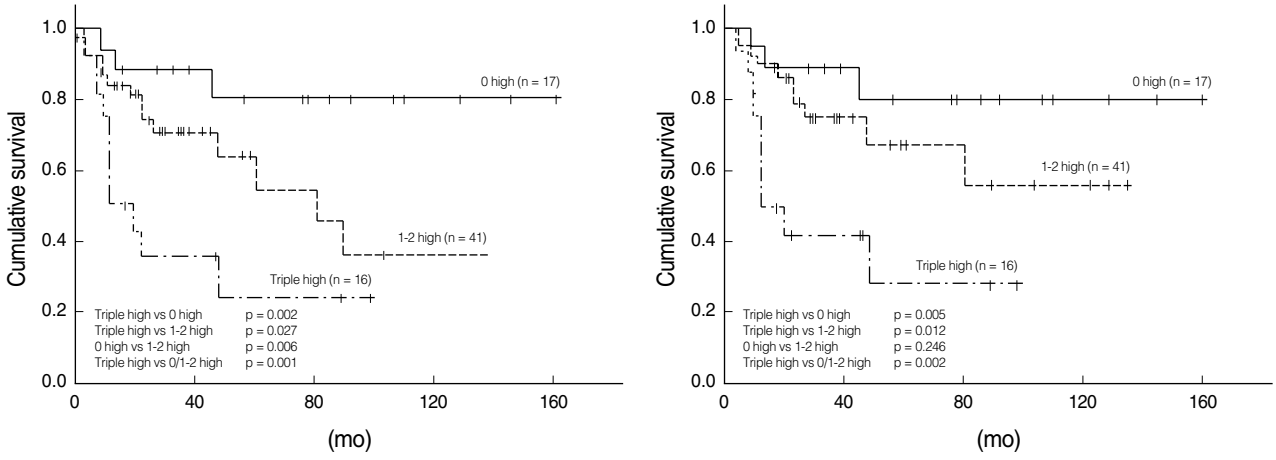

Fig. 2. Kaplan-Meier survival curves are calculated by using combined expression patterns of hypoxia-inducible factor (HIF)-1α, pyruvate dehydrogenase kinase (PDK) 1 and lactate dehydrogenase (LDH) 5 for (A) disease free survival and (B) overall survival (1 high, high expression of one marker among HIF-1α, PDK 1 or LDH 5; 2 high, high expression of two markers among HIF-1α, PDK 1 and LDH 5).

tion mediated by HIF-1α was reported.[19] Epithelial-mesenchymal transition is a decisive stage in tumor invasion and metastasis.[19] Further research on the association of invasiveness of tumor cells with HIF-1α in oral SCC is required.

In our study, expression of PDK 1 expression was associated with lymph node metastasis ($p = 0.026$) and LDH 5 with lymph node metastasis and lymphatics invasion ($p = 0.020$ and $p = 0.028$, respectively). The relationships between metabolites of PDK 1, LDH 5 or their metabolites and lymphangiogenesis or lymph node metastasis are yet to be established. Association of lymph node metastasis with LDH 5 and PDK 1 expression may be part of the metabolic adaptation process that occurs during the progression of carcinogenesis.[20] However, in our experiments, high HIF-1α, PDK 1, and LDH 5 expression patterns were significantly correlated to each other (Table 4). In recent studies on head-and-neck SCC cell lines, expression of tumor LDH and LDH-A mRNA increased during hypoxia,[10] and lactate was produced via HIF-1α-mediated PDK 1 regulation.[11] Lactate elevated during anaerobic glycolysis maintains HIF-1α activation via inhibition of prolyl hydroxylase,[21] possibly supporting an association with molecules known to mediate Warburg effects. HIF-1α-induced increase in lymphangiogenesis through vascular endothelial growth factor (VEGF)-C in esophageal cancer[22] and an association of VEGF-C with HIF-1α in oral SCC were reported.[23] Thus, changes in the glycolytic metabolism via increases in the levels of PDK 1 and LDH 5 may lead to amplification of HIF-1α expression.

In our study, high expression of LDH 5 was associated with tumor invasiveness in addition to lymph node metastasis ($p = 0.015$), and showed significantly decreased DFS ($p = 0.005$). LDH 5 is reported as a poor prognostic factor in colorectal[9] and non-small cell lung cancers.[24] LDH 5 is most directly associated with lactate, the end product of anaerobic glycolysis. Since acidosis and hypoxia augment the invasive capacity of SCC cell lines,[25] lactate production induces peritumoral acidosis and changes in the microenvironment. Acidosis, in turn, increases acid-mediated tumor invasion by degrading the extracellular matrix.[25] In addition to carbonic anhydrase IX investigated for the evaluation of tumor acidosis,[9] expression of LDH 5 in head-and-neck SCC may be a significant factor in association with tumor microenvironment acidosis.

Among the tumor samples examined, all three markers (HIF-1α, PDK 1, and LDH 5) were highly expressed in 16 specimens. Elevated expression of all three markers was significantly associated with increased tumor invasiveness ($p = 0.043$). A statistically significant decrease in DFS was additionally observed in cases where all three markers were expressed (Table 5, Fig. 2). Increased expression of all three markers mediates a synergistic effect on hypoxia-mediated metabolic adaptation. Such tumors show aggressive behavior and are linked to poor prognosis. Furthermore, in leukemia cell lines, the Akt-mediated pathway is associated with an increase in glycolysis and lactate production, independently of HIF-1α.[26] In a head-and-neck SCC cell line, persistent activation of Akt was observed.[27] HIF-1α was additionally affected by oncogenic signals, such as Akt.[28] Thus, changes in the glycolytic metabolism may not only indicate the underlying hypoxic state but also serve as a marker representing the level of change in oncogenic signals.

In univariate analysis, high expression of HIF-1α, PDK 1 or LDH 5 was associated with poorer survival in terms of DFS. However, in multivariate analysis, compared with the advanced N stage, these three markers did not display a statistically significant association with survival. HIF-1α is known to be a marker for poor prognosis in DFS of head-and neck-SCC, including oral cavity and oropharyngeal cancers.[13,29] In our analyses, high expression of HIF-1α was a significant prognostic factor of DFS, together with lymphatic invasion in stage I and II patients ($n = 43$, $p = 0.010$, data not shown). However, in 31 patients in stage III and IV patients, no statistical significance was evident. The discrepancy between our findings and those of Aebersold *et al*.[13] and Winter *et al*.[29] may be due to differences in the proportion of patients treated with radiation therapy; specifically, all the patients considered by Aebersold *et al*.[13] and 85% of those analyzed by Winter *et al*.[29] received radiotherapy, whereas in our study only 11 of 31 patients in an advanced stage could be treated with postsurgical radiotherapy.

There are some limitations in our study. In our study, we employed tissue microarray (TMA). Despite the small diameters of the tissue samples, the data obtained typically offer a high degree of concordance with whole-section analysis. However, if the hypoxic regions were focal, it is possible that the TMA section would not represent hypoxia of the entire tumor, leading to underestimation of the hypoxic state. Also, other already known prognostic biomarkers such as epidermal growth factor receptor and human papilloma virus of oral SCC were not evaluated in this study, thus these prognostic factors, in addition to HIF-1α associated metabolic markers, may have an effect on the results of our study.

In conclusion, we show that oral cavity and oropharynx SCCs with altered glycolytic metabolism display a more invasive and aggressive phenotype, and these alterations are effectively associated with poor prognosis. The association of oncogenic signals

with glycolytic metabolism should be further investigated in oral SCC in order to develop novel drugs and therapeutic modalities on the basis of molecular genetic findings of the present study.

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