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Prognostic Value of Carbonic Anhydrase IX and Ki-67 Expression in Squamous Cell Carcinoma of the Tongue

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Background: Hypoxia-induced changes may allow tumor cells to survive under sustained hypoxic microenvironments resulting in achievement of more aggressive phenotypes. The purpose of this study is to determine the prognostic relevance of the expression of carbonic anhydrase IX (CA IX), a hypoxia-related protein in surgically resected squamous cell carcinoma of the tongue. We also relate CA IX to Ki-67 expression representing tumor cell proliferation to provide a prognostic model.
Methods: We analysed the expression of CA IX and Ki-67 with immunohistochemistry in 60 patients with squamous cell carcinoma of the tongue.
Results: The percentage of CA IX-positive tumor cells had a wide variation from 0.0 to 77.5%, and the Ki-67 expression was 1.50–75.1%. High CA IX and Ki-67 expression ($\geq$10.0% of tumor cells positively stained with CA IX and Ki-67) was associated with a poorer overall survival ($P < 0.05$). High CA IX and Ki-67 expression showed shorter disease-free survival (DFS), although they are not statistically significant. To make a risk model based on the expression of CA IX and Ki-67, we divided the patients into three groups: high risk (high CA IX and Ki-67), low risk (low CA IX and Ki-67) and intermediate risk (either high CA IX or Ki-67). Being in the high-risk group was found to be an independent prognostic factor for overall survival and DFS in multivariate analysis ($P < 0.05$).
Conclusion: The expression of CA IX and Ki-67 may be useful for predicting prognosis in squamous cell carcinoma of the tongue.

Key words: carbonic anhydrase IX – Ki-67 – hypoxia – squamous cell carcinoma – tongue

INTRODUCTION

Hypoxia is a common pathophysiological consequence of a disturbed microcirculation in tumors as tumor cells outgrow their blood supply (1). In the past, tumor hypoxia was regarded as mainly associated with poor response to radiotherapy or chemotherapy, because adequate blood supply is generally required for their anti-cancer effects (2,3). However, the relationship between tumor hypoxia and the aggressive phenotype of tumors has also been suggested. Thus, a variety of genetic changes within tumor cells may occur under hypoxia. These changes may help tumor cells survive under hypoxic microenvironment. Therefore, a clone of tumor cells achieving these hypoxia-induced changes can have more aggressive phenotype resulting in invasion and metastasis (1,4). Thus, the hypoxia-induced changes may be indirect indicators for more aggressive phenotype and worse prognosis.

Hypoxia can stabilize and increase the hypoxia-inducible factor-1α, which is a major transcription factor activating >40 target genes including carbonic anhydrases (5). Therefore, the products of these genes can be used to assess tumor hypoxia in routine clinical biopsy samples such as paraffin-embedded blocks. Carbonic anhydrase IX (CA IX) is a transmembrane enzyme frequently overexpressed in a variety of tumors, and associated with hypoxia and tumor aggressiveness (6–8). CA IX catalyzes the reversible

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hydration of carbon dioxide to carbonic acid (9,10). This leads to intracellular alkalosis and extracellular acidosis in the tumor microenvironment, which allows tumors to survive under hypoxic conditions (11,12). Furthermore, extracellular acidosis facilitates the breakdown of the extracellular matrix promoting local invasion and metastasis (13). Thus, the expression of CA IX in tumor samples may stand for the presence of hypoxia and tumors cells with more aggressive phenotype due to hypoxia-induced cellular changes.

In the past, cells were thought to proliferate slowly or not at all under hypoxic conditions. However, previous studies have suggested the possibility of continuous cellular growth in the hypoxic compartment of tumors (14,15). Although the exact relationship remains unclear, the presence of cells proliferating in the hypoxic compartment may represent more aggressive clones selected by hypoxia. Therefore, pretreatment characterization of tumor hypoxia and proliferation may be useful for predicting prognosis, and this approach may help establish a treatment strategy. In this study, we evaluated a series of squamous cell carcinomas of the tongue with the primary treatment of surgery to assess the prognostic value of CA IX, which was known as a marker for tumor hypoxia. We also analysed the Ki-67 expression representing the fraction of cellular proliferation to prove its prognostic relevance in patients surgically treated tongue squamous cell carcinoma. Thus, we have determined their prognostic value in univariate and multivariate analyses focussing on squamous cell carcinomas developed from the tongue.

PATIENTS AND METHODS

Patients and Tissues

Sixty patients with squamous cell carcinoma of the tongue at the Korea University Medical Center were included in this study from 1997 to 2004. All patients underwent primary surgery as a frontline treatment. All of the patients were staged at the time of surgery following the guidelines of the American Joint Committee on Cancer Staging. Patients received postoperative adjuvant radiation therapy in a multidisciplinary setting. Thus, 41 patients received radiation therapy after surgery (68.3%) and 19 patients received surgery alone (31.7%). Among formalin-fixed, paraffin-embedded archival tissue blocks of squamous cell carcinoma of tongue, we selected the best section from each block representing tumor areas after review of all hematoxylin slides. The included samples were originated from complete resection material. Follow-up data were obtained from medical records. Survival times were measured from the date of surgery. This study was approved by the Institutional Review Board of Korea University Medical Center.

Immunohistochemistry

Immunohistochemistry for CA IX and Ki-67 was performed using consecutive 4 μm serial sections of formalin-fixed, paraffin-embedded tissues placed onto positively charged glass slides using a single-staining procedure. Two observers reviewed the immunostained slides, and disagreement between the two observers was resolved by consensus. All of the interpretations of the immunohistochemistry were without knowledge of the patient clinical outcome.

Carbonic Anhydrase IX (CA IX)

We used the anti-CA IX mouse monoclonal antibody clone M75 (gift from Dr E.O.). Tissue sections were dewaxed and rehydrated followed by antigen retrieval by incubation with proteinase K (S3028, Dako, Copenhagen, Denmark) for 5 min. Staining was performed using an Envision kit (K4007, Dako). After blocking with 10% human serum to prevent non-specific binding for 30 min, the slides were incubated with the primary antibody (10 μg/ml) for 1 h at room temperature. After the incubation with labeled HRP of Envision kit for 30 min, color was developed by 5 min incubation in 3,3′-diaminobenzidine (DAB) solution. A positive control was used with human cervical squamous cell carcinoma tissue that was previously established to be positive for CA IX (16,17). Two batch controls were included in each run to assess variability of immunohistochemistry. After the slides were scanned at low magnification (×40), three areas (per case) of maximum CA IX expression were selected as previously described (15). The degree of CA IX staining on each selected field was assessed at high magnification (×200) based on the semiquantitative scale of 0–3: grade 0, no staining; grade 1, light staining; grade 2, moderate staining; grade 3 intense staining. Grade 3 staining means the equal intensity to positive control (cervical squamous cell carcinoma). The percentage of tumor cells stained for CA IX was measured at low magnification (×40). The mean value of the examined fields was designated as the final value for each case.

Ki-67

For the detection of Ki-67, we performed immunostaining with the anti-Ki-67 mouse monoclonal antibody (clone MIB-1, DakoCytomation, CA, USA) based on the above-mentioned procedure. When tumor cells showed intranuclear DAB staining, they were considered positive for the Ki-67 antigen. After the slides were scanned at low magnification (×40), three areas (per case) of maximum Ki-67 expression were selected. The cells with positively stained nuclei were counted in there fields at high magnification (×400). The percentage of Ki-67 expression was quantified by determining the number of positive cells expressing nuclear Ki-67 among the total number of tumor cells per high power field.

Statistical Analysis

The Fisher's exact test was applied to assess the association between categorical variables. Thus, the correlation of each

marker expression with clinical and pathological characteristics was tested in univariate analysis. The coefficient of correlation (r) between expressions of markers was calculated using the Spearman's rank test. The period of overall survival (OS) was measured from the date of surgery to the date of death or the last follow-up visit. Disease-free survival (DFS) was measured from the date of surgery to the date of disease relapse or the date of the last follow-up visit. OS and DFS curves were calculated using the Kaplan–Meier method and compared by the log-rank test. The Cox proportional hazards regression model was used for multivariate analyses. All the statistical analyses were performed using a statistical software package (SPSS, Version 10.0, Inc., Chicago, IL, USA). Statistical significance was defined as p-values <0.05. All p-values were two-sided.

RESULTS

Clinical and Pathological Patient Characteristics

The clinical and pathological characteristics of the patients enrolled in this study are summarized in Table 1. The median follow-up duration at the time of analysis was 29.51 months (range 4.10–117.13 months). The median age of the patients at diagnosis was 54.9 years (range 20.0–79.4 years), and the ratio of males to females was 2:1. Twenty-nine patients had Stages I and II disease (48.4%), and 20 cases developed from the tongue base. There were 30 patients with relapse during follow-up; among them 22 deaths occurred; 19 were cancer-related deaths and 3 were not related to the cancer: 1 acute myocardial infarction, 1 subarachnoid hemorrhage and 1 pneumonia. There were 30 patients alive without evidence of disease relapse, and eight patients were alive with disease.

CA IX and Ki-67 Expression

The immunostaining for CA IX expression showed a membranous and/or cytoplasmic staining pattern. We designated positive CA IX expression as those with membranous and/or cytoplasmic staining with an intensity of grade 3 (Fig. 1A) to exclude false positive cases such as non-specific or vaguely immunostained cases. The distribution of positive CA IX expression was focal and predominantly found around necrotic areas. The percentage of CA IX-positive tumor cells had a wide variation from 0.0 to 77.5%. The intensity of CA IX immunostaining was significantly correlated with the percentage of CA IX positive tumor cells ($P < 0.05$). Positive Ki-67 expression was defined by nuclear staining for Ki-67 antigen, and the range of percentage of Ki-67 expression was 1.50–75.1% (Fig. 1B).

To assess the inter-relationships between CA IX and Ki-67 expression, the percentage of CA IX-positive tumor cells was compared with Ki-67 expression using the Spearman's rank test. CA IX expression showed a weak correlation with Ki-67 expression ($r = 0.373$, $P = 0.0008$,

Table 1. Characteristics of patients

Characteristics	No. of patients (%)
Gender	
Male	40 (66.7)
Female	20 (33.3)
Age	
Median, range (54.9, 20.0–79.4)	
<60	43 (71.7)
≥60	17 (28.3)
Primary site of involvement	
Anterior	6 (10.0)
Lateral	34 (56.7)
Base	20 (33.3)
Degree of differentiation	
Well differentiated	35 (58.3)
Moderately/poorly differentiated	25 (41.7)
T stage	
T1/T2	18/28 (30.0/46.6)
T3/T4	7/7 (11.7/11.7)
N stage	
N0	33 (55.0)
N1	9 (15.0)
N2	18 (30.0)
Stage	
I/II	13/16 (21.7/26.7)
III/IV	10/21 (16.6/35.0)
Treatment	
Surgery followed by radiotherapy	41 (68.3)
Surgery alone	19 (31.7)
Smoking	
Yes	30 (50.0)
No	30 (50.0)
Resection margin	
Negative	50 (83.3)
Positive	10 (16.7)
Relapse	
Yes	30 (50.0)
No	30 (50.0)
Health status	
Alive without disease	30 (50.0)
Alive with disease	8 (13.3)
Cancer-related death	19 (31.7)
Non-cancer-related death	3 (5.0)

T, tumor; N, lymph node.

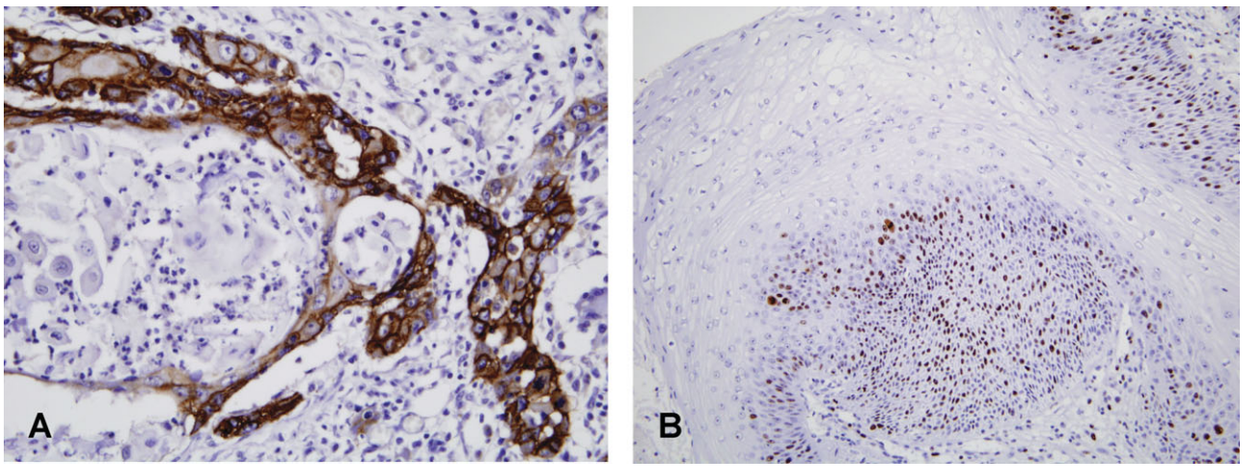

Figure 1. Representative staining of carbonic anhydrase IX and Ki-67 (magnification ×200). (A) CA IX expression, brown colored cytoplasmic and membranous staining. (B) Ki-67 expression, brown colored nuclear staining. CA, carbonic anhydrase.

Fig. 2). When we compared the CA IX expression with Ki-67 expression in the same field, tumor areas positive for CA IX also showed the presence of tumor cells positively stained for Ki-67 (Fig. 2). These findings suggest that the proliferating tumor cells may exist in the tumor areas with high CA IX expression representing hypoxic microenvironment. This is consistent with the possibility of continuous cellular growth in the hypoxic compartment of tumors.

Association with Clinical and Pathological Characteristics

We used the percentage of tumor cells positively stained with CA IX and Ki-67 to designate high and low expression instead of the staining intensity. Thus, we designated 10.0% as a cut-off value for high CA IX expression because 22 cases with $<10.0\%$ showed the range of 0.0–2.5%. For Ki-67 expression, 10.0% was used as a cut-off value to dichotomize patients into high and low expression group because it is a median value.

High CA IX expression was more frequently observed in moderately or poorly differentiated tumors compared with the well-differentiated tumors ($P = 0.007$), whereas Ki-67 expression did not correlate with the degree of differentiation. When we compared their expression based on the site of involvement, tongue base showed high CA IX and Ki-67 expression than other sites of involvement such as lateral or anterior tongue ($P < 0.05$). Nodal involvement was

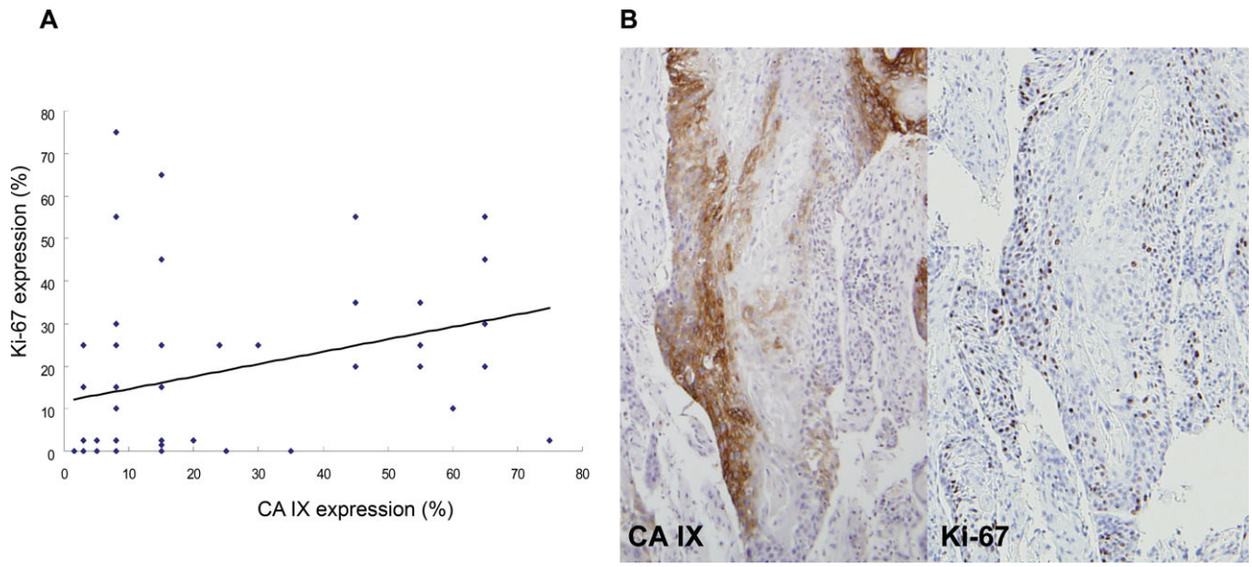

Figure 2. Inter-relationship of CA IX and Ki-67 expression. (A) The percentage of CA IX-positive cells showed a weak correlation with Ki-67 expression ($r = 0.373$, $P = 0.0008$). (B) Immunohistochemistry was performed (magnification ×200), and CA IX expression was compared with Ki-67 expression.

correlated with Ki-67 expression, and however, stage was not associated with CA IX or Ki-67 expression. Smoking status was significantly associated with CA IX expression ($P = 0.003$). Patients treated with adjuvant radiotherapy had higher CA IX and Ki-67 expression than patients treated with surgery alone (Table 2). Because we have decided to perform postoperative radiotherapy for patients with high probability of relapse or unfavorable histological characteristics, these findings suggest that CA IX and Ki-67 expression might be related with an aggressive tumor phenotype.

Relationship with Prognosis

Among the 30 cases with relapse, 27 were loco-regional and 3 were at distant sites: lung and bone metastasis. Tumor relapse was not significantly related with the expression of CA IX and Ki-67, although 22 cases with relapse showed high CA IX expression (Table 2). When the overall survival was analysed in the univariate analysis, high CA IX and Ki-67 expression was significantly associated with a poorer overall survival as illustrated by Kaplan–Meier curves (Fig. 3A). The DFS ($P > 0.05$, Fig. 2B) was not significantly different based on these markers' expression. However, high CA IX and Ki-67 expression showed shorter DFS than low CA IX and Ki-67, although they are not statistically significant (Fig. 3B).

Thus, we made an expression profile with CA IX and Ki-67 expression status for risk-stratification model. We divided the patients into three groups depending on the expression of CA IX and Ki-67: the group with a high expression of CA IX and Ki-67 (designated as high risk), the group with a high expression of CA IX or Ki-67 (designated as intermediate risk) and the group with low expression of CA IX and Ki-67 (low risk). When we analysed DFS and overall survival based on these risk groups, there was a significant association with poorer overall and DFS (Fig. 4A and B). This risk-stratification model was also significantly associated with the site of involvement, the degree of differentiation and relapse rate (Table 3).

To examine the independent prognostic significance of this expression profile with CA IX and Ki-67, multivariate analysis was performed with clinical pathological characteristics including age, stage, location of primary site, presence of tumor cells in the resection margin, degree of differentiation and treatment modality. The risk model based on the expression profile of CA IX and Ki-67 was an independent prognostic factor for overall survival and DSF ($p = 0.005$, 0.007, respectively, Table 4).

Table 2. Correlations of CA IX and Ki-67 with clinical and pathological characteristics

Characteristics	CA IX			Ki-67		
	<10%	≥10%		<10%	≥10%	
Gender						
Male	12	28		17	23	
Female	10	10	0.161	12	8	0.275
Age						
<60	16	27		20	23	
≥60	6	11	1.000	9	8	0.777
Location						
Anterior	1	5		2	4	
Lateral	17	17		22	12	
Base	4	16	0.049	5	15	0.014
Differentiation						
Well	18	17		20	15	
Moderately/ poorly	4	21	0.007	9	16	0.124
T stage						
T1/T2	17	29		23	23	
T3/T4	5	9	1.000	6	8	0.763
N stage						
N0	13	20		21	12	
N1–2	9	18	0.789	8	19	0.011
Stage						
I/II	12	17		17	12	
III/IV	10	21	0.593	12	19	0.196
Surgery + radiotherapy	12	29		16	25	
Surgery alone	10	9	0.094	13	6	0.052
Smoking						
Non-smoker	17	13		18	12	
Smoker	5	25	0.003	11	19	0.120
Relapse						
Yes	8	22		12	18	
No	14	16	0.180	17	13	0.301

CA, carbonic anhydrase.

DISCUSSION

Squamous cell carcinoma of the oral cavity is estimated to be the twelfth most common cancer (18), among which the tongue is one of the most frequent sites. In most cases of squamous cell carcinoma of the tongue, the primary treatment is surgical resection alone or combined with postoperative radiotherapy (19). Despite advances in surgical techniques, the overall prognosis remains poor and loco-regional relapse accounts for most treatment failures (19). Our results showed 27 loco-regional relapsed cases among 30 cases with relapse emphasizing the need for better local control of this disease. Although the resection margin is one of the most important factors closely related to relapse, our study demonstrated a limited number of cases with a positive resection margin; when a positive margin was present it was not significantly associated with relapse or survival ($P > 0.05$, data not shown); only 5 cases had a

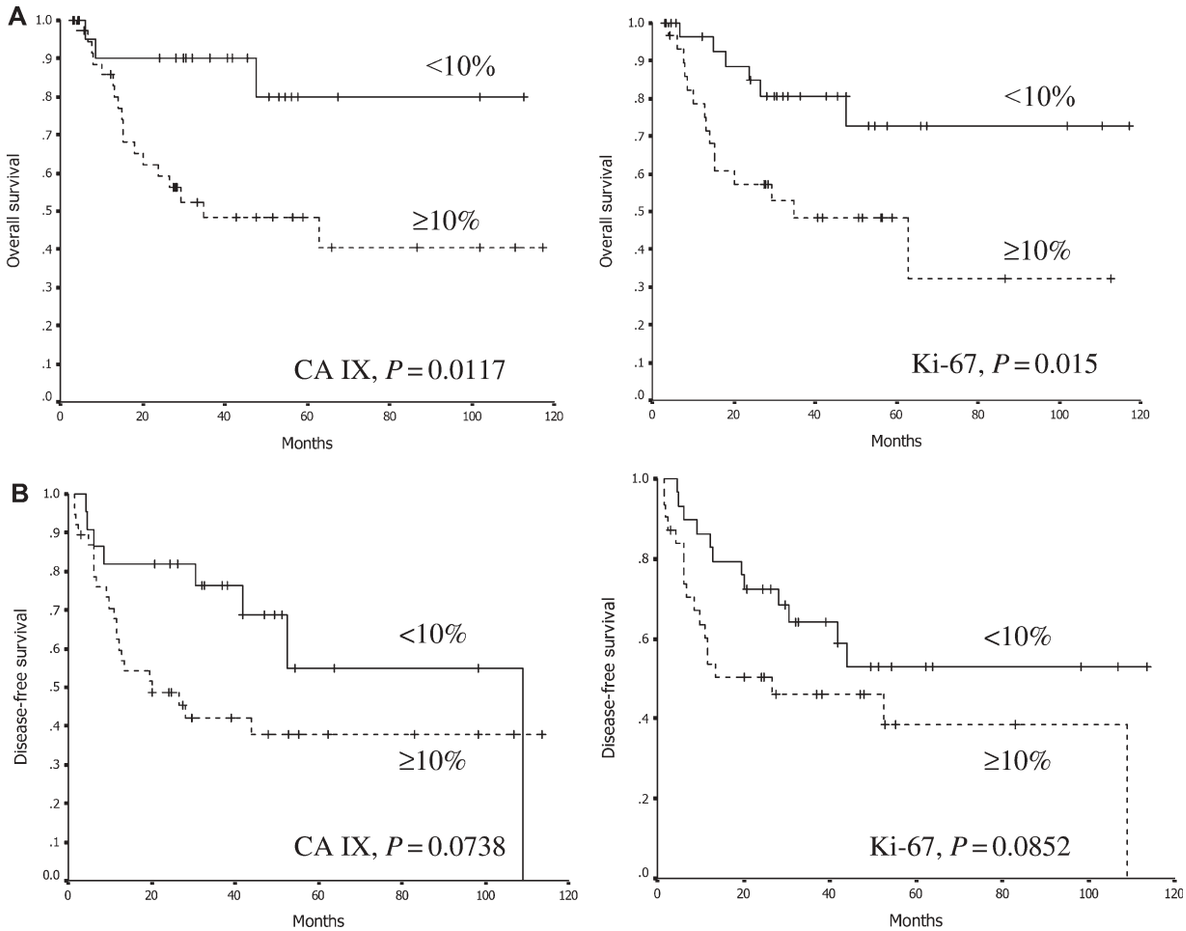

Figure 3. Overall and disease-free survival analysis. (A) High CA IX and Ki-67 expression (the percentage of positively stained tumor cells ≥10.0%) was significantly associated with poorer overall survival in the univariate analysis as illustrated by Kaplan–Meier curves. (B) Disease-free survival was not associated with high CA IX and Ki-67 expression, although they showed a trend towards better disease-free survival.

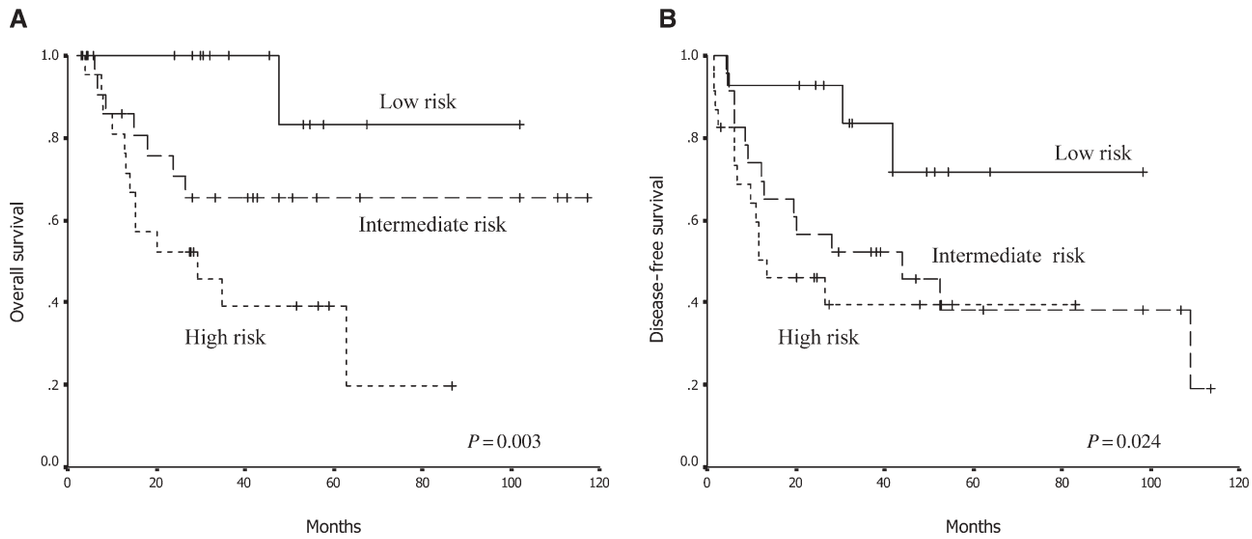

Figure 4. Survival analysis based on the profile of CA IX and Ki-67. The group with a high expression of CA IX and Ki-67: high-risk group, the group with a high expression of CA IX or Ki-67: intermediate-risk group and the group with low expression of CA IX and Ki-67: low-risk group. The high-risk group shows a significant association with poorer overall survival (A) and disease-free survival (B).

Table 3. Correlations of risk stratification model with clinical and pathological characteristics

Characteristics	Risk			
	Low	Intermediate	High	*P* value
Gender				
Male	7	15	18	0.206
Female	7	8	5	
Age				
<60	11	14	18	0.343
≥60	3	9	5	
Location				
Anterior	1	1	4	0.013
Lateral	12	15	7	
Base	1	7	12	
Differentiation				
Well	12	14	9	0.020
Moderately/poorly	2	9	14	
T stage				
T1/T2	12	16	18	0.516
T3/T4	2	7	5	
N stage				
N0	11	12	10	0.108
N1–2	3	11	13	
Stage				
I/II	10	9	10	0.136
III/IV	4	14	13	
Surgery + radiotherapy	6	16	19	
Surgery alone	8	7	4	0.041
Smoking				
Non-smoker	12	11	7	
Smoker	2	12	16	0.005
Relapse				
Yes	3	9	13	
No	11	14	10	0.049

positive resection margin among 30 relapses. The extent of the primary tumor and the state of the regional lymph nodes are known to correlate with prognosis (19,20). However, a substantial number of patients with early stage disease have relapse and a poor prognosis. In our study, the stage including T stage and N stage correlated with overall survival. However, there were 12 relapsed cases among 29 patients with Stage I/II (41.4%). Thus, there is a need to identify additional prognostic parameters that can complement the current TNM staging system.

Our study showed a significant relationship between the expression of CA IX and Ki-67 and overall survival. Although the results were not statistically significant

Table 4. Cox proportional hazards regression model analysis of disease free survival and overall survival

Variables	Overall survival			Disease-free survival		
	Coefficient	SE	*P* value	Coefficient	SE	*P* value
Sex	0.561	0.586	0.324	0.703	0.457	0.440
Age ≥60	3.014	0.560	0.049	2.236	0.435	0.064
Location: base	1.815	0.419	0.155	1.678	0.430	0.229
Moderately/poorly differentiated	0.383	0.562	0.088	0.730	0.454	0.487
Positive resection margin	1.333	0.591	0.627	0.876	0.573	0.818
Surgery + radiotherapy	0.847	0.910	0.855	0.258	0.594	0.023
Stage III/IV	3.360	0.653	0.063	2.457	0.520	0.084
CAIX/Ki-67 risk: high	4.040	0.494	0.005	2.386	0.324	0.007

SE, standard error of the coefficient.

($P > 0.05$), the group with high CA IX and Ki-67 expression had a poorer DSF. These findings are consistent with previous reports on the prognostic value of CA IX for oral squamous cell carcinoma (8,21). CA IX has been extensively studied as a prognostic factor in a variety of tumors especially in squamous cell carcinoma. This is because squamous cell carcinoma is frequently accompanied by necrosis and CA IX is expressed predominantly around necrosis. This perinecrotic expression reflects the association of CA IX with hypoxia (15,22). In this study, we also observed dominant expression of CA IX around necrotic areas. Co-localization of an exogenous marker of hypoxia, pimonidazole, with CA IX supports the association of CA IX with hypoxia in the tumor microenvironment (6,23). However, the relationship of CA IX with hypoxia and prognosis is debated because some reports have not shown an association (17,24). But our results showing the correlation of CA IX expression with prognosis may become indirect evidence that hypoxia is a poor prognostic sign.

In this study, a weak correlation was observed between CA IX and KI-67 expression. Although their correlation was not strong, these findings suggest at least the presence of proliferating tumor cells in the tumor areas with high CA IX expression representing hypoxic microenvironment. These are consistent with prior reports about the correlation of cellular proliferation with hypoxic microenvironment (15,25,26). The proliferating tumor cell clones under hypoxic conditions may represent tumor cells developing a more aggressive phenotype (15,27), and the co-expression of hypoxia markers and Ki-67 may represent tumor aggressiveness. Thus, when we combine the expression of CA IX and Ki-67, the group of patients with CA IX and Ki-67 designated as a high-risk group had a poorer overall survival and DSF, although each factor failed to show a correlation with DSF.

Our study population was confined to squamous cell carcinoma of the tongue, because most prior studies on the prognostic value of CA IX and Ki-67 have been in head and neck squamous cell carcinoma including various sites of the head and neck such as larynx, pharynx, nasal cavity and floor of the mouth (8,21). This is the first study on the prognostic value of CA IX and Ki-67 in patients with squamous cell carcinoma of tongue. Although the staging system of the American Joint Committee on Cancer Staging is the accepted predictor of prognosis, the survival data for each stage shows heterogeneous populations; patients with the same stage disease vary, and some have a poorer prognosis than others do. Therefore, identifying patients at high risk for a poor prognosis would allow for the development of treatment strategies. Considering the prognostic significance of the profile combining CA IX and Ki-67 expression demonstrated in our study, these factors might be useful for selecting patients who require additional treatment before or after primary treatment.

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Conflict of interest statement

None declared.

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