Predictive Markers for Late Cervical Metastasis in Stage I and II Invasive Squamous Cell Carcinoma of the Oral Tongue

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ABSTRACT

Purpose: **Patients with oral tongue carcinoma treated by intraoral excision only should be followed up carefully for cervical lymph node metastasis and salvaged immediately if found, because some patients have a more aggressive clinical course. The purpose of this study was to find useful markers for predicting late cervical metastasis in patients with stage I and II invasive squamous cell carcinoma of the oral tongue.**

Experimental Design: **We investigated clinicopathologic factors and immunohistochemical biomarkers predicting late cervical metastasis in surgical specimens from 56 patients with $T_{1-2}N_0M_0$ invasive squamous cell carcinoma of the oral tongue who did not undergo elective neck dissection. Histopathologic factors including tumor thickness, mode of invasion, Broders grade, total score of three different malignancy grading systems, eight other clinicopathologic parameters, and immunohistochemical expression of p53, cyclin D1, Ki-67, epidermal growth factor receptor, microvessel density, cyclooxygenase-2, MUC1, laminin-5 γ2, E-cadherin, and β-catenin were examined. All of the clinicopathologic factors and immunohistochemical expression of biomarkers were compared in terms of survival.**

Results: **In the univariate analysis, tumor thickness ($P = 0.009$), Broders grade ($P = 0.017$), nest shape ($P = 0.005$), mode of invasion ($P < 0.001$), Anneroth score ($P = 0.029$), Bryne score ($P < 0.001$), and E-cadherin expression ($P = 0.003$) were correlated with late cervical metastasis. Multivariate analysis on late cervical metastasis revealed that tumor thickness >4 mm, mode of invasion grade 3 or 4, and E-cadherin expression were independent factors. Late cervical metastasis was the only prognostic factor for overall survival ($P = 0.002$).**

Conclusions: **Our results indicate that patients with stage I and II invasive squamous cell carcinoma of the oral tongue with tumor thickness >4 mm, mode of invasion grade 3 or 4, and low expression of E-cadherin should be considered a high-risk group for late cervical metastasis when a wait-and-see policy for the neck is adopted.**

INTRODUCTION

The presence of lymph node metastasis is the most important prognostic factor in head and neck carcinoma. Survival rates may decrease by ~50%, and the possibility of distant metastasis may increase when there is cervical node involvement (1, 2). Many methods are used to detect cervical lymph node metastasis clinically, and the imaging studies used include computed tomography, magnetic resonance imaging, ultrasound, and positron emission tomography. Despite negative imaging study, late cervical metastasis develops in some clinically N_0 patients. The sensitivity of preoperative imaging by computed tomography or magnetic resonance imaging and clinical examination is only ~70% (3), and there are no imaging studies capable of detecting micrometastasis in cervical lymph node. The current standard of care is to perform a neck dissection even in the clinically negative neck when the chance of occult metastasis exceeds 20%, but there is controversy as to whether patients with stage I and II oral tongue carcinoma should undergo elective neck dissection or not.

The process of metastasis is very complex and is considered a late event in tumorigenesis. Cells proliferate, lose contact with neighboring cells, migrate through the interstitial matrix, invade blood and lymph vessels, and grow out again in lymph nodes or distant organs. Gene products in these steps can be used as predictive markers of lymph node metastasis, but it is unknown which markers are predictive for cervical lymph node metastasis in clinically N_0 necks in early invasive squamous cell carcinoma of the oral tongue.

The present study sought to identify clinicopathologic factors and immunohistochemical biomarkers predicting late cervical metastasis in stage I and II invasive squamous cell carcinoma of the oral tongue. Molecular factors including p53, cyclin D1, Ki-67, epidermal growth factor receptor, microvessel density (MVD), cyclooxygenase-2 (COX-2), MUC1, laminin-5 γ2, E-cadherin, and β-catenin were selected as candidates. Tumor suppressor gene *p53* has been shown to be a useful predictor of regional recurrence in stage I tongue carcinoma (4). Epidermal

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Table 1 Panel of primary antibodies

Antibody	Clone	Dilution	AR[a]	City/nation	Source
p53		1:200	MW	Tokyo/Japan	Nichirei
Ki-67	MIB-1	1:200	MW	Glostrup/Denmark	DAKO
EGFR	EGFR.113	1:20	MW	Newcastle/UK	Novocastra
Cyclin D1	P2D11F11	1:50	MW	Newcastle/UK	Novocastra
CD31	JC/70A	1:50	MW	Glostrup/Denmark	DAKO
COX-2		1:50	MW	Gunma/Japan	IBL
MUC1	Ma695	1:100	MW	Newcastle/UK	Novocastra
Laminin-5	D4B5	1:1,000	protease	Temecula/CA	Chemicon
E-cadherin	36	1:100	MW	Lexington/KY	Transduction laboratories
β-catenin	14	1:200	MW	Lexington/KY	Transduction laboratories

[a] AR, antigen retrieval; MW, microwave; protease, 0.05% protease XXIV.

growth factor receptor (EGFR) is a potent membrane-bound tyrosine kinase that promotes cellular proliferation, and overexpression of EGFR has been reported to be of prognostic value in head and neck carcinoma (5). Cyclin D1 is a cell cycle regulatory molecule in the G_1 to S-phase control and has been shown to be associated with increased lymph node stage in anterior tongue cancer (6, 7). Ki-67 is a proliferation marker, and the Ki-67 index has been reported to be correlated with poor prognosis in oral tongue squamous cell carcinoma (8). E-cadherin and β-catenin complex are important in cellular adhesion, and E-cadherin has been reported to be a significant prognostic factor for recurrence and survival in oral tongue carcinoma (9) and to be correlated with nodal metastasis in laryngeal carcinoma (10). Laminin-5 γ2 is an invasion-associated molecule and has been shown to be correlated with poor outcome in squamous cell carcinoma of the tongue (11). MVD has been reported to be correlated with regional recurrence in $T_{1-3}N_0$ oral cavity squamous cell carcinoma (12). MUC1 is a mucin-like glycoprotein encoded by the *MUC1* gene and has been reported to be correlated with lymph node metastasis in oral squamous cell carcinoma (13). COX-2 has been found to be up-regulated in head and neck carcinoma (14) and has been shown to increase tumor metastasis potential (15). In addition to the aforementioned molecular factors, we also assessed histopathologic parameters, including tumor thickness, mode of invasion, malignancy grading systems that have been used to evaluate prognosis, and cervical lymph node metastasis of oral cancer (4, 16–25) in the present study.

PATIENTS AND METHODS

Patients. We reviewed the medical records of 402 patients treated for squamous cell carcinoma of the oral tongue at the National Cancer Center Hospital East between 1992 and 2000. The subjects of this study were limited to patients with stage I/II ($T_{1-2}N_0M_0$) invasive squamous cell carcinoma of the oral tongue. Cervical lymph node of the patient was considered N_0 when there is no palpable lymph node by physical examination and size of the lymph node is <1 cm by both computed tomography and magnetic resonance imaging without any area suggesting central necrosis or metastasis. Patients with stage III/IV cases (191 cases) carcinoma *in situ* and microinvasive squamous cell carcinoma (56 cases), presence of multiple carcinoma with other sites of tongue (32 cases), verrucous carcinoma (11 cases), and recurrent carcinoma cases (78 cases) were excluded to evaluate cervical lymph node metastasis of primary invasive squamous cell carcinoma of the oral tongue. Ultimately, 56 patients were selected for this retrospective study: all of the patients underwent partial glossectomy through the mouth without elective neck dissection, and no patients had positive surgical margin, preoperative or postoperative treatment, and recurrence at the primary site. Of the 56 patients, 18 developed late cervical metastasis within 2 years postoperatively, and 38 had no evidence of disease after surgery. The mean age of the patients was 61 years (range, 26–87 years), and the male:female ratio was 1.2:1 (31 men and 25 women). The preoperative clinical American Joint Committee on Cancer (1997) stage was $cT_1N_0M_0$ in 24 patients and $cT_2N_0M_0$ in 32 patients. The follow-up period was at least 2 years.

Methods. Paraffin-embedded specimens were retrieved from the files of the pathology department and cut into serial sections 4-μm thick. Immunohistochemical staining and H&E staining were performed.

Histopathologic Evaluation. All of the slides were reviewed at the maximum cross-section by two observers (S-C. L. and G. I.) without knowledge of the clinical data. The histological evaluation of the invasive front of the tumor was based on the grading system of Bryne *et al.* (Ref. 23; Table 1). The Anneroth score (24) and Martinez-Gimeno score (25) were calculated. Mode of invasion was graded from 1 to 4 based on the criteria used by Bryne *et al.* (23) and Anneroth and Hansen (24). Grade 1 tumors had well-delineated, pushing borders. Grade 2 tumors had an advancing edge of the tumor that infiltrated in solid cords, bands, or strands. Grade 3 lesions had a margin that contained small groups or cords of infiltrating cells. In grade 4, the host/tumor interface showed marked cellular dissociation into small groups or even single cells. Tumors were grouped into mode of invasion 1, 2 and 3, 4 (26). Lymphatic invasion, vascular invasion, and perineural invasion were also recorded. Tumor thickness was measured from the surface of the normal mucosa to the deepest portion of the tumor regardless of whether the growth type was exophytic or ulcerative (17). The shape of tumor nest is also discussed. Tumors that had oval-shaped or sheet-like nests with a round margin (with $>80\%$ of the tumor area showing these features) were classified as type-A tumors. Tumors that had asteroid-shaped tumor nests with a spiculated margin or scattered small tumor nests (with $>20\%$ of tumor area showing these features) were classified as type-B tumors (27).

Immunohistochemical Staining. Immunohistochemical staining was performed by the avidin-biotin-peroxidase complex method. Formalin-fixed, paraffin-embedded whole specimens were cut into 4-μm sections. The sections were then deparaffinized and dehydrated, and endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min, followed by microwaving (750 W, 20 min in citrate buffer) for 20 min or incubation with 0.05% protease XXIV for 10 min at room temperature. After treatment with 2.0% BSA and 5% skim milk in PBS for 20 min to block nonspecific binding of primary antibodies, the slides were then incubated with primary antibodies according to the manufacturer's recommendations (Table 1). After washing in PBS, the slides were incubated with biotin-labeled antimouse or antirabbit secondary antibodies (Vector Laboratories Inc., Burlingame, CA) at room temperature for 30 min, and then washed in PBS and incubated with peroxidase-labeled streptavidin (DAKO, Glostrup, Denmark) for 30 min. The reaction products were visualized by immersing the slides in freshly prepared diaminobenzidine (Dojindo, Kumamoto, Japan) solution for 10–20 min, and the slides were counterstained with Mayer's hematoxylin before dehydration and mounting.

Evaluation of Immunohistochemistry. The percentages of p53, cyclin D1, and Ki-67-positive tumor cells were calculated by counting the number of brown-stained tumor nuclei among total number of tumor cells in the most highly stained area on each slide in the selected microscopic field at ×200. The area of each microscope fields was 0.391 mm^2. In each specimen, ~1000 tumor cells were examined. According to previous literature (28) and histogram patterns of staining, we classified the sample as positive if >10, >40, and >30% of the tumor nuclei were stained by anti-p53, anticyclin D1, and Ki-67 antibodies, respectively. EGFR, E-cadherin, and β-catenin were expressed specifically on the cell membrane. According to the intensity of cell membrane EGFR staining, all of the specimens could be divided into the following four groups: (*a*) group 0+, fainter staining than normal epithelium; (*b*) group 1+, the same as normal; (*c*) group 2+, moderately stronger than normal; and (*d*) group 3+, markedly stronger staining. Groups 0+ and 1+ were defined as negative for EGFR expression, and groups 2+ and 3+ were defined as positive for EGFR expression (29). Expression of E-cadherin and β-catenin was defined as high when membrane staining of >50% of the cells was observed and low when membrane staining ≤50% of the cells stained (9). Small blood vessels were visualized by staining endothelial cells for CD31 antibody. Hot spot was identified on each slide, and the MVD was calculated as the highest numbers of vessels on a ×200 field and patients were then divided into two groups with the median value as the dividing line. In calculating the MVD, areas of inflammation, sclerotic tumor, and adjacent benign tissue were excluded. Laminin-5 γ2 was expressed in the cytoplasm of cancer cells, and the staining pattern were categorized as: (*a*) few or no tumor cells positive; (*b*) part of the tumor nest periphery positive; (*c*) the entire circumference of the tumor nest periphery positive; and (*d*) almost all of the tumor cells positive. Specimens were then grouped into pattern a, b and pattern c, d (11). COX-2 expression was in the cytoplasm of the cancer cells. Cytoplasmic staining with COX-2 was recorded as positive when the staining was stronger than that of normal epithelial cells. Sample was defined as COX-2-positive when cytoplasmic staining of >10% of the cells was observed (30). Membranous and/or cytoplasmic staining of MUC1 was graded as positive if immunoreactivity was present.

Statistical Analysis. All of the data were tabulated, and statistical tests were performed with SPSS 10.0 statistical software. The χ^2 test was used to assess associations between late cervical metastasis, and both clinicopathologic factors and immunohistochemical biomarkers. The predictive significance of the clinicopathologic factors and immunohistochemical biomarkers for late cervical metastasis was assessed by multivariate logistic regression analysis. The correlations between clinicopathologic factors, and immunohistochemical biomarkers and survival curves were plotted by the Kaplan-Meier method. The log rank test and Breslow test were used to compare difference in survival between two groups. The prognostic significance of clinicopathologic factors and immunohistochemical biomarkers for overall survival was assessed by Cox's multivariate proportional regression analysis. A $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Clinicopathologic and Immunohistochemical Findings. The patients ($n = 56$) were divided into two groups according to the value for each parameter. Clinicopathologic and immunohistochemical findings are shown in Tables 2 and 3. Nuclear expression of p53, cyclin D1, and Ki-67 ranged from 0% to 95%, 3% to 91%, and 8% to 77%, respectively. Membranous staining of EGFR was group 0+ in 4 (7%), group 1+ in 22 (39%), group 2+ in 24 (43%), and group 3+ in 6 (11%). E-cadherin and β-catenin expression ranged from 2% to 82% and 2% to 74%, respectively (Fig. 1). MVD ranged from 27 to 158 microvessels/field (= 0.391 mm^2), and the median value was 74.5. Cytoplasmic expression of laminin-5 γ2 were pattern a in 1 (2%), pattern b in 19 (35%), pattern c in 29 (52%), and pattern d in 7 (11%). COX-2 expression was noted in the cytoplasm of cancer cells and varied in intensity. Focal strong expression was mainly found in the superficial portion of the cancers. MUC1 was focally expressed in the parakeratin layer around keratin pearls.

Univariate Analysis of Clinicopathologic Factors and Immunohistochemical Biomarkers in Late Cervical Metastasis. The associations between clinicopathologic factors and late cervical metastasis are shown in Table 2. Late cervical metastasis correlated with Broders grade ($P = 0.017$), nest shape ($P = 0.005$), tumor thickness ($P = 0.009$), mode of invasion ($P < 0.001$), Anneroth score ($P = 0.029$), and Bryne score ($P < 0.001$), but not with age, sex, cT stage, lymphatic invasion, vascular invasion, perineural invasion, or Martinez-Gimeno score. The results of univariate analysis of immunohistochemical biomarkers are summarized in Table 3. There was a significant association between late cervical metastasis and E-cadherin expression ($P = 0.003$), and E-cadherin expression was correlated with Broders grade ($P = 0.001$) but not with β-catenin expression. None of the immunohistochemical biomarkers except E-cadherin were significant.

Logistic Regression Analysis of Clinicopathologic Factors and Immunohistochemical Biomarkers in Late Cervical Metastasis. Multivariate logistic regression analysis of clinicopathologic factors and immunohistochemical biomarkers revealed that mode of invasion, tumor thickness, and E-cadherin

Table 2 Clinicopathologic factors and late cervical metastasis

Factors			No. of cases	No. of nodal metastasis (%)	*P*
Age	>mean 61		27	7 (26%)	0.500
	<mean 61		29	11 (38%)	
Sex	Male		31	12 (38%)	0.377
	Female		25	6 (24%)	
cT	T_1		24	6 (25%)	0.483
	T_2		32	12 (38%)	
Broders grade	Well		33	6 (18%)	0.017
	Moderately, poorly		23	12 (52%)	
Keratinization	>50%		17	3 (18%)	0.222
	<50%		39	15 (38%)	
Nest shape	Type A		26	3 (12%)	0.005
	Type B		30	15 (50%)	
Tumor thickness	>4 mm		38	17 (45%)	0.009
	<4 mm		18	1 (5%)	
Lymphatic invasion	Negative		47	14 (30%)	0.636
	Positive		9	4 (44%)	
Vascular invasion	Negative		35	11 (31%)	>0.999
	Positive		21	7 (33%)	
Perineural invasion	Negative		49	16 (20%)	>0.999
	Positive		7	2 (28%)	
Mode of invasion	Grade 1, 2		31	3 (10%)	<0.001
	Grade 3, 4		25	15 (60%)	
Grading system	Bryne	>7	23	14 (60%)	<0.001
	Martinez	<7	33	4 (12%)	
	Martinez	>17	23	9 (39%)	0.520
	Gimeno	<17	33	9 (27%)	
	Anneroth	>11	27	13 (50%)	0.029
		<11	29	5 (17%)	

had predictive value for late cervical metastasis ($P = 0.027$, $P = 0.016$, and $P = 0.049$, respectively; Table 4). To establish a more effective method for the prediction of late cervical metastasis, three significant factors in the multivariate analysis were combined in a linear discrimination analysis, and the combination of these three factors was found to be more predictive on late cervical metastasis (Table 5). It had 77.8% and 89.5% in sensitivity and specificity, respectively, and 77.8% and 89.5% in the positive predictive value and negative predictive value, respectively.

Kaplan-Meier Analysis of Overall Survival. The cumulative 5-year survival rate in stage I and II invasive squamous cell carcinoma of the oral tongue was 82.1%. Late cervical metastasis was significant in both the log rank test ($P = 0.0011$)

Table 3 Immunohistochemical biomarkers and late cervical metastasis

Biomarker		No. of cases	No. of nodal metastasis (%)	*P*
E-cadherin	>50%	18	1 (5%)	0.003
	<50%	38	17 (45%)	
β-Catenin	>50%	18	3 (17%)	0.161
	<50%	38	15 (39%)	
EGFR	0, 1+	26	8 (31%)	>0.999
	2+, 3+	30	10 (33%)	
p53	>10%	31	10 (32%)	>0.999
	<10%	25	8 (32%)	
Cyclin D1	>40%	25	7 (28%)	0.758
	<40%	31	11 (35%)	
Laminin-5 γ2	Pattern A, B	20	4 (20%)	0.249
	Pattern C, D	36	14 (38%)	
Ki-67	>30%	35	14 (40%)	0.184
	<30%	21	4 (19%)	
MVD	>median 74.5	28	8 (28%)	0.775
	<median 74.5	28	10 (36%)	
COX-2	>10%	39	10 (26%)	0.205
	<10%	17	8 (47%)	
MUC1	>10%	31	11 (35%)	0.758
	<10%	25	7 (28%)	

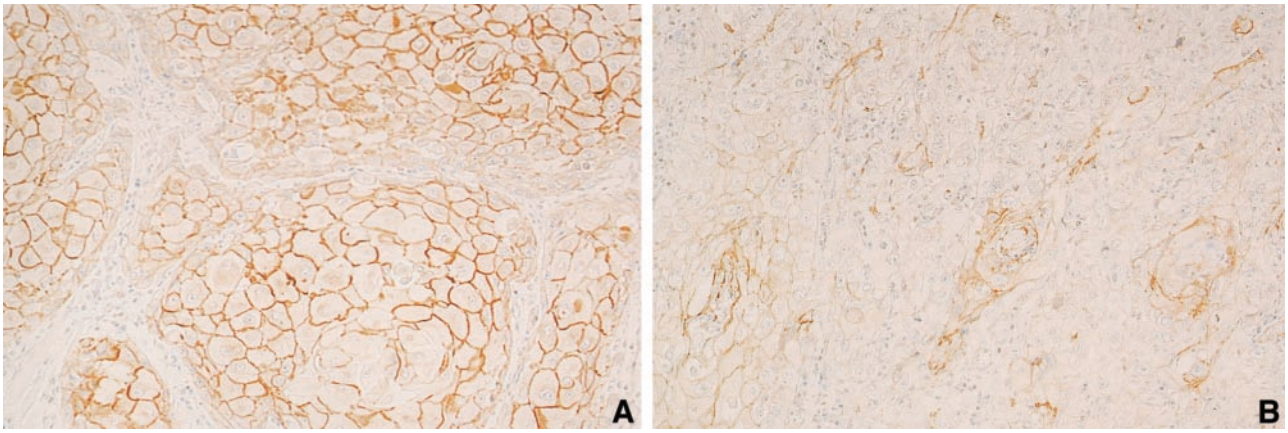

Fig. 1 Immunohistochemical findings of E-cadherin showing high expression (*A*) and low expression (*B;* ×200).

Table 4 Multivariate logistic regression analysis on late cervical metastasis

Factors	*P*	Risk ratio	95% confidence interval
Mode of invasion	0.027	6.523	1.242–34.274
Tumor thickness	0.016	16.616	1.685–163.859
E-cadherin	0.049	0.098	0.10–0.998

and the Breslow test ($P = 0.0004$; Table 6). Nest shape ($P = 0.0366$), Broders grade ($P = 0.0336$), mode of invasion ($P = 0.0429$), and Bryne score ($P = 0.0266$) were significant in the Breslow test. When the Cox proportional hazard regression model was used to analyze these factors for overall survival, late cervical metastasis was demonstrated to be the only independent factor for overall survival ($P = 0.002$).

DISCUSSION

There are two options for treatment of the neck in early oral cavity carcinoma. One is elective neck dissection policy and the other is wait-and-see policy. Elective neck dissection itself entails low morbidity and allows accurate N staging, but it may be an unnecessary operation in some patients, and adjuvant therapy such as radiation is required when the dissected cervical nodes turn out to be metastatic lymphadenopathy. On the other hand, previous studies have reported that wait-and-see group had better survival than elective neck dissection group (31–33). The answer as to which policy should be adopted in stage I and II squamous cell carcinoma of the oral tongue will require a large prospective randomized multicenter study, and early detection of cervical lymph node metastasis is obviously very important to improving survival.

In addition to conventional imaging studies, ultrasound-guided fine-needle aspiration cytology has been used to detect occult metastasis. Its sensitivity for the clinically N_0 neck has been reported to be as high as 73% and to have a specificity of 100% (34), although others have reported sensitivities in the 42–50% range (35–37). A combination of sentinel node lymphoscintigraphy and ultrasound-guided fine-needle aspiration cytology has been reported, but adding sentinel lymphoscintigraphy did not improve the results (31). Because it is difficult technically, ultrasound-guided fine-needle aspiration cytology is not generally used. Because occult cervical metastases in the wait-and-see group may develop more unfavorable characteristics such as extracapsular spread and multiple lymph node involvement if careful follow-up is not done, in this study, we attempted to find useful predictive markers of late cervical metastasis in early invasive squamous carcinoma of the oral tongue by assessing clinicopathologic factors and immunohistochemical biomarkers with which we are familiar.

Several studies have focused on the tumor thickness of oral tongue cancer (17, 19–22, 38–42), and although it has been found to be an important factor predicting cervical metastasis, its cutoff value has varied. In this study, we have shown that thickness >4 mm predicts late cervical metastasis, and Kurokawa *et al.* (20) suggested that a tumor depth of ≥4 mm and moderately differentiated squamous cell carcinoma of the tongue are associated with a higher rate of late cervical metastasis. In the study by Yuen *et al.* (40), tumor thickness from >3 mm to 9 mm was associated with a 50% nodal metastasis rate and a 11% local recurrence, whereas Hosal *et al.* (19) found that thickness of ≥9 mm was the only variable that predicted occult lymph node metastasis in carcinoma of the oral tongue. Although there is consensus about the importance of tumor thickness, the most valid cutoff value is not decided yet. These differences in tumor thickness cutoff value may be attributable to many factors, such as the definition of tumor thickness and oblique section in processing surgical specimen.

Table 5 Linear discrimination analysis for late cervical metastasis

Combination of factors	Probability (%)
TT >4 mm + MOI 3/4 + E-cad <50%[a]	77.8% (14/18)
TT >4 mm + MOI 3/4	70.0% (14/20)
MOI 3/4 + E-cad <50%	65.2% (15/23)
TT >4 mm + E-cad <50%	61.5% (16/26)
MOI 1/2 + E-cad >50%	6.2% (1/16)
TT <4 mm + E-cad >50%	0% (0/6)
TT <4 mm + MOI 1/2	0% (0/13)
TT <4 mm + MOI 1/2 + E-cad >50%	0% (0/6)

[a] TT, tumor thickness; MOI, mode of invasion; E-cad, E-cadherin.

Table 6 Kaplan-Meier analysis and clinicopathologic factors

Factors			*P*	
			Log-rank test	Breslow test
Age	>61		0.9150	0.9338
	<61			
Sex	Male		0.3717	0.3746
	Female			
cT	T_1		0.1488	0.2094
	T_2			
Broders grade	Well		.1196	0.0336
	Moderately, poorly			
Keratinization	>50%		0.3334	0.2997
	<50%			
Nest shape	Type A		0.0950	0.0366
	Type B			
Tumor thickness	>4 mm		.7760	0.5959
	<4 mm			
Lymphatic invasion	Negative		.9904	0.7581
	Positive			
Vascular invasion	Negative		0.0539	0.0637
	Positive			
Perineural invasion	Negative		0.8225	0.5846
	Positive			
Mode of invasion	Grade 1, 2		0.1325	0.0429
	Grade 3, 4			
Grading system	Bryne	>7	0.0883	0.0266
		<7		
	Martinez	>17	0.5187	0.5661
	Gimeno	<17		
	Anneroth	>11	0.1719	0.0654
		<11		
Late cervical metastasis	Negative		0.0011	0.0004
	Positive			

The importance of mode of invasion and the malignancy grading system has been reported frequently (26, 39, 43). We found grade 3 or 4 mode of invasion in stage I and II invasive squamous cell carcinoma of the oral tongue to be correlated significantly with late cervical metastasis in the multivariate analysis and this finding was consistent with the study by Spiro *et al.* (26). We also evaluated correlations between three malignancy grading systems (Bryne, Anneroth, and Martinez-Gimeno score) and late cervical metastasis and found that both Bryne score and Anneroth score were significant in the univariate analysis alone. The combination of tumor thickness and mode of invasion yielded a predictive rate of 70%, and it can be used as a marker during follow-up, because evaluation of mode of invasion and measurement of tumor thickness are simple and easy.

The E-cadherin and β-catenin system is closely related to invasion and metastasis. The results of our multivariate analysis showed that E-cadherin expression was the only immunohistochemical biomarker associated with late cervical metastasis, although its correlation with cervical metastasis in head and neck carcinoma is debatable. In contrast to our findings, the study by Okamoto *et al.* (22) reported no correlation between E-cadherin expression and late cervical metastasis, but that Flt-4, a lymphatic marker, was correlated in stage I and II tongue cancer. This discrepancy can be attributed to their heterogeneous treatment modalities and the smaller sample size in their study compared with our study design. Although we performed Flt-4 immunohistochemical staining in our study, we were very disappointed with the nonspecific staining pattern of commercially available Flt-4 antibody. No specific marker of lymphatic vessels is yet available, and there is a need to find reliable one.

The only significant factor affecting overall survival in the multivariate analysis was late cervical metastasis, and the importance of late cervical metastasis cannot be too overemphasized. In this study we found that tumor thickness, mode of invasion, and low level of E-cadherin expression were associated significantly with increased risk of late cervical metastasis and that combination of these three independent factors was more predictive. It seems reasonable to stratify the patients into a high- and low-risk group, and place patients with tumor thickness >4 mm, mode of invasion grade 3 or 4, and low expression of E-cadherin in the high-risk group. Combination of these factors may be a useful marker for deciding on salvage treatment during wait-and-see follow-up.

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