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ORIGINAL PAPER

Integrin Expression Levels Correlate with Invasion, Metastasis and Prognosis of Oral Squamous Cell Carcinoma

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Abstract The present study evaluated the relationship between alpha 3, alpha 6A, and beta 1 integrin expression in cancer cells at the invasive front of oral squamous cell carcinoma (OSCC) and survival rates, as well as the clinical and pathological characteristics. Sections of 100 specimens of primary OSCC were immunostained to assess alpha 3, alpha 6A, and beta 1 integrin expression in cancer cells at the invasive front. OSCC patients with higher expression levels of alpha 3, alpha 6A, and beta 1 integrin had significantly better prognosis than those with lower expression levels (median survival at low *vs.* high expression levels: alpha 3, 37.1 months *vs*. 55.7 months; alpha 6A , 38.3 months *vs*. 47.9 months; and beta 1, 26.1 months *vs*. 46.1 months) ($P<0.05$). In addition, beta 1 integrin expression showed the highest correlation with clinical and pathological characteristics. This study concludes that alpha 3, alpha 6A, and beta 1 integrin expression in cancer cells at the invasive front are related to the mode of invasion and prognosis in OSCC.

Keywords Integrin · Oral squamous cell carcinoma · Invasion · Metastasis · Prognosis

Abbreviations

ECM	extracellular matrix
OSCC	oral squamous cell carcinoma
SCC	squamous cell carcinoma

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Introduction

Squamous epithelium comprises intra- as well as extracellular matrix adhesion molecules that show robust binding interactions including intercellular binding involving desmosomes and cadherins and that between the matrix layer and extracellular matrix (ECM) involving fibronectin and integrins. Several studies have been conducted on adhesion between cancer cells and the ECM [1, 2]. In fact, studies on cell—ECM adhesion began before the role of integrins was conceptualized in 1986 [1–3]. Integrins are cellular transmembrane proteins that have recently drawn attention as bi-directional signaling molecules, *i.e.*, they transmit signals from the outside to the inside of cells and vice versa. Eighteen alpha and eight beta subunits combine into around 25 different integrins, each of which is capable of binding to a specific type of cell surface and ECM protein ligand [4, 5].

Until date, involvement of integrins in vascular neogenesis [6] as well as the expression and distribution of various integrins in pancreatic [7, 8], lung [9], and colon cancers [10], have been investigated. These studies indicate that alpha 3, alpha 6A, and beta 1 integrins are significantly involved in the invasive metastasis of cancers [11–15]. Metastasis of squamous cell carcinoma (SCC) in the oral cavity occurs following a decrease or loss of the ability of cells to adhere by E-cadherin [16]. Few studies, however, there are limited studies integrins on parameters predicting prognoses that are related to invasion and metastasis of oral cancers [17]. We, therefore, investigated the expression of alpha 3, alpha 6A, and beta 1 integrin subunits in oral SCC (OSCC), the extent to which integrins induce adhesion of cancer cells to the ECM, and its correlation with cancer invasion and lymph node metastasis.

Materials and Methods

Patients

Pre-treatment biopsy specimens obtained from 100 patients (51 males and 49 females; mean age 67 years) with primary OSCC who had been treated in Kanazawa University Hospital between April 1988 and December 2005 were examined. The origins of the cancers were tongue (n=42), gingiva (n=29), oral floor (n=12), buccal mucosa (n=9), soft palate (n=4), lips (n=2), and mandible (n=2).

Pathological features, *i.e.*, differential type and mode of invasion, of each specimen were assessed by histological examination with hematoxylin and eosin stain. The grade of tumor differentiation was determined according to the criteria proposed by the World Health Organization. In this study, the mode of cancer invasion was evaluated using the criteria reported by Yamamoto et al. [18]. Clinical information including the presence or absence of regional lymph node metastases and prognosis was obtained from the patients' medical records.

Staining

Each specimen was fixed in 10% buffered formalin and embedded in paraffin to prepare serial sections (4 μm). The sections were then dewaxed in xylene, rehydrated in ethanol, and treated with 3% methanolic hydrogen peroxide for 10 min to block endogenous peroxidase activity and reduce nonspecific background staining. The slides were heated up to 95°C in citrate buffer in an oil bath for 40 min for antigen retrieval, and then incubated with monoclonal antibodies. The primary antibodies used were anti-alpha 3 (MAB1952Z), anti-alpha 6A (MAB1356), or anti-beta 1 integrin antibodies (MAB1951) at × 200 dilution (CHEMICON International, USA). They were reacted at 4°C for 24 h, incubated with biotinylated anti-mouse immunoglobulin (Dako Japan, Kyoto, Japan), and reacted at room temperature for 60 min. After reacting with peroxidase-conjugated streptoavidin (Dako Japan) for 60 min, the slides were washed with PBS. Immunohistochemical reactions were performed in 3, 3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), followed by counterstaining with hematoxylin. Negative controls were treated with all reagents except the primary antibodies.

Criteria for Alpha 3, Alpha 6A, and Beta 1 Integrin Subunit Expressions

In each area of advanced cancer invasion, three regions were image-captured under a light microscope at 200 × magnification. Cells in the images were counted, and the ratios of positive tumor cells to all tumor cells were calculated. Correlations between positive rates and clinical as well as pathological parameters were examined.

The alpha 3 and 6A integrin subunits were divided into ≥50% and <50% positive rate groups, respectively. Since the positive rates for all beta 1 subunits were ≥50%, they were divided into ≥75% and <75% positive rate groups. Clinicopathological factors and survivals were compared between these groups.

Statistical Analysis

Spearman's rank correlation coefficient tests were used to analyze the statistical relationship between integrin expression, type of differentiation, and mode of invasion. Chi-square tests were used for statistical comparison of the prevalence of these expressions between the presence and absence of lymph node metastasis. Survival rates were calculated by the Kaplan–Meier method, and examined for statistical significance using the log-rank test in each group. To determine prognostic parameters related to survival, multivariate analysis was performed with Cox proportional analysis. Statistical analyses were conducted using Stat View version 4.5 software (SAS Institute Inc, Cary, NC, USA).

Results

Correlation Between Various Clinicopathological Factors and Survival

A statistical analysis using the log-rank test was performed for age, sex, tumor origin, histopathological classification, integrin subunit, and survival period (Table 1). Prognosis was poorest for patients aged ≤55 years (mean observation period, 23 months), followed by >70 (31 months) and

Table 1 Clinicopathology parameters and survival months

		Total *n*	Median survival months	log-rank *P*
Age,years	−55	14	23.86	n.s. (P=0.164)
	56–70	46	46.93	
	>70	40	31.38	
Sex	Male	51	36.86	n.s. (P=0.476)
	Female	49	36.96	
Origin	tongue	42	33.69	n.s. (P=0.323)
	gingival	29	32.82	
	oral floor	12	35.16	
	buccal mucosa	9	40.66	
	soft palate	4	28.25	
	lips	2	53.66	
	mandible bone	2	15.5	
Integrin	α3 Integrin <50%	80	37.096	0.0014*
	50%≤ α 3 Integrin	20	55.69	
	α6a Integrin <50%	57	38.27	0.048*
	50% ≤ α 6a Integrin 43	43	47.85	
	β1 Integrin <75%	46	26.09	0.0005*
	75%≤ β1 Integrin	54	46.13	
T category	1	28	43.29	0.0063*
	2	48	34.21	
	3–4	24	34.88	
N category	Negative	62	40.76	0.0059*
	Positive	38	30.63	
M category	Negative	95	38.07	0.0015*
	positive	5	14.8	
Differentiation	Well	66	37.71	0.0005*
	Moderate	22	45	
	Poor	12	17.66	
Mode of invasion	1	16	58.31	0.0071*
	2	19	42.79	
	3	35	33.8	
	4C	22	28.45	
	4D	8	17	

*P< 0.05, clinicopatholigical parameters vs. survival months (Chi-square)

56–70 (46 months) years, the last age group having the longest observation period. Although there was no statistically significant difference, the survival period tended to be shorter with tumor onset in the younger age group. The mean survival period in males and females was 36.8 months and 36.9 months, respectively, with no statistically significant difference. With regards to tumor origin, the mean survival period was shortest (15.5 months) for mandible tumors, followed by gingiva and tongue (approximately 33 months), buccal mucosa (40.6 months), and lip (53.6 months) tumors. However, there were no statistically significant differences in survival periods by tumor origin.

With regards to histopathological classification, the survival period shortened as tumor size increased in the T category, while in the N class, the presence of lymph node metastases shortened the survival period by 10 months. Delayed metastasis shortened the survival period significantly. In the case of tumor grades, the survival period tended to be shorter in low grade tumors. Regarding the mode of invasion, the survival period tended to shorten in a step-wise manner as invasion progressed from grade 1 (low aggravation) to grade 4 (high aggravation). Statistically, there were significant differences in survival among the histopathological categories of TNM as well as in the grades of differentiation and modes of invasion.

Correlation Between Integrin Expression and Clinicopathological Factors

1) Immunohistochemical expression

Observation of expression of the various integrin subunits inside the cancer lesion and along the periphery in advanced tumor areas revealed that the beta 1 subunit

was expressed in the periphery of the cancer lesion, whereas the alpha 3 and 6A subunits were expressed in the periphery of the cancer lesion and to a lesser extent inside the cancer lesion. In normal oral and buccal mucosa, the alpha 3, alpha 6A, and beta 1 integrin subunits mainly were expressed in the region between the basal epithelial layer and stroma. This suggested that integrin expression in the basal membrane is important for adhesion. There was continuous expression of the various integrin subunits in the periphery of cancer lesions (Fig. 1).

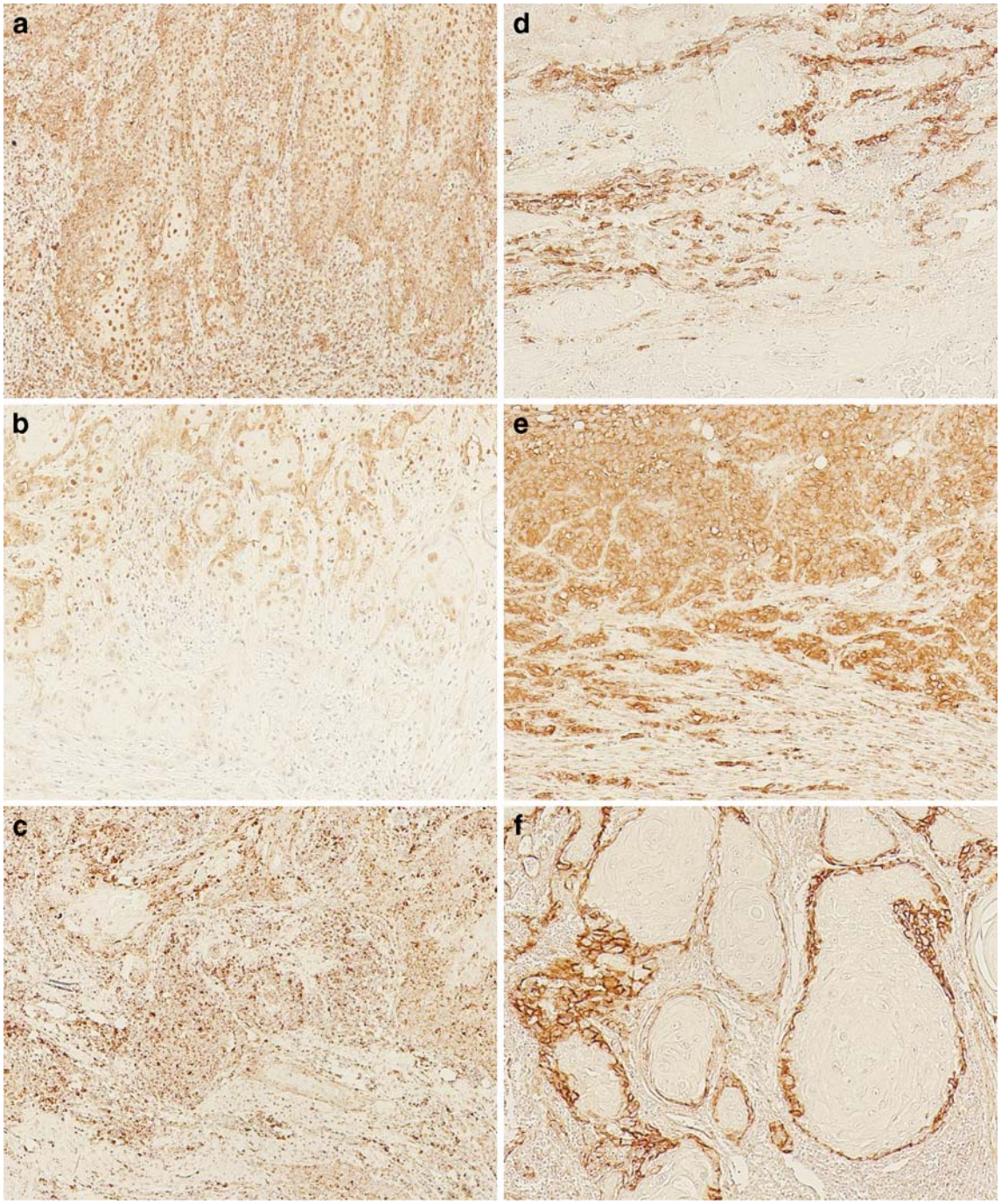

Fig. 1 **a**, **b** Immunohistochemical expression of alpha 3 integrin at the invasive front of oral squamous cell carcinoma. (a≥50%, b<50%, Magnification: ×200). **c**, **d** Immunohistochemical expression of alpha 6A integrin at the invasive front of oral squamous cell carcinoma. (c≥50%, d<50%, Magnification: ×200). **e**, **f** Immunohistochemical expression of beta 1 integrin at the invasive front of oral squamous cell carcinoma. (e≥75%, f<75%, Magnification: ×200)

2) Integrin expression and T classification

A positive rate of ≥50% for alpha 3 integrin was shown in 19 (25%) of 76 T1 and T2 patients, but in only 1 (4.2%) of 24 T3 and T4 patients, most of whom exhibited a positive rate of <50%. Statistically, a significant correlation was observed between alpha 3 integrin and T category. A positive rate of ≥50% for alpha 6A integrin was shown by 38 (50%) of T1 and T2 patients but only 5 (20.8%) of T3 and T4 patients. A significant correlation was observed between alpha 6A integrin and T category. A positive rate of ≥75% for beta 1 integrin was shown by 47 (61.8%) of T1 and T2 patients but only 7 (29.2%) of T3 and T4 patients. A significant correlation was observed between beta 1 integrin and T category. The above findings indicate that positive rates for alpha 3, alpha 6A, and beta 1 integrins tend to decrease with an increase in tumor size (Table 2).

3) Integrin expression and N classification

Among 38 patients with regional lymph node metastases, 37 (97%) showed a <50% positive rate for alpha 3 integrin. The presence of regional lymph node metastases resulted in a decrease in the positive rate for alpha 3 integrin with a statistically significant correlation. Among the 38 regional lymph node metastases patients, 33 (87%) showed a positive rate of <50% for alpha 6A integrin, indicating that the presence of regional lymph node metastases results in a decrease in the positive rate for alpha 6A integrin. Among the 62 patients without regional lymph node metastases, 47 (76%) showed a positive rate of ≥75% for beta 1 integrin. However, 7 (18%) of the 38 patients with regional lymph node metastases also showed a beta 1 integrin positive rate of <75%. A significant correlation was observed between beta 1 integrin and N category. Five patients with distant metastases showed a positive rate of <50% for the alpha 3 and 6A integrins, and a positive rate of <75% for beta 1 integrin.

The above findings showed that the number of patients showing positive rates for alpha 3, alpha 6A, and beta 1 integrin subunits decreased with an increase in the rate of lymph node and distant metastases.

Table 2 Integrin α 6A, β1 and clinicopathological parameters

		Total	α3 Integrin		*P*	α6A integrin		*P*	β1 Integrin		*P*
		n	<50%	≥50%		<50%	≥50%		<75%	≥75%	
Age, years	−55	14	11	3	n.s.	10	4	n.s.	9	5	n.s.
	56–70	46	35	11	(*P*=0.582)	21	25	(*P*=0.098)	19	27	(*P*=0.315)
	>70	40	34	6		26	14		18	22	
Sex	Male	51	37	14	n.s.	32	19	n.s.	28	23	n.s.
	Female	49	43	6	(*P*=0.057)	25	24	(*P*=0.236)	18	31	(*P*=0.068)
Origin	tongue	42	31	11	0.0496**	21	21	n.s.	19	23	n.s.
	gingival	29	28	1		20	9	(*P*=0.576)	14	15	(*P*=0.793)
	oral floor	12	10	2		7	5		7	5	
	buccal mucosa	9	6	3		4	5		3	6	
	soft palate	4	3	1		2	2		2	2	
	lips	2	1	1		2	0		1	1	
	mandible bone	2	1	1		1	1		0	2	
T category	1	28	16	12	0.0011**	12	16	0.0266**	8	20	0.0207**
	2	48	41	7		26	22		21	27	
	3–4	24	23	1		19	5		17	7	
N category	Negative	62	43	19	0.0007**	24	38	<0.0001**	15	47	<0.0001
	Positive	38	37	1		33	5		31	7	
M category	Negative	95	75	20	n.s.	52	43	0.046**	41	54	0.0129
	Positive	5	5	0	(*P*=0.251)	5	0		5	0	
Differentiation	Well	66	50	16	n.s.	35	31	n.s.	23	43	0.0009**
	Moderate	22	19	3	(*P*=0.313)	12	10	(*P*=0.144)	12	10	
	Poor	12	11	1		10	2		11	1	
Mode of invasion	1	16	10	6	0.0291**	6	10	n.s.	0	16	<0.0001**
	2	19	12	7		8	11	(*P*=0.056)	1	18	
	3	35	30	5		24	11		21	14	
	4C	22	20	2		12	10		16	6	
	4D	8	8	0		7	1		8	0	

**$P<0.05$, clinicopathological parameters vs. integrin expression (Chi-square test)

4) Integrin expression and histopathological differentiation

There was no statistically significant difference between expression of alpha 3 or 6A integrins and histopathological differentiation. However, the positive rate for beta 1 integrin decreased at lower levels of histopathological differentiation with a significant correlation between beta 1 integrin expression and histopathological differentiation.

5) Integrin expression and mode of cancer invasion

Alpha 3 integrin expression in cancer cells was compared with mode of invasion, and a positive rate of ≥50% for alpha 3 integrin was shown by 13 (37%) of 35 grade 1 and 2 patients, and by 7 (10%) of 65 grade 3, 4C, and 4D patients, indicating that a positive rate for alpha 3 integrin tended to decrease with an increase in invasion with a statistically significant correlation (χ^2 test: P=0.029). With regard to alpha 6A integrin, a similar level of ≥50% positive rate was shown by 21 (60%) of grade 1 and 2 patients, and by 22 (34%) of grade 3, 4C, and 4D patients. This showed that the positive rate tended to decrease with an increase in invasion. However, there was no statistically significant correlation (χ^2 test: P=0.056). Regarding the beta 1 integrin subunit, there was a high correlation with mode of invasion. In grade 1 invasions, the lowest mode of invasion, all patients showed a beta 1 integrin positive rate of ≥75%. In contrast, in grade 4D invasions, the highest mode of invasion, the positive rate was <75%. A positive rate of ≥75% was shown by 34 (60%) of 35 grade 1 and 2 patients, and by 20 (31%) of 65 grade 3, 4C and 4D patients. This showed that the positive rate tended to decrease with an increase in the mode of invasion, with a statistically significant correlation (χ^2 test: P<0.01).

6) Integrin expression and survival rate

In the Kaplan–Meier analysis of integrin expression in tumor cells in advanced tumor areas and cumulative survival rate, the 5-year survival rate of patients with a positive rate of ≥75% for beta 1 integrin was 66%, whereas for <75%, the survival rate was significantly lower at 29% (P<0.01). Similarly, the 5-year survival rate of patients with a positive rate of ≥50% for alpha 3 integrin was 79%, whereas that of patients with a positive rate of <50% was 42% (P<0.01). The 5-year survival rate of patients with a positive rate of ≥50% for alpha 6A integrin was 64%, whereas that of patients with a positive rate of <50% was 38% (P<0.01). All 5-year survival rates decreased with decreases in integrin positive rates (Fig. 2). Cox proportional hazard model analysis detected the influences of beta 1 integrin expression and survival (Table 3).

Discussion

Tumor TNM classification, differentiation, and mode of invasion for the alpha 3, alpha 6A, and beta 1 integrin subunits were investigated. These factors have been reported to be important prognosis predicting factors, and all of them are reported to be negatively correlated with the results of integrin staining [19–21]. Thus, attenuation of the degree of positivity of integrin staining is associated with poor survival prognosis. Until date, there have been several reports of attenuation of integrin expression in oral squamous cell cancer and the results of this study support those findings. According to Bottger et al. [19], beta 1

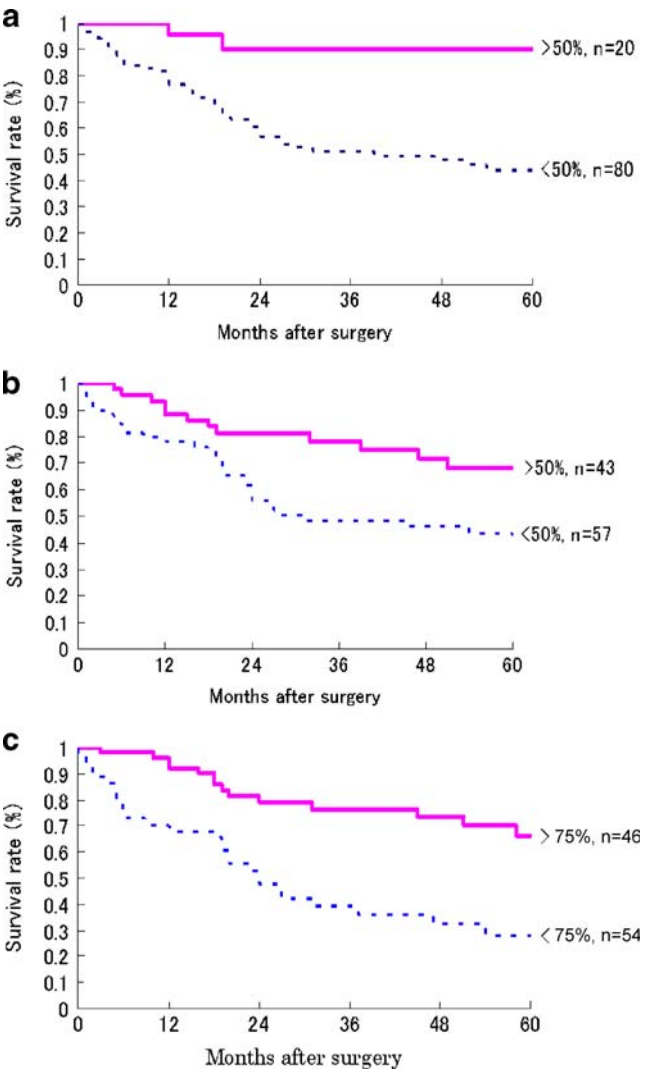

Fig. 2 Survival rate curves for patients with oral squamous cell carcinoma. **a** Correlation between alpha 3 integrin expression and survival. **b** Correlation between alpha 6A integrin expression and survival. **c** Correlation between beta 1 integrin expression and survival

Table 3 Multivariate analyses for pathological parameters to survival of patients with oral squamous cell carcinoma

Pathological parameters	Category	Risk ratio (95% CI)	Cox regression *P*-value
Mode of cancer onvasion	1, 2, 3 vs. 4C,4D	1.32	0.509
Cancer cell differentiation	Well vs. Mod, Poor	1.10	0.955
Expression of alpha 3 integrin	≥50% vs. <50%	1.12	0.805
Expression of alpha 6A integrin	≥50% vs. <50%	1.17	0.731
Expression of beta 1 integrin	≥75% vs. <75%	[a]3.53	[a]0.002

[a] the result showed that expression of beta 1 integrin was the independent factor on the survival. *CI* confidence interval

integrin expression in SCC of the throat tends to be attenuated. Fujita et al. [20] reported attenuation of staining of beta 1 integrin as metastasis increased in colon cancer, a finding common with the present study; however, in our study, different observation sites were used and the evaluations were based on the range of the sites staining positive rather than the strength of staining. We observed differing degrees of positive reaction for beta 1 integrin between the basal lateral membrane and stroma. As a positive reaction was observed in most patients, we concluded that it was effective to rate the degree of staining from the range of the sites that stained positive.

There are many reports on the alpha integrin subunits; however, variation in the present staining results suggests that their use as a diagnostic standard is questionable. With regard to the alpha 6 integrin subunit, Satake et al. [21] reported attenuation in the staining results of SCC in the head and neck. Alternatively, Waes et al. [15] reported alpha 6 integrin expression in seriously advanced cancers by employing the Western blot technique. Integrin expression itself is not limited to the periphery of cancer lesions. Even in our present study, integrin expression was observed in cell areas that corresponded to the granular cell layer in cancer lesions. Therefore, the results on sites of positive reaction seen in cells described by Satake et al. [21] were assumed to be identical to those reported by Waes et al. [15].

In this study, the rate of positive reactions in adhesion to the ECM tended to increase as the cancer worsened, in contrast to the expression in the cellular periphery. The alpha 6A integrin subunit, in particular, exhibited this tendency, which was consistent with the results of Waes et al. [15] as mentioned above. Protein eluted from larger tumor masses reflected significant expression of alpha 6 integrin within the lesions, resulting in an increased expression of the alpha 6 subunit.

We statistically examined the relationship between the proportions of alpha 3, alpha 6A and beta 1 integrin positive cells and the survival rate. When establishing the cut-off values for the proportions of alpha 3 and alpha 6A integrin positive cells as 50%, and that for the proportion of beta 1 integrin positive cells as 75%, the patients could be divided the most accurately. In TNM differentiation, mode of invasion, and survival rate, the staining results for the beta 1 subunit showed significant differences. This suggests that beta 1 subunit staining results can be an effective prognosis rating factor, and may be a useful parameter for rating postsurgical prognosis of patients with OSCC. Similar differences were observed in the alpha 3 and 6A integrin subunits. However, in the alpha 6A integrin subunit, a correlation was only seen with differentiation. The alpha 6A integrin subunit expresses differently from other integrin subunits, in that it not only expresses between the basal layer and ECM (as do the alpha 2 and 3 integrin subunits), but also within cells. Therefore, the alpha 6A subunit has a different distribution and action, and is not likely to be involved in adhesion to the ECM. On the other hand, the alpha 3 integrin subunit is mostly expressed between the basal layer and ECM. According to the report by Matsurra et al. [12], the number of patients with cervical lymph node metastasis was significantly high among those with attenuated positive rates for alpha 3 integrin. Similarly, in this study, the positive rate decreased with a worsening degree of cancer.

In this study, integrin expression in advanced cancer areas was examined and the diagnostic criteria of prognosis were investigated. All parameters of TNM differentiation, mode of invasion, and survival rate showed that in OSCC, the positive rate for integrin expression in the lesion periphery was attenuated as the degree of cancer worsening increased. The positive rate for some alpha integrins tended to increase with increased degree of worsening in the lesions; however, as they are involved with cell-to-cell adhesion rather than adhesion with the ECM, they were excluded from this investigation.

Integrins are important membrane protein mediators of ECM adhesion. The integrin subunits present in the epithelial basal layer, in particular, are similar to the integrin subunits that are distributed in areas with advanced cancer invasion. A statistical correlation was observed between the 5-year survival and positive stain rates for each integrin subunit. We, therefore, suggest that in OSCC,

integrin expression in the periphery of cancer lesions can be used to rate the prognosis of cancer. In the strongly stained beta 1 integrin subunit, there was a significant statistical correlation with various histopathological parameters. This confirmed that prognosis rating through immunostaining may be reliable, particularly when the beta 1 integrin subunit is the target. As the degree of positive staining for the beta 1 integrin subunit is high, we suggest that staining is also a good method for investigating the degree of cleavage in the lesion periphery for prognosis rating that does not depend on lesion size. Thus, degree of integrin staining can be a useful factor for determining the prognosis and deciding treatment strategies.

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