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A Valuable System with High Specificity for Evaluating both Metastasis and Prognosis of Oral Squamous Cell Carcinoma

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OBJECTIVE A system was established to evaluate the metastasis and prognosis of oral squamous cell carcinoma by analyzing the tumor differentiation, the TNM stage, the mode of invasion, and the expression of E-cadherin and S100A4.
METHODS Squamous cell carcinoma of the oral cavity of 86 cases was the focus of our study. In this system, the histopathological grade and the histochemical patterns were estimated on a 0-3 point scale, the total points graded from 0 to 13.
RESULTS The incidence of metastasis and prognosis in the cases with total points more than 8 was significantly higher than that with total points less than 7 ($P < 0.05$, $\chi^2 = 22.0658$ and $P < 0.05$, $\chi^2 = 10.7047$). The system had a significant higher specificity than that of 'DIAGS index' system (Differentiation, Invasion mode, Adhesion molecules, Glycosaminoglycan, and the Sugar chain) in the evaluation of metastasis ($P < 0.05$, $u = 2.2339$). Moreover, the specificity for evaluation of metastasis in the system was significantly higher than that of E-cadherin ($P < 0.05$, $u = 2.4996$) or S100A4 ($P < 0.05$, $u = 2.4289$) only. Furthermore the specificity for evaluation of unfavorable prognosis in the system was also significantly higher than that of E-cadherin ($P < 0.05$, $u = 2.1313$) or S100A4 only ($P < 0.05$, $u = 2.0301$).
CONCLUSION This is a valuable evaluation system with high specificity to predict metastatic potential and prognosis of oral squamous cell carcinoma.

KEY WORDS: metastasis, prognosis, squamous cell carcinoma, E-cadherin, S100A4.

Introduction

Squamous cell carcinoma (SCC) with a poor clinical outcome, is the most frequent malignancy present in the oral cavity. The goal of surgical treatment is to prevent local and regional recurrence and instant metastasis to the regional lymph node so as to increase the survival rate[1-3]. If the potential of local invasion, the status of regional lymph nodes metastasis and prognosis of SCC were recognized before an operation, it would increase the options in determining the best therapy methods for the patients.

Many reports suggest the histopathologic parameters such as tumor differentiation, TNM stage and mode of invasion are the useful prognostic factors for oral SCC[4-9]. In recent years, it has been reported the reduced expression of E-cadherin, a member of the cadherin super family that mediates calcium-dependent cell-to-cell adhesion, is associated with invasion and metastasis in the oral SCC[10-12] and other tumors[13-15]. In addition S100A4, a member of the S100 calcium-

binding protein family, has also been suggested to be a metastasis-associated molecule in human tumor tissues, such as colorectal adenocarcinoma[16], breast cancer[17], bile duct adenocarcinomas[18], non-small cell lung cancer[19], esophageal and squamous cell carcinoma in others parts of the body[20,21]. Furthermore, S100A4-induced invasiveness in malignant tumor cells is partially caused by down-regulation of E-cadherin[22]. Therefore, analyzing all information of tumor differentiation, TNM stage, mode of invasion of carcinoma and expression of E-cadherin and S100A4 in oral SCC is helpful in evaluating the tumor behavior of oral SCC.

In the present study, we focused on establishing a system to evaluate the potential metastasis and prognosis of oral SCC. Also, we analyzed whether or not the system revealed a more accurate measure which predicts the potential metastasis and prognosis of the carcinoma than the way of detecting the expression E-cadherin or S100A4 alone and other system.

Patients and Methods

Patients

Squamous cell carcinoma of the oral cavity of 86 cases surgically treated at the Tianjin Medical University Stomatology Hospital and Hospital of the Kobe University (Japan) from September 1991 to July 2001 was the focus of our study. The cases consisted of 64 males and 22 females with an average age of 59.6 years old ranging from 37 to 81. The original sites of the tumors in the 86 cases were different, 60 cases in tongue, 8 in buccal mucosa 8 in lower gingival, 2 in mandibular gingival, 2 in the floor of mouth and 6 in soft palate. Tumor differentiations were classified into well differentiated (n = 46), moderately differentiated (n = 34), and poorly differentiated (n = 6), according to the International Histological Classification of Tumors (WHO 97)[23]. According to the TNM stage classification of the tumor[24], our cases were classified as the following, 10 cases in Stage I, 26 cases in Stage II, 18 cases in Stage III and 32 cases in Stag IV. Metastases to lymph node occurred in 48 cases (55.81%); distant metastases occurred in 14 cases (16.28%) and local recurrence occurred in 22 cases (25.58%). At the end of the follow-up period (62 months to 129 months, average of 95.5 months), 22 cases (25.58%) died of the disease; 20 cases (23.26%) still had the disease and 44 cases (51.16%) had no evidence of the disease.

Immunohistochemical staining

Immunohistochemical studies were performed using an indirect streptavidin-biotin immunoperoxidase technique. Sections were dewaxed and rehydrated in a concentration of sodium citrate buffer (20 ×), and heated at 750 w output in a microwave oven for 10 min. Sections were also treated with methanol for 20 min in order to inhibit endogenous peroxide, and then blocked with serum blocking solution to reduce nonspecific labeling. The sections were incubated with 1:400 diluted HECD-1 (McCabe to human E-cadherin, TAKARA SHUZO, Japan) and 1:100 diluted anti-S100A4 polyclonal antibody (that was obtained from rabbit serum immunized with recombinant S100A4 protein subcutaneously as described previously[25], was manufactured by ourselves) at room temperature for 1 h, respectively. As negative controls, the sections were treated with an irrelevant primary antibody. Thereafter, the sections were incubated with diluted biotinylated anti-mouse and anti-rabbit Ig antibody for 15 min, and then reacted with horseradish peroxidase-conjugated streptavidin (Dako Japan, Kyoto, Japan) for 15 min. After each step, the sections were rinsed in phosphate-buffered saline (PBS) for 15 min. To visualize the immunoreactivity, the sections were treated with DAB. After hematoxylin counterstaining, slides were permanently mounted. Scoring of the immunostaining was performed by a doctor who knew nothing about the patients' clinical status.

The degree of staining for E-cadherin was scored using the formula of Hiraki et al.[26]:

3+ = extensive staining comparable to control epithelium at the invasion front of SCC towards the connective tissues.

2+ = staining reduced from the control level but more than 50 % of positive staining.

1+ = staining positive but less than 50 % of the control level.

− = very little or no staining.

The degree of staining for S100A4 was undertaken using a semi quantitative scale by Franchi et al.[27] as follows:

3+ = diffuse, more than 75% of positive tumor cells.

2+ = moderate, 25% to 75% of positive tumor cells.

1+ = low, less than 25% of positive tumor cells.

− = absent, 0%.

The invasions of carcinoma were classified according to the mode of invasion by Yamamoto et al.[28], as follows: mode 1, well defined margin; mode 2, cords, less obvious margin; mode 3, groups of cells, no distinct margin; mode 4c, diffuse invasion of cord-like type; mode 4d, diffuse invasion of diffuse type. A total of 86 cases in the study were divided into mode 1 (n = 0), mode 2 (n = 14), mode 3 (n = 34), mode 4 c (n = 30), and mode 4 d (n = 8).

Setting up of a system

Based on the examination of the tumor differentiation, the TNM stage, the mode of invasion, and the expression of E-cadherin, S100A4 in the oral SCC, a valuable system was set up to evaluate metastasis potential and prognosis of the carcinoma. The system was estimated on a 0-3 point scale according to degree of differentiation, type of TNM stage, mode of invasion and pattern of E-cadherin and S100A4 staining. A total point for

every patient was achieved by plusing the point of every single item together. The evaluation system permits a grading with a total of points from 0 to 15. The cases examined in this study remained within the range of 3-13 points, so the system (tumor differentiation, TNM stage, invasive mode, E-cadherin and S100A4 expression) was established (Table 1).

Table 1. Grading of malignancy on basis of histopathological and histochemical of oral squamous cell carcinoma.

	Point			
	0	1	2	3
Differentiation	-	W	M	P
T-classification	1	2	3	4
Invasion mode	1-2	3	4 c	4 d
E-cadherin	+++	++	+	–
S100A4	–	+	++	+++

Statistical analysis

The χ^2 test was used to assess the statistical significance of the system's points in relation to the metastasis and prognosis. The *u* test was used to evaluate the significance of rate difference. The *P* value of less than 0.05 was considered as statistically significant.

Results

Immunohistochemical staining in oral SCC

The previous report showed that immunostainings for E-cadherin in tumor cells were recognized not only in the membrane but also in the cytoplasm and S100A4 distributed in the cytoplasm in tumor cells. We also found that the strong expression of E-cadherin and the negative expression of S100A4 (Fig.1A,B). Furthermore, the negative expression of E-cadherin and the strong expression of S100A4 (Fig.1C,D) were observed in the same region of the section. Simultaneously, the staining intensity of S100A4 and E-cadherin among the tumor cell or nests of the same tissue were observed, which indicated that the heterogeneity was stained in the tumor.

A system for evaluation of both metastasis and prognosis

In our evaluation system, the number of the metastasis cases with a total point less than 7 was only 4 out of 86 cases (4.65%). However 44 cases with a total point more than 8 showed metastasis (51.16%). The incidence of metastasis in cases with total points more than 8 was

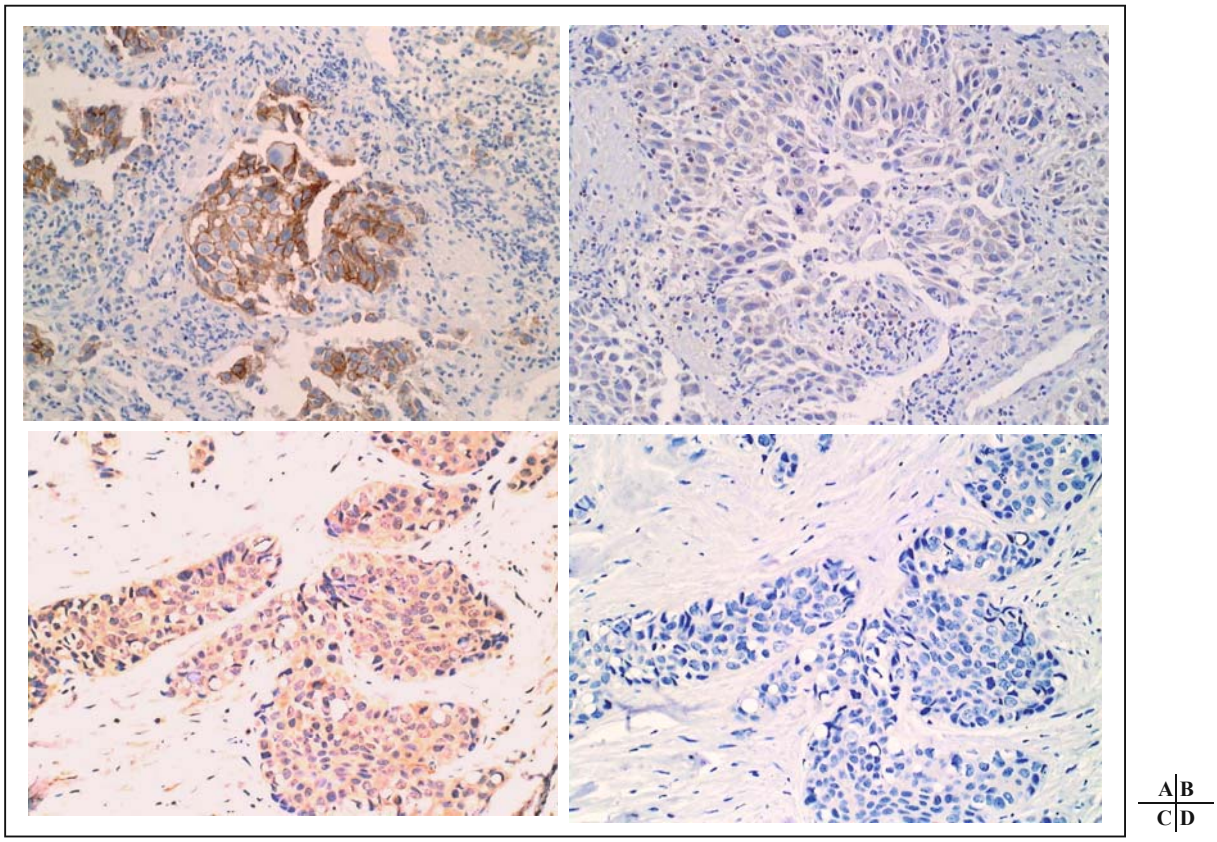

Fig.1. E-cadherin and S100A4 immunohistochemical staining of oral SCC. Strong expression of E-cadherin and the negative expression of S100A4 were observed in the same region: A, E-cadherin (3+) expression (× 200); B, S100A4 (-) expression (× 200). Negative expression of E-cadherin and the strong expression of S100A4 were observed in the same region: C, S100A4 (3+) expression (× 200); D, E-cadherin (-) expression (× 200).

significantly higher than that of cases with the points less than 7 ($P < 0.05$, $\chi^2 = 22.0658$). On the other hand, the cases that had a total of more than 8 points have an unfavorable prognosis while the cases with a total of less than 7 points have a favourable prognosis. The local recurrences, instance metastasis and death are considered as unfavourable prognosis. The incidence of unfavorable prognosis in the cases with a total point more than 8 was significantly higher than that in the cases with the points less than 7 ($P < 0.05$, $\chi^2 = 10.7047$, Table 2). These results indicated that a total point of more than 8 evaluated by the system had a higher incidence of metastasis and unfavourable prognosis.

Table 2. Total of points of the system, the incidence of metastasis to the regional lymph nodes and prognosis.

	Metastasis		Prognosis	
Total point	+	–	+	–
≥ 8	44	6	14	36
≤ 7	4	32	30	6
P	< 0.05		< 0.05	
χ^2	22.0658		10.7047	

Metastasis (+), metastasis to the regional lymph nodes; (–), no metastasis to the regional lymph nodes; Prognosis (+), favorable prognosis; (–), unfavorable prognosis. Incidence of metastasis and unfavorable prognosis in the cases with a total score of more than 8 was significantly higher than that with a total score of less than 7.

Higher specificity of the system for evaluation of both metastasis and prognosis

We compared the results of the system in our study for evaluation of metastasis and prognosis with those obtained by the way of detection of E-cadherin or S100A4 only. Reduced expression of E-cadherin was recognized in 72 cases and 42 of them were observed to have metastasis to the regional lymph nodes (58.33%). The expression of S100A4 was recognized in 74 cases and 44 of them were observed to have metastasis to the regional lymph nodes (59.46%). The specificity of the both is significantly lower than that of the result obtained by using the system in our study ($P < 0.05$). However, reduced expression of E-cadherin, the expression of S100A4 and a total point more than 8 in the system were recognized in 42 cases (87.5%), and 44 cases (91.76%) in total of 48 cases with lymph nodes metastasis, respectively. Statistical analysis showed there was no significant difference between them (Table 3).

On the other hand, 32 cases with poor prognosis were recognized from 72 cases with reduced expression of E-cadherin (44.44%). Thirty-four cases with poor prognosis were recognized from 74 cases with the expression of S100A4 (45.94%). Thirty-six cases that had poor prognosis were recognized from 50 cases with the total points of more than 8 (70%) as indicated by the system. The specificity of the result obtained by using the system was significantly higher than that obtained by the way of detection of E-cadherin ($P < 0.05$, $u = 2.1313$) and S100A4 only ($P < 0.05$, $u = 2.0301$), respectively. Reduced expression of E-cadherin, the expression of S100A4 and a total of more than 8 points as indicated in the system were recognized in 32 cases (76.19%), 34 cases (80.95%) and 36 cases (85.71%) in total of 42 cases with poor prognosis, respectively. Statistical analysis showed that there was no significant difference between them (Table 3).

Our results were also compared with those by the Mori et al.[29] 'DIAGS index' system. In their system, the incidence of metastasis in the group with a total score of more than 6 points was significantly higher than that in the group with a total score of less than 5 (total point grade from 0 to 12). Twenty-seven with a total score of more than 6 points in the 86 cases showed metastasis to the regional lymph nodes (31.4%). There is no significant difference when comparing the incidence of metastasis in our results. Furthermore, in our system, 44 cases with total points of more than 8 were detected from 48 cases with lymph nodes metastasis. The sensitivity of our system is 91.67%, which also showed no significant difference to that of the 'DIAGS index' system (87.09%)[29]. However, 44 cases of more than 8 points in our study were observed with lymph nodes metastasis. The specificity of our system is 88%, which

Table 3. Sensitivity, specificity of E-cadherin, S100A4 and our system about prognosis and metastasis of oral squamous cell carcinoma.

	Metastasis to regional lymph nodes			Prognosis		
	Reduced expression of E-cadherin	Expression of S100A4	System ≥ 8	Reduced expression of E-cadherin	Expression of S100A4	System ≥ 8
Specificity (%)	58.33 (42/72)	59.46 (44/74)	88.00 (44/50)	44.44 (32/72)	45.94 (34/74)	72.00 (36/50)
u	2.4996	2.4289		2.1313	2.0301	
P	< 0.05	< 0.05		< 0.05	< 0.05	
Sensitivity (%)	87.50 (42/48)	91.67 (44/48)	91.67 (44/48)	76.19 (32/42)	80.95 (34/42)	85.71 (36/42)
P	> 0.05 NS	> 0.05 NS		> 0.05 NS	> 0.05 NS	

The specificity of the system was significantly more accurate than E-cadherin and S100A4 criteria alone in the prediction of the metastatic potential and prognosis of oral squamous cell carcinoma.

has a significant difference when compared with that of the 'DIAGS index' system in which 27 out of the 43 cases with total points of more than 6 showed metastasis (62.79%) ($P < 0.05$, $u = 2.2339$, Table 4).

Table 4. Comparison of 'DIAGS index' system and our system.

	'DIAGS index' system	Our system
Specificity (%)	62.79 (27/43)	88.00 (44/50)
u	2.2339	
P	< 0.05	
Sensitivity (%)	87.09 (27/31)	91.67 (44/48)
P	> 0.05 NS	

The specificity of our system was significantly more accurate than that of 'DIAGS index' system in the prediction of metastatic potential of oral squamous cell carcinoma.

Discussion

Several histological factors associated with the prognosis of patients with oral SCC have been reported in previous studies. However, a single factor is not sufficient to accurately predict the prognosis of patients with oral SCC. Consequently we developed a system with high specificity, which can evaluate both metastasis potential and prognosis of the oral SCC.

Although the TNM stage system used alone is not enough to determine the prognosis of the patient with carcinoma, it can still be considered to be the most important prognostic factor for oral SCC[7,8]. An advanced degree of differentiation of the carcinoma is regarded as a feature of favorable significance, while lack of differentiation is considered to be an ominous indication[8]. On the other hand, Yamamoto et al.[28] reported that the mode of invasion was one of the most important factors of the tumor-host relationship in the prediction of prognosis for the patients. In the present study, we believe the tumor differentiation, TNM stage and the mode of invasion might be a useful parameter for the prediction of the metastatic potential and prognosis. However, 30 cases from 32 cases with Stage IV showed metastasis. In the unfavorable prognosis, 30 out of 42 cases were found to be in Stage IV and Stage III (71.43%). When looking at the mode of invasion, we found that 24 cases from 38 cases of the '4c and 4d' showed metastasis (63.16%). In 42 cases unfavorable prognosis, 26 cases were the '4 c and 4 d' (61.9%). In our study, 22 of 40 cases (55%) with moderately or poorly differentiated and 26 of 46 cases (56.52%) with well differentiated showed lymph node metastasis. However 24 of 40 cases (60%) with moderately or poorly differentiated and 18 of 46 cases (39.13%) with well differentiated had an unfavorable prognosis. These results support our theory and let us to select these parameters in the evaluation system of the present study.

In addition to the histopathological parameters two histochemical parameters were used in the prediction of the metastasis and prognosis in our study. The cell-cell adhesion molecule E-cadherin has been shown to suppress invasive growth of epithelial cells in vitro, and the loss of its expression is thought to be important in the invasion and metastatic potential of carcinomas in vivo and in oral SCC[29,30]. On the other hand, increased expression of S100A4 correlates with increased invasiveness and metastatic potential of the carcinomas[16-19,22]. It was also reported that there was statistically significant inverse correlation between expression of S100A4 and that of E-cadherin in oral SCC[22], therefore, S100A4 may be more useful as a marker of prognosis in combination with adhesion molecules of the E-cadherin. Many researchers support that expression of E-cadherin and S100A4 can predict metastasis potential and prognosis of the cancer.

In the present study, we examined the expression of E-cadherin, S100A4 in oral SCC and used them as parameters to predict the lymph node metastasis and prognosis. Based on their expression pattern and other histopathological factors, a valuable system with high specificity was set up to evaluate the metastatic potential and prognosis of oral SCC. Further, we also analyzed weather or not the system revealed a more accurate measure of metastatic potential and prognosis of the carcinoma than that of the detection of the expression E-cadherin or S100A4 only. The specificity of the system was significantly higher than that of E-cadherin ($P < 0.05$) or S100A4 ($P < 0.05$) only in the evaluation of the metastatic potential ($P < 0.05$) and prognosis ($P < 0.05$) of oral SCC. But the sensitivity of the system showed no significant difference with them. It was observed that the system has a statistically significant correlation with lymph node metastasis ($P < 0.05$) and prognosis ($P < 0.05$), which revealed a more accurate measure of metastatic potential and prognosis of the oral SCC than that of a single criteria.

In 1998, Mori et al.[29] established a system (DIAGS index) to evaluate the metastasis potential of oral SCC. To determine weather or not our system is a more accurate measure in prediction of metastasis potential of the carcinoma than 'DIAGS index' system, we compared the sensitivity and specificity of the two systems for evaluation of the metastasis potential of carcinoma. The specificity of our system was significantly higher than that of 'DIAGS index' system ($P < 0.05$). However, the sensitivity of our system was the same as that of the 'DIAGS index' system in evaluation of the metastatic potential of oral SCC. These results strongly indicated that our system has a high specificity for evaluation of both metastasis and prognosis of oral SCC. As the results of our research and findings, we believe our system may be one of the most useful systems for the prediction of the metastasis potential and prognosis of patients with oral SCC. Further, the system will be of great value to clinical oncologist in order for them to determine what

cancers can be treated by the best therapy methods possibly leading to a more desirable prognosis.

In conclusion, we set up a valuable evaluation system with high specificity for evaluating metastatic potential and prognosis of oral SCC.

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