The Evaluation of both Metastasis and Prognosis of Oral Squamous Cell Carcinoma by S100A4 and E-cadherin Immunostaining

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Using immunohistochemistry, we studied the expression of S100A4 and E-cadherin in 43 oral squamous cell carcinomas (SCC) to evaluate the metastatic potential and patient survival. Expression levels of S100A4 correlated with lymph node metastasis (P=0.024) and poor prognosis (P=0.0136). Reduced E-cadherin expression was also associated with lymph node metastasis (P=0.023) and poor prognosis (P=0.0146) and was inversely correlated with S100A4 expression (P=0.011). We also found that S100A4(2+,3+)/E-cadherin(-,+) expression was significantly associated with lymph node metastasis (P=0.04) and poor prognosis (P=0.0021). Furthermore, we systematized the evaluation of oral SCC metastasis and prognosis by analyzing the expression of S100A4, E-cadherin, and other histopathological factors. Cases with total points greater than 8 were associated with lymph node metastasis (P=0.000) and poor prognosis (P=0.0022). These results indicate that S100A4, as both a single factor and in combination with E-cadherin, may be one of the most useful markers predicting the metastatic potential and prognosis of oral SCC patients.

Keywords: S100A4; E-cadherin; metastasis; prognosis; squamous cell carcinoma

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Introduction

Squamous cell carcinoma (SCC), the most frequent malignancy present in the oral cavity, is associated with a poor clinical outcome. The prevention of local regional recurrence, instant metastasis, and regional lymph node metastasis is the goal of surgical treatment seeking to increase survival rates (1-3). Investigation of the molecules associated with regional lymph nodes metastasis and poor prognosis in patients with SCC would be useful in recognizing tumor behavior, accurately predicting prognosis, and increasing the ability to determine the most appropriate therapies for each patient. In recent years, S100A4, a member of the S100 calcium-binding protein family, has been associated with increased metastases; S100A4 expression correlates strongly with invasive and metastatic behavior of cultured tumor cells, including colorectal adenocarcinoma (4), bile duct adenocarcinoma (5), and human squamous cell carcinoma (6). In clinical research, elevated expression of S100A4 has been connected with metastasis and poor prognosis in breast cancer (7,8), non-small cell lung cancer (NSCLC) (9), gallbladder cancer (10), and esophageal squamous cell carcinoma (11). There have not been any reports detailing the expression of S100A4 in surgical specimens of oral SCC. In contrast, reduced expression of E-cadherin, a member of the cadherin super family mediating calcium-dependent cell-cell adhesion, is associated with invasion and metastasis in the oral SCC (12-14) and other tumors (9,15,16). Furthermore, S100A4-induced invasiveness in malignant tumor cells follows the downregulation of E-cadherin (17). In addition, the histopathologic parameters, such as tumor differentiation, TNM stage and mode of invasion, continue to serve as useful prognostic factors for oral SCC (2,18-23). In this study, we investigated both the correlation of S100A4 expression with clinicopathological parameters and the relationship between S100A4 and E-cadherin in oral SCC. Also, we combine an analysis of S100A4 and E-cadherin expression with additional histopathological factors, including tumor differentiation, TNM stage, and mode of invasion, to create a novel method evaluating metastatic potential and predicting prognosis in oral SCC.
Materials and Methods

Patients:

Forty-three cases of squamous cell carcinoma of the oral cavity were surgically treated at Kobe University Hospital (Japan) from September 1991 to July 1997. The cases consisted of 32 males and 11 females, ranging in age from 41 to 81 years (average age of 59.6 years). The original tumor sites in the 43 cases were tongue (30 cases), buccal mucosa (4 cases), lower gingival (4 cases), mandibular alveolus (1 case), floor of mouth (1 case), and soft palate (3 cases). Tumors were classified into well differentiated (n=22), moderately differentiated (n=18), or poorly differentiated (n=3), according to the International Histological Classification of Tumors (24). According to the TNM tumor stage classification (25), our cases were classified as stage I (5 cases), stage II (14 cases), stage III (9 cases), and stage IV (15 cases). Lymph node metastases
occurred in 24 cases (55.81%), distant metastases occurred in 7 cases (16.28%), and local recurrence occurred in 11 cases (25.58%). At the end of the follow-up period (62 months to 129 months, average 95.5 months), 21 patients (48.84%) had died, one case (2.32%) retained active disease, and 21 cases (48.84%) had no evidence of disease.

All patients were treated initially with surgery, while 17 received postoperative radiotherapy or chemotherapy. Resected specimens were examined in our study.

**Immunohistochemical staining:**

Immunohistochemical studies were performed using an indirect streptavidin-biotin immunoperoxidase technique. Sections were dewaxed and rehydrated in concentrated sodium citrate buffer (20x), then heated for 10 minutes at 750w output in a microwave oven. Sections were treated with methanol for 20 minutes to inhibit endogenous peroxide activity, then blocked with serum blocking solution to reduce nonspecific labeling. Sections were incubated with 1:400 diluted HECD-1 (McCabe to human E-cadherin, TAKARA SHUZO, Japan) or 1:100 diluted anti-S100A4 polyclonal antibody (that was obtained from rabbit serum immunized with recombinant S100A4 protein subcutaneously as described (26)) for 1 hour at room temperature. Sections were also treated with irrelevant primary antibody as a negative control. Sections were treated with diluted biotinylated anti-mouse and anti-rabbit Ig antibody for 15 minutes, then reacted with horse-radish peroxidase-conjugated streptavidin (Dako Japan, Kyoto, Japan) for 15 minutes. After each step, sections were rinsed in phosphate-buffered saline (PBS) for 15 minutes. To visualize immunoreactivity, the sections were treated with DAB. Following hematoxylin counterstaining, slides were permanently mounted. Immunostaining scores were determined by a doctor with no knowledge of the patient clinical status.

The degree of staining for E-cadherin was scored as described by Hiraki et al. (13):

- **3+** = extensive staining comparable to control epithelium at the invasion front of SCC.
- **2+** = staining reduced from control levels, but greater than 50 percent of the level of positive staining.
- **1+** = positive staining, but reduced to less than 50 percent of control levels.
- **-** = very little or no staining.

Normal epithelium proximal to the tumor within the specimen was used as a control.

The degree of staining for S100A4 was evaluated using a semi quantitative scale by Franchi et al. (27) as follows:

- **3+** = diffuse, more than 75% positive tumor cells.
- **2+** = moderate, 25% to 75% positive tumor cells.
- **1+** = low, less than 25% positive tumor cells.
- **-** = absent, 0%.

Carcinoma invasion was classified according to the mode of invasion by Yamamoto et al. (20): 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. The 43 cases in this study were divided into mode 1 (n=0), mode 2 (n=6), mode 3 (n=18), mode 4c (n=15), and mode 4d (n=4).

**Statistical Analysis:**

The $\chi^2$-test was used to assess the statistical significance of S100A4 and E-cadherin expression in relation to clinicopathological parameters and the correlation of the S100A4 and E-cadherin expression. Survival curves were obtained using the Kaplan-Meier method. Differences in the probabilities of survival were calculated using the log-rank test (Cox-Mantel test). P values less than 0.05 were considered significant.
Results

Immunohistochemical staining in oral SCC

In tumor cells, S100A4 protein was distributed throughout the cytoplasm (Fig. 1A-D). As in previous reports, E-cadherin immunostaining in tumor cells was observed not only in the membrane but also in the cytoplasm. We also find strong expression of E-cadherin and negative expression of S100A4 (Fig. 2A, B), furthermore, negative expression of E-cadherin and strong expression of S100A4 (Fig. 3A, B) 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, it means that the heterogeneity was stained in the tumor.

Association of the S100A4 and E-cadherin expression status with lymph node metastasis and prognosis

S100A4 expression was scored as diffuse in 10 cases (23.3%), moderate in 13 (30.2%), low in 6 (13.9%), and negative in 14 cases (32.5%). The expression of S100A4 was not associated with differentiation status (P=0.877), tumor stage (P=0.099), or invasive pattern (P=0.166). The incidence of lymph node metastasis in tumor exhibiting expression(2+) or (3+) was significantly higher than cases demonstrating expression(+) or (-) (P=0.024) (Table 1). According to the Kaplan-Meier method, the cumulative survival curve demonstrated a significant difference between cases with S100A4 expression(2+) or (3+) and those with expression(+) or (-) (P=0.0136) (Fig. 4).

E-cadherin expression(3+) was observed in 10 cases (23.3%), expression(2+) in 11 (25.6%), expression(+) in 13 (30.2%), and no expression in 9 cases (20.9%). E-cadherin expression(+) and (-) was significantly associ-

Fig. 4: Kaplan-Meier survival curves according to the tissue status of S100A4 in oral SCC. The cases with S100A4 expression (2+,3+) were significantly poorer in prognosis than the S100A4 expression (-,+) populations. (P=0.0136)

Fig. 5: Kaplan-Meier survival curves according to the tissue status of E-cadherin in oral SCC. The cases with E-cadherin expression (-,+) were significantly poorer in prognosis than those with E-cadherin expression (2+,3+)(P=0.0146)

Fig. 6: Kaplan-Meier survival curves according to the tissue status of group of oral SCC. Group A: S100A4(2+,3+)/E-cadherin(-,+); Group B: S100A4(-,+)/E-cadherin(2+,3+); Group C: S100A4(2+,3+)/E-cadherin(2+,3+); Group D: S100A4(-,+)/E-cadherin(-,+). The A group had a significantly poorer prognosis than the B group (P=0.0021) but did not differ significantly from the C (P=0.6842) and D groups (P=0.5002).

Fig. 7: Kaplan-Meier survival curves according to the system points in oral SCC. The cases with total points ≥8 had a significantly poorer prognosis than those with total points ≤7. (P=0.0022)

ated with more advanced stage tumors (P=0.024), demonstrating a diffusely invasive pattern (P=0.009) and lymph node metastasis (P=0.023). No relationship was observed between E-cadherin expression and tumor differentiation (P=0.877) (Table 1). The survival curve demonstrated a significant difference between the cases with expression (-) or (+) of E-cadherin from the expression (2+) or (3+) population (P=0.0146) (Fig.5). We also analyzed the association of S100A4 expression with the downregulation of E-cadherin expression. Our results demonstrated the inverse correlation between S100A4 and E-cadherin expression in oral SCC (P=0.011) (Table 2). We observed a significant inverse correlation between S100A4 and E-cadherin expression in oral SCC

Table 1: Relationship between the expression status of S100A4 and E-cadherin and clinicopathological parameters of oral SCC

<table>
<thead>
<tr>
<th>factor</th>
<th>Differentiation</th>
<th>Tumor stage</th>
<th>Invasive pattern</th>
<th>Lymph node metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W</td>
<td>I~II</td>
<td>1~3</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>M,P</td>
<td>III~IV</td>
<td>4c,4d</td>
<td>Positive</td>
</tr>
<tr>
<td>S100A4(2+,3+) (%)</td>
<td>11 (50%)</td>
<td>12 (63.16%)</td>
<td>10 (41.67%)</td>
<td>6 (31.58%)</td>
</tr>
<tr>
<td>S100A4(-,+) (%)</td>
<td>11 (50%)</td>
<td>13 (65%)</td>
<td>14 (58.33%)</td>
<td>13 (68.42%)</td>
</tr>
<tr>
<td>E-cadherin(2+,3+) (%)</td>
<td>11 (50%)</td>
<td>7 (35%)</td>
<td>16 (66.67%)</td>
<td>13 (68.42%)</td>
</tr>
<tr>
<td>E-cadherin(-,+) (%)</td>
<td>11 (50%)</td>
<td>8 (34.78%)</td>
<td>8 (33.33%)</td>
<td>8 (33.33%)</td>
</tr>
<tr>
<td>p</td>
<td>0.877</td>
<td>0.099</td>
<td>0.166</td>
<td>0.024</td>
</tr>
</tbody>
</table>

Table 2: Correlation between S100A4 and E-cadherin expression levels in oral SCC

<table>
<thead>
<tr>
<th>S100A4</th>
<th>E-cadherin</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100A4(2+,3+) (%)</td>
<td>E-cadherin(2+,3+) (%)</td>
<td>E-cadherin(-,+) (%)</td>
</tr>
<tr>
<td>7 (30.33%)</td>
<td>16 (69.57%)</td>
<td>0.011</td>
</tr>
</tbody>
</table>

We observed a significant inverse correlation between S100A4 and E-cadherin expression in oral SCC. Lymph node metastasis was detected in the A (13/16=81.3%), B (4/14=28.6%), C (4/7=57.1%), and D groups (3/6=50%), at the indicated frequencies. Statistical analysis demonstrated a significant difference between the 4 groups in the incidence of lymph node metastasis (P=0.04) (Table 3). According to the Kaplan-Meier survival curve, the A group had a significantly poorer prognosis than the B group (P=0.0021), but had no worse prognosis than the C and D groups (Fig.6).

Evaluation of both metastatic potential and prognosis by expression of S100A4 and E-cadherin and histopathological factors

Based on examination of tumor differentiation, the TNM stage, mode of invasion, and the expression of S100A4 and E-cadherin in the oral SCC, we set-up a system to evaluate the metastatic potential and prognosis of the carcinoma. This system estimated each of the individual criteria, degree of differentiation, type of TNM...
stage, mode of invasion, and pattern of S100A4 and E-cadherin staining, on a 0-3 point scale (Table 4). A total point value for each patient was determined by summing the point values for the criteria. The evaluation system permits a gradation of values ranging from 0 to 15. The cases examined in this study remained within the range of 2-13 points, establishing the validity of the system (tumor differentiation, TNM stage, invasive mode, S100A4 and E-cadherin expression).

According to our evaluation system (we named it as DTISE system), the number of the metastatic cases with a total point value less than 7 numbered only 2 of 43 cases (4.65%). Twenty-two cases with a total point greater than 8 exhibited metastases (51.16%). The incidence of metastasis in cases with total points more than 8 was significantly higher than the cases with fewer than 7 points (P=0.000) (Table 5). In addition, Kaplan-Meier analysis demonstrated that the cases with a score of more than 8 points had a significantly poorer prognosis than the cases with less than 7 points (P=0.0022) (Fig.7).

**Discussion**

S100A4 is a calcium-binding protein belonging to the S100 family. This molecule has been linked to increased metastasis; its expression levels strongly correlate with the invasive and metastatic behavior of cultured tumor cells (4-6,8). In addition, S100A4 expression correlated with lymph node metastasis and prognosis in clinical studies of multiple cancers (7-10). In this study, we examined the expression of S100A4 by immunohistochemistry in oral SCC specimens; our results showed S100A4 expression correlated with lymph node metastasis (P=0.024) and poor prognosis (P=0.0136), but not with either differentiation (P=0.877), tumor stage (P=0.099), or invasive pattern (P=0.166). Although only limited information detailing the relationship between S100A4 expression and clinicopathological parameters has been reported in clinical studies, our findings are agreement with previous studies of breast cancer and NSCLC. The cell-cell adhesion molecule E-cadherin suppresses invasive growth of epithelial cells in vitro. Loss of expression is important in lymph node metastasis (28), leading to poor prognosis in oral SCC (28,29). In this study, reduced expression of E-cadherin correlated with increased lymph node metastasis (P=0.023) and poorer prognosis (P=0.0146), as seen in previous reports. Furthermore, reduced expression of E-cadherin correlated with tumor stage (P=0.024) and in-

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**Table 3: Relationship between the cooperative expression of S100A4 and E-cadherin and lymph node metastasis in oral SCC**

<table>
<thead>
<tr>
<th>Cooperative expression of S100A4 and E-cadherin</th>
<th>Lymph node metastasis</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (%)</td>
<td>Positive (%)</td>
</tr>
<tr>
<td>A (16) S100A4(2+,3+) E-cadherin(-,+)</td>
<td>3 (18.75%)</td>
<td>13 (81.25%)</td>
</tr>
<tr>
<td>B (14) S100A4(-,+) E-cadherin(2+,3+)</td>
<td>10 (71.43%)</td>
<td>4 (28.57%)</td>
</tr>
<tr>
<td>C (7)  S100A4(2+,3+) E-cadherin(2+,3+)</td>
<td>3 (42.86%)</td>
<td>4 (57.14%)</td>
</tr>
<tr>
<td>D (6)  S100A4(-,+) E-cadherin(-,+)</td>
<td>3 (50%)</td>
<td>3 (50%)</td>
</tr>
</tbody>
</table>

There was significant difference between the 4 groups in the incidence of lymph node metastasis.

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**Table 4: Grading of malignancy on the basis of histopathology and histochemistry of oral squamous cell carcinoma**

<table>
<thead>
<tr>
<th>Point</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiation</td>
<td>W</td>
<td>M</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td>Invasion mode</td>
<td>1~2</td>
<td>3</td>
<td>4C</td>
<td>4D</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>3+</td>
<td>2+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>S100A4</td>
<td>−</td>
<td>+</td>
<td>2+</td>
<td>3+</td>
</tr>
</tbody>
</table>

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**Table 5: Correlation of the total points for each group with the incidence of metastasis to the regional lymph nodes**

<table>
<thead>
<tr>
<th>Lymph nodes metastasis</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (%)</td>
<td>Negative( % )</td>
</tr>
<tr>
<td>Total point ≥ 8</td>
<td>22 (88%)</td>
</tr>
<tr>
<td>Total point ≤ 7</td>
<td>2 (11.11%)</td>
</tr>
</tbody>
</table>

Incidence of metastasis in the cases with a total score of more than 8 was significantly higher than those with a total score of less than 7.
vasive pattern (P=0.009), but not with differentiation (P=0.877), similar to the results of Shinohara et al., reporting no significant relationship between the downregulation of E-cadherin and differentiation in oral SCC (13).

A statistically significant inverse correlation between the expression of S100A4 and of E-cadherin was recently reported in murine cells (17). An inverse correlation between the expression of S100A4 and the members of the cadherin-catenin complex occurs in gastric cancer and NSCLC (9,30). No correlation, however, could be observed between S100A4 immunoreactivity and expression of E-cadherin or α- or β-catenin in breast cancer (31). In our study, chi-square analysis indicated the importance of both S100A4 and E-cadherin in evaluation of lymph node metastases in oral SCC. In terms of the prognosis determined by Kaplan-Meier method, the S100A4(2+,3+)/E-cadherin(-,+)+ group had a significantly poorer prognosis than the S100A4(-,+)/E-cadherin(2+,3+) group (P=0.0021), the S100A4(2+,3+)/E-cadherin(2+,3+) and S100A4(-,+)/E-cadherin(-,+)+ groups exhibit similarly poor prognosis as the S100A4(2+,3+)/E-cadherin(-,+)+ group. Only S100A4(-,+)/E-cadherin(2+,3+) group demonstrated an increased survival amongst these groups (P=0.007). These data suggest that both E-cadherin and S100A4 expression influence the outcome of disease; aberrant expression of either molecule leads to poor prognosis.

Several histological factors have been associated with the prognosis of oral SCC patients in previous studies. We determined the combination of S100A4 and E-cadherin expression and histopathological factors may provide a more accurate evaluation of metastatic potential and prognosis for patients with oral SCC. Consequently, we developed a system that can evaluate both metastasis potential and prognosis of the oral SCC using tumor differentiation, TNM staging, mode of invasion, and E-cadherin and S100A4 expression as criteria.

Although the TNM stage system alone is not sufficient to determine the prognosis of carcinoma patients, it is still considered the most important prognostic factor for oral SCC in clinical use (2,21-23,32). An advanced degree of differentiation of the carcinoma is regarded as a favorable feature, while the lack of differentiation is an ominous indication (19,21). Some researchers, however, have reported that the mode of invasion is an equally important factor of the tumor-host relationship in patient prognosis prediction (18,20,21). In this study, Twenty-two of 23 cases of with stage 3 and stage 4 carcinomas possessed lymph node metastasis (95.65%). The 5-year survival rate for stage 3-4 and stage 1-2 tumors were 52.17% and 75%, respectively. Examination of the mode of invasion determined that 14 of 19 cases staged as ‘4c’ or ‘4d’ exhibited metastasis (73.68%), while 10 of 24 cases staged as ‘1’, ‘2’ and ‘3’ showed lymph node metastasis (41.67%). The 5-year survival rate for ‘4c’ and ‘4d’ cases was only 31.58%, while the ‘1’, ‘2’ and ‘3’ cases has a survival rate of 87.5%. Furthermore, 14 of 21 moderately or poorly differentiated cases (66.67%) and 10 of 22 well differentiated cases (45.45%) showed lymph node metastases. The 5-year survival rates of the cases with moderately or poorly differentiated and well differentiated tumors were 47.62% and 77.27%, respectively. These results demonstrate that tumor differentiation, TNM stage, and the mode of invasion are useful parameters in the prediction of the metastatic potential and prognosis. These results support our selection of these parameters in our DTISE evaluation system. Statistical analysis demonstrated that our DTISE system accurately evaluates the lymph node metastasis of oral SCC (P=0.000); cases with a score greater than 8 points were associated with poor survival (P=0.0022).

In the present study, we examined the expression of S100A4 and E-cadherin in oral SCC as useful parameters in the evaluation of lymph node metastasis and prognosis prediction. We also analyzed the correlation between S100A4 and E-cadherin expression. Subsequently, based on expression pattern and other histopathological factors, we have established a system to evaluate oral SCC metastases and prognosis. As a result, we believe S100A4, both as a single factor and in combination with E-cadherin and other histopathological factors, may be a highly useful marker in the prediction of metastatic potential and prognosis of oral SCC patients. S100A4 status will be of great value to clinical oncologists in determining the best therapy methods for patients to obtain an improved outcome.

References


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