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Osteopontin as biomarker in early invasion by squamous cell carcinoma in tongue

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BACKGROUND: Osteopontin (OPN) expression in squamous cell carcinoma (SCC) of the tongue has not been clearly elucidated.
METHODS: We selected 46 cases of tongue SCC and investigated the expression of OPN by immunohistochemical staining. The immunopositive reaction and score for each case were semiquantitatively evaluated.
RESULTS: Scores were significantly higher in carcinoma nests than in neighboring normal epithelium or epithelial dysplasia. The OPN was expressed clearly in the cytoplasm of carcinoma cells. In cases of early invasive carcinoma, in particular, expression of OPN showed a remarkable increase at the invasion front compared with the non-invaded regions. However, there was no significant correlation between expression of OPN in the primary tumor nest and lymphatic metastasis, recurrence, or survival rate.
CONCLUSION: This suggests that OPN is a useful biomarker of early invasion by SCC in tongue.
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Keywords: tongue carcinoma; osteopontin; immunohistochemistry

Introduction

Oral squamous cell carcinoma (OSCC) is not a rare disease, and the 5-year cancer survival rate is $< 50\%$ if the cancer progresses from the localized to the regional stage (1). Therefore, early detection and treatment are extremely important for the patient's prognosis. Although, specific biomarkers and various factors related to OSCC have been investigated (2–4), a useful biomarker for OSCC remains to be found.

Many kinds of extracellular matrix (ECM) have been investigated in malignant tumors as potential factors concerned with proliferation, invasion and metastasis. Recently, the expression of osteopontin (OPN), an ECM, has been examined in relation to the carcinogenesis and metastasis of various malignant tumors such as the lung, esophageal, breast, and salivary gland (5–8), and the results have suggested that OPN is a candidate biomarker of malignant tumors (9, 10).

Osteopontin was first described as a secreted transformation-specific phosphoprotein (11), and it has also been recognized as a major sialoprotein of the ECM that binds calcium and functions in early-stage mineralization in bone and dentin (12). The OPN has many other functions, including being an early component of type-1 immunity (13) in activated T lymphocytes and macrophages, mediating angiogenesis and inhibiting apoptosis in soft tissues (10). The OPN is also involved in cell adhesion and cell migration, as it has the Arg–Gly–Asp (RGD) sequence that binds certain integrins, including integrin$\alpha_v\beta_1$, integrin$\alpha_v\beta_3$, and integrin$\alpha_v\beta_5$ (10). OPN also influences cell migration via interaction with CD44 (14) and activation of the epidermal growth factor pathway (7).

Although the relationship between expression of OPN and malignancy has already been studied in head and neck squamous cell carcinoma (SCC) (15, 16), only a few cases of OSCC have been investigated. No studies have semiquantitatively analyzed the immunoreactive score of OPN or investigated the relationship between OPN expression and prognosis such as in lymph node metastasis and recurrence in tongue SCC.

We have already elucidated the function of OPN in OSCC cell line. Proliferation and invasion showed a reduction, by inhibition of OPN antisense in BSC-OF derived from oral basaloid SCC cells (17). Therefore, it is important to clarify the expression of OPN in tissue from OSCC patients. In the present study, we immunohistochemically evaluated the expression of OPN in dissected tongue SCC tissue. Tumor progression and route of metastasis differ depending on the site of the primary origin of OSCC. Therefore, we examined only primary tongue SCC, and analyzed the correlation between OPN expression and recurrence, metastasis and survival.

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Materials and methods

Materials

Data related to forty-six patients with tongue SCC between the years 1994 and 2000 were selected from the files of the Departments of Pathology and Oral and Maxillofacial Surgery at the Chiba Hospital of Tokyo Dental College. Twenty-five patients were male, and 21 were female. Age ranged from 25 to 96 years (average 58). Pre-operative treatment was performed in 37 cases; chemotherapy in 34 cases and chemo-radiotherapy in three cases. Eight of nine cases were early invasive carcinoma. None of these patients had received pre-operative treatment.

Immunohistochemical detection of osteopontin

Dissected tissues were fixed with 10% neutral buffered formalin and embedded in paraffin. Immunohistochemical staining was performed with the Ventana HX system (Ventana, Yokohama, Japan) according to the manufacturer's protocol. For antigen retrieval, deparaffinized 4 μm paraffin sections were incubated in 0.01 M buffered citric acid and microwaved at 60°C for 10 min. To block endogenous peroxidase, sections were exposed to 0.3% hydrogen peroxide in methanol, and then incubated with 10% normal goat serum (IBL, Gunma, Japan) for 10 min to avoid non-specific reaction. Mouse monoclonal anti-OPN antibody (IBL) diluted 1:50 with 0.01 M phosphate-buffered saline (PBS) was applied as the primary antibody for 30 min at 37°C. As a negative control, 0.01 M PBS was used instead of the primary antibody. After incubation with biotinylated secondary antibody and peroxidase-conjugated horseradish streptavidin, staining was visualized using 3,3′-diaminobenzidine in 0.05 M Tris–HCl (pH 7.6) with hydrogen peroxide. The sections were then counterstained with hematoxylin and examined using a conventional light microscope. Immunopositive reaction was judged with a 450 ∼ 600 nm green interference filter (Zeiss, München, Germany).

Evaluation of immunohistochemical staining

Immunopositive reaction was semiquantitatively evaluated with reference to the method of Grizzle et al., altering the classification of the immunopositive staining level (18). Evaluation was carried out at three areas in each case: in normal epithelium (NE), epithelial dysplasia (ED) and the nest of invasive carcinoma (NC). First, the cells were classified individually from level 0 to 3 based on the intensity of immunostaining for OPN determined from several views selected at random in each area (Table 1). Next, we counted cell number for each value of intensity. The total product of intensity and proportion of all levels in one view was taken as the score in each case. For example, a cell showing a level 1 intensity accounting for 29% of one field would score 0.29 (1 × 0.29), yielding a value of 2.24 (Table 2).

Table 1 Intensity of immunostaining

Level	*Intensity*
0	Negative
1	Weak staining of cytoplasm
2	Distinct staining of cytoplasm
3	Remarkable staining of cytoplasm

Table 2 Scoring of osteopontin expression for the case 902–98, NC

Intensity	*0*	*1*	*2*	*3*
Population	0	0.29	0.18	0.53
Score	0	0.29	0.36	1.59

Total score: 0 + 0.29 + 0.36 + 1.59 = 2.24.

Correlation with clinico-pathological factors

All cases were divided into low- and high-score groups using the median of the total score (1.47 in NC area) as the cut-off value. In particular, histopathological differentiation, tumor size (T classification) at primary region (19), and lymphatic metastasis were compared between the two groups.

Cumulative lymphatic metastasis, recurrence and survival rate were also compared between the two groups.

Statistical analysis

We analyzed correlation of OPN expression among the three above-mentioned areas with a one-way factorial ANOVA and multiple-comparison tests. Analyses of the correlation between OPN expression and clinico-pathological factors and prognosis were performed using the chi-squared test, the Kaplan–Meier method and the Log-rank test.

Results

The average total score for OPN expression in the 46 tongue SCC cases was 0.07 ± 0.13 in NE, 0.28 ± 0.39 in ED, and 1.23 ± 0.79 in NC. There were significant differences between NE and NC, and ED and NC ($P < 0.01$, Fig. 1).

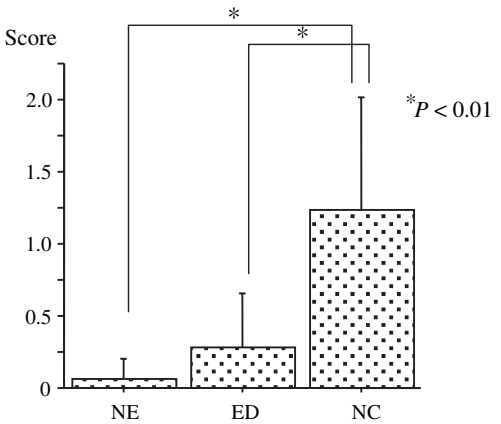

Figure 1 Average total score was 0.07 ± 0.13 in normal epithelium (NE), 0.28 ± 0.39 in epithelial dysplasia (ED), and 1.23 ± 0.79 in nest of invasive carcinoma (NC). There were significant differences between NE and NC, ED and NC ($P < 0.01$, Fig.1).

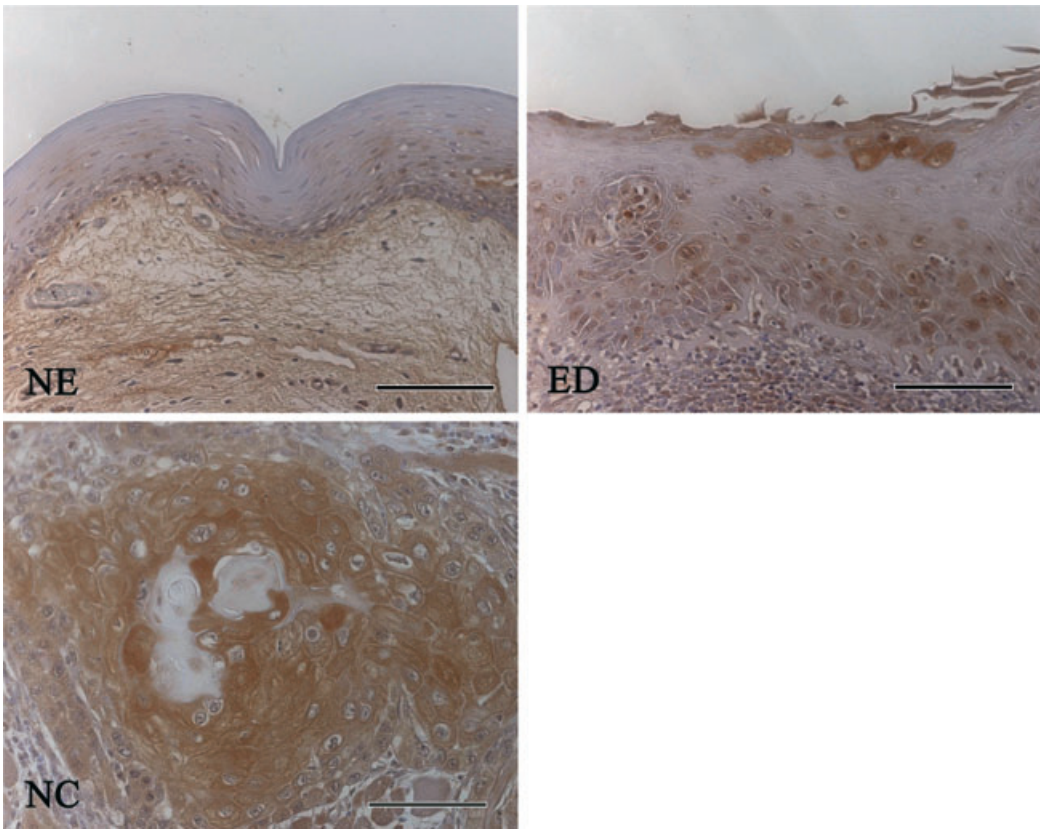

Figure 2 Normal epithelium (NE), Very weak osteopontin (OPN) expression was found in basal cell layer; epithelial dysplasia (ED), OPN localized in cytoplasm of basal and prickle cell layers, with more distinct staining than in NE; invasive carcinoma (NC), almost all cells, except keratinized cells, showed remarkable staining in cytoplasm (bar = 100 μm).

In many cases, OPN expression was negative or very weak in the basal cell layer of NE, whereas it was localized in the basal and prickle cell layers of ED, with a more distinct staining than that in NE. Almost all cells, except keratinized cells, showed remarkable staining in the cytoplasm in the NC area (Fig. 2). In early invasive carcinoma without pre-operative treatment, in particular, OPN showed a definite, more increased expression at the invasion front than the neighboring ED (Fig. 3A). In areas where the basement membrane of squamous epithelium was unclear, OPN showed an increase (Fig. 3B).

There were no significant differences between the two groups (high- and low-score group) in terms of histopathological differentiation, tumor size or lymphatic metastasis. However, although there was a tendency for OPN expression to be higher in NE in lymphatic metastasis positive cases than in negative cases, the difference was not significant (Table 3). Cumulative lymphatic metastasis, recurrence and survival rate showed no significant differences between the high- and low-score groups.

Discussion

The purpose of this study was to determine the OPN expression in SCC of the tongue. Semiquantitative evaluation of immunopositive reaction of OPN and scoring for each case revealed that OPN expression was significantly higher in carcinoma cells than in normal or dysplastic epithelia. This suggests that OPN expression increases with malignant transformation of the tongue. In those cases which received pre-operative treatment, OPN expression was similar to that in those which did not. These results are consistent with those of our previous *in vitro* study showing that proliferation and invasion were reduced by inhibition of OPN in BSC-OF cells (17).

In this study, abundant OPN was detected in the cytoplasm of the carcinoma cells in the tumor nest in the tongue. Under hypoxia, OPN expression is up-regulated via a Ras-activated enhancer (20). This suggests that OPN expression is significantly and consistently high during tumor progression in the carcinoma nest in solid tumors, including OSCC. Generally, secreted OPN binds to integrins and functions in cell migration and chemotaxis as an ECM (12). Furthermore, Zohar et al. (21) found that intercellular OPN bound the CD44-ezrin/radixin/moesun (ERM) complex in fibroblasts. The OPN in the cytoplasm of tumor cells binds to CD44 on the plasma membrane, transducing the intercellular signal for cell motility (9). Teramoto et al. (22) reported that the OPN-CD44 Rac autocrine pathway was enhanced by H-Ras in NIH3T3 cells. The ERM binds

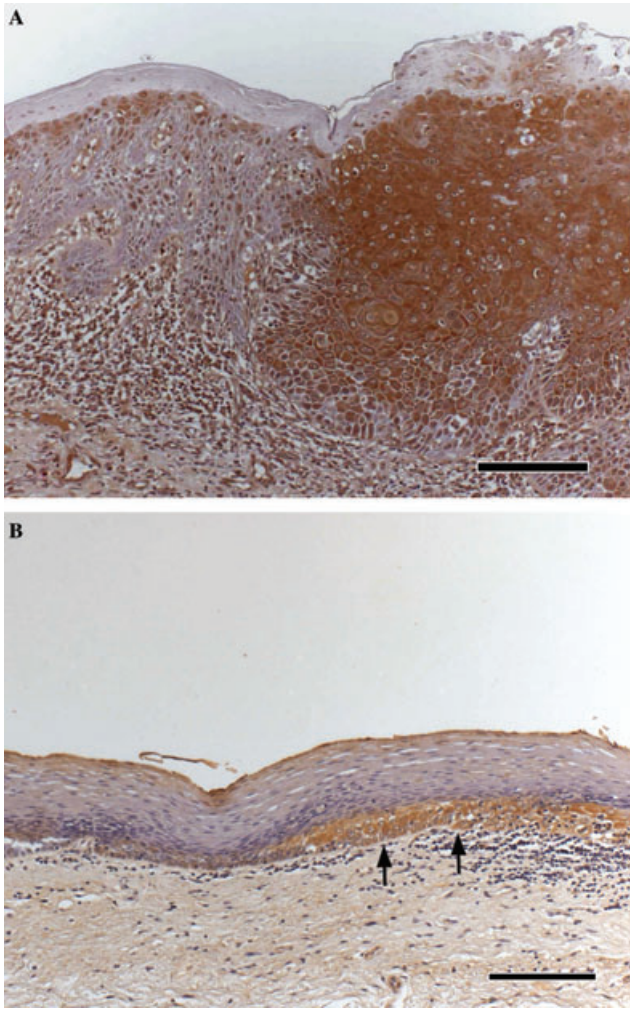

Figure 3 (A) Osteopontin (OPN) expression increased remarkably at invasion front compared with the neighboring epithelial dysplasia in early invasive carcinoma (bar = 150 μm). (B) In specimen of early invasive carcinoma, OPN was found in region where basement membrane was destroyed (arrow, bar = 75 μm).

Table 3 Relationship between osteopontin expression and clinico-pathological parameters

	No. of cases	*Low-score group*	*High-score group*
Histology			
Early invasive	8	5	3
Well differentiated	22	12	10
Moderately differentiated	5	2	3
Poorly differentiated	11	6	5
Tumor size			
T1–2	31	15	16
T3–4	15	10	5
Lymphatic metastasis			
Negative	32	19	13
Positive	14	6	8

to actin filaments and induces cell motility (12, 21). These findings indicate that OPN functions in invasion via both extra- and intercellular pathways in the autocrine system in carcinoma cells, including SCC in the tongue.

The SIBLING (small integrin-binding ligand N-linked glycoproteins) family currently includes five members, i.e. bone sialoprotein, OPN, dentin matrix protein 1, dentin sialophosphoprotein and matrix extracellular phosphoglycoprotein. These are conserved gene clusters and have RGD sequences and shown to bind and activate some kind of matrix metalloproteinases (23–25). Therefore, the family is considered to have an important role about tumor invasion and metastasis. Expressions of the SIBLING members have been investigated as a biomarker in prostate, lung and other cancers (24, 26, 27). Furthermore, OPN expression showed a significant correlation with tumor size in breast, rectal and lung cancers (24). In the present study, we showed no significant correlation between OPN expression and prognostic parameters including lymphatic metastasis, recurrence or cumulative survival rate. No significant differences were also found between OPN expression and histopathological differentiation or tumor size. These results suggest that expression and value as biomarker of SIBLING family including OPN are different due to the primary region of cancers as reported by Fisher et al. (24). On the other hand, we distinctly observed OPN expression at the region where the basement membrane was destroyed rather than at dysplastic epithelium. The result suggests that OPN is more an early invasion marker than a prognostic marker in OSCC.

It is often difficult to distinguish early invasive carcinoma and severe ED in OSCC in pathological diagnosis. It is hard to determine whether destruction of the basement membrane has taken place, due to abundant lymphocyte infiltration of connective tissue beneath the epithelium. From the clinical point of view, the results of this study suggest that OPN expression offers a useful biomarker for distinguishing severe ED and early invasive carcinoma in tongue.

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