

CLINICAL SIGNIFICANCE OF OSTEOPONTIN EXPRESSION IN T1 AND T2 TONGUE CANCERS

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Abstract: *Background.* Osteopontin (OPN) is considered to be a tumor-related protein associated with tumor aggressiveness and metastasis.

Methods. Immunohistochemistry was used to study the clinical significance of OPN expression in T1 and T2 tongue cancers.

Results. Positive OPN expression significantly correlated with higher tumor classification (T) ($p = .004$), positive nodal classification (N) ($p < .001$), greater tumor thickness ($p < .001$), and presence of tumor necrosis ($p = .016$), respectively. The unfavorable cumulative 5-year disease-free survival rate significantly correlated with positive OPN expression ($p < .001$), T2 ($p = .024$), positive N ($p < .001$), greater tumor thickness ($p = .023$), and positive tumor necrosis ($p = .003$). However, taking CD105 into consideration, only CD105 expression was the independent prognostic factor for survival by Cox's regression analysis.

Conclusion. Overexpression of OPN in the tumors implicated a more aggressive tumor behavior and was an important factor

for survival. In addition, there might be relationship between OPN and CD105 expressions in angiogenesis. © 2008 Wiley Periodicals, Inc. *Head Neck* **30**: 776–781, 2008

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Osteopontin (OPN) was first described by Senger et al in 1979 by analyzing the proteins secreted by the transformed epithelial cells.¹ It is a calcium-binding glyco-phosphoprotein functioning as a ligand of alpha versus beta integrin and CD44 receptors.^{2,3} The receptor binding allows OPN to mediate adhesive cell–matrix interaction.³ The OPN gene is located on the chromosome 4q13 and has been reported to be expressed and secreted by many kinds of cancers.^{4–10} It was also linked to tumor hypoxia and has been shown to be related to tumor progression.⁹

OPN is considered to be a tumor-associated protein that may promote tumor development and

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metastasis.^{11,12} Overexpression of OPN was found to be related to tumor progression and lymphatic metastasis of human gastric cancer.⁵ Tuck et al^{13,14} have shown that recombinant OPN could induce migration and invasion of human mammary epithelial cells. Furthermore, high level of plasma OPN in patients with malignancies is significantly related to metastatic diseases.^{4,15}

Oral cancer is the sixth most common cancer worldwide and is the most frequently observed head and neck cancer in Southeast Asia.¹⁶ Among the cancer-related death rates in Taiwan, oral cancer was approximately 8.8 per 100,000 persons in the general population in 2004, and ranked the fourth place in men (<http://www.doh.gov.tw/statistic/data/>). Besides imaging studies, identification of promising biomarkers in the tumor tissue among patients with oral cancer could be helpful for prognosis evaluation and treatment planning. Our study aimed to evaluate the clinicopathological significance of OPN expression among patients with T1 and T2 tongue cancers by immunohistochemical analysis.

PATIENTS AND METHODS

Study Population. The study cohort was the same as in the previous study.¹⁷ The study included 94 patients who underwent primary surgical resection between July 1996 and August 2005 by 2 surgeons (C.-Y. S. and C.-Y. C.) for treatment of squamous cell carcinoma of the oral tongue in early tumor classification (T1 and T2) without subsequent radiotherapy and/or chemotherapy. The clinicopathologic information, including, sex, age, tumor classification (T), nodal classification (N), histological grade, tumor thickness, tumor necrosis, perineurial invasion, vascular invasion, and disease-free survival, was obtained from the clinical records and the pathologic reports, retrospectively. Positive tumor necrosis was defined as at least 1 definitely necrotic cell cluster or a necrotic area in the tumor nests microscopically, but single cell necrosis or apoptosis was excluded. The TNM status was classified according to 2002 American Joint Committee on Cancer (AJCC) system. This study was approved by the Medical Ethics and the Human Clinical Trial Committee at Chang Gung Memorial Hospital.

Immunohistochemical Study. The representative blocks of the formalin-fixed, paraffin-embedded

tissues were retrieved and sectioned for the immunohistochemical study. The OPN (AKm2A1) monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and was diluted 1:100 in phosphate buffered saline (PBS) according to the manufacturer's recommendation. The sections were incubated overnight in a 37°C oven, deparaffinized, treated with 3% hydrogen peroxide for 10 minutes to deprive the endogenous peroxidase activity, and then microwaved in 10 mM citrate buffer, pH 6.0, to unmask the epitopes. After antigen retrieval, the sections were incubated with diluted OPN antibody for 1 hour followed by PBS wash. Horseradish peroxidase/Fab polymer conjugate (PicTure-Plus kit; Zymed, South San Francisco, CA) was then applied to the sections for 30 minutes followed by PBS wash. Finally, the sections were incubated with peroxidase substrate diaminobenzidine for 5 minutes to develop the signals. Negative control was done simultaneously by omitting the primary antibody.

Osteopontin Status Evaluation. All the sections were evaluated by a pathologist (C.-C. H.) who did not know the clinical data. To evaluate the expression of OPN, the sections were examined under microscope with 200× magnification. Positive OPN expression was defined as detectable immunoreactivity in the perinuclear and/or other cytoplasmic regions in at least 10% of the cancer cells.¹⁸

Statistical Analysis. Clinicopathologic factors that were evaluated including sex, age (<60 y/o vs ≥ 60 y/o), T1 versus T2, N classification, histological grade, the greatest tumor thickness (≤4 mm vs >4 mm),¹⁴ and presence of tumor necrosis, perineurial invasion and vascular invasion. Fisher's exact test was used to evaluate the correlation between the clinicopathologic variables and the expression of OPN. A *p* value less than .05 was considered significant in all the statistical analyses. The clinicopathologic variables and the expression of OPN were taken into account for the analysis of survival based on Kaplan–Meier method and the statistical significance defined as *p* < .05 was assessed by log-rank test. To determine the effect of distinct prognosis factors on survival, a multivariate analysis was performed according to the Cox's regression model.

RESULTS

There were 11 women and 83 men, with an average age of 50.3 years (range, 26–84 years). Thirty-nine patients (41.5%) were classified as T1, 55

Table 1. Clinical profile and correlation between the clinicopathologic features and expression of OPN.

Variables	No. of patients	OPN (-)	OPN (+)	<i>p</i> value
Sex				
Male	83	55	28	.493
Female	11	9	2	
Age				
≤59 y	69	50	19	.142
≥60 y	25	14	11	
T classification				
T1	39	33	6	.004*
T2	55	31	24	
N classification				
Negative	66	62	4	<.001*
Positive	28	2	26	
Histological grade				
Well differentiated	69	48	21	.624
Moderately differentiated	25	16	9	
Tumor necrosis				
Negative	79	58	21	.016*
Positive	15	6	9	
Tumor thickness				
≤4 mm	38	35	3	<.001*
>4 mm	56	29	27	
Perineurial invasion				
Negative	76	55	21	.092
Positive	18	9	9	
Vascular invasion				
Negative	74	51	23	.79
Positive	20	13	7	

Note: OPN (-), negative expression of OPN; OPN (+), positive expression of OPN.
*Significant.

patients (58.5%) as T2. Sixty-six patients (70.2%) were classified as N0, 18 (19.1%) as N1, 6 (6.4%) as N2b, 4 (4.3%) as N2c (Table 1).

The OPN expression in the tongue cancers was mainly present in the cytoplasm of the malignant squamous epithelial cells (Figure 1). Sixty-four cases were negative and 30 cases were positive for the OPN immunostaining. The underlying skeletal muscle and surrounding inflammatory cells were also focally immunoreactive with OPN. The result of the OPN immunostaining in the cancer cells and its correlation with the clinicopathologic variables are summarized in Table 1. The positive expression of OPN significantly correlated with relatively advanced T (T2 vs T1) ($p = .004$), positive N (positive vs negative) ($p < .001$), the presence of tumor necrosis ($p = .016$), and greater tumor thickness ($p < .001$). However, no correlation was found between the expression of OPN and sex, age, histological grade, perineurial invasion, and vascular invasion.

The cumulative 5-year disease-free survival rate was 86.6%, with the mean follow-up of

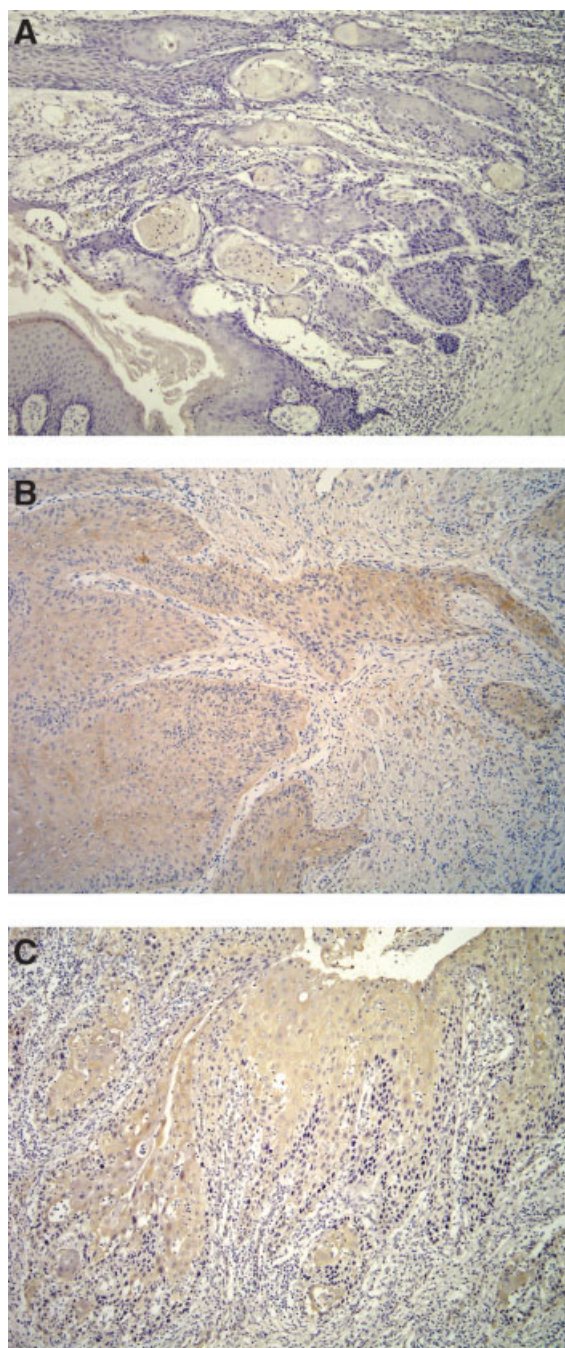


FIGURE 1. Osteopontin immunostaining of representative cases of early tongue cancers. (A) The well-differentiated squamous cell carcinoma (T2) without nodal metastasis shows only focal faint staining for osteopontin and is classified as negative for osteopontin expression. (B) Another well-differentiated squamous cell carcinoma (T2) with nodal metastasis shows distinct staining for osteopontin in most of the tumors cells, and is classified as positive for osteopontin expression. Focal increase in staining intensity is noted in the deeper invasive cells (right side). (C) The moderately differentiated squamous cell carcinoma (T2) with nodal metastasis shows distinct staining for osteopontin in most of the tumors cells and is classified as positive for osteopontin expression.

44.1 months. The disease-free survival rates for patients with negative expression of OPN (92.8%) was significantly higher than for those patients with positive expression of OPN (63.4%) ($p < .001$, log-rank test). According to our previous study,¹⁷ the disease-free survival rates for those patients in T2, with positive N, with tumor necrosis, and having greater tumor thickness were significantly lower than for those in T1, with negative N, without tumor necrosis, and having smaller tumor thickness ($p = .024$, $p < .001$, $p = .003$, and $p = .023$, respectively). However, Cox regression analysis revealed that only the expression of OPN (95% confidence interval = 1.899–20.107, relative risk = 6.179, $p = .002$) and the presence of tumor necrosis (95% confidence interval = 1.09–10.604, relative risk = 3.4, $p = .035$) were the independent prognostic factors for survival.

To delineate the relationship among the expressions of OPN, vascular endothelial growth factor (VEGF), and CD105, OPN was analyzed to the same cohort as that in our previous study for VEGF and CD105.¹⁷ The p values were both less than .001 (Spearman rank-correlation coefficient). Because all the expressions of OPN, VEGF, and CD105 were shown to be important prognostic factors in our study cohort of the early tongue cancer,¹⁷ these markers were included in survival analysis again by Cox's regression model. The result showed that the expression of CD105 but not the expression of OPN was the only independent prognostic factor for survival.

DISCUSSION

OPN has been known to be related to chemotaxis, cell adhesion, macrophage-directed interleukin-10 suppression, angiogenesis, and prevention of apoptosis.^{12,19–21} However, the role of OPN in tumorigenesis has not yet been well understood. Some studies revealed that overexpression of OPN could protect tumor cells by escaping from the host immune system through suppressing production of nitric oxide in the activated macrophages via inhibition of nitric oxide synthetase.^{22,23} This is believed to be a key step for tumor cell survival in human tissues. OPN also binds with CD44 or alpha versus beta 3 integrin to initiate many signal pathways that are associated with cell adhesion, migration, and tumor metastasis.^{2,12}

It has been found that OPN plays important roles in angiogenesis that is essential for tumor growth and metastasis. VEGF may induce OPN expression, and OPN stimulates endothelial cell

migration in cooperation with VEGF.²⁴ In addition, OPN also functions through the signal pathway by alpha versus beta integrins to regulate endothelial cell survival and the process of angiogenesis.^{24–28} The present study showed that positive expression of OPN in early tongue cancers was related to T2, positive N, and greater tumor thickness, all of which suggest a more aggressive tumor status. Furthermore, positive expression of OPN was also related to tumor necrosis, a sign of tumor hypoxia caused by ongoing tumor growth outpacing the blood supply. Accordingly, OPN expression might be induced by the hypoxia signals of the aggressive tumors to enhance tumor angiogenesis for tumor growth and metastases. This postulate was also supported by the findings of the previous reports that the expressions of OPN and other angiogenic factors such as CD105 and VEGF significantly correlated with nodal metastasis among head and neck cancers.^{29–32}

The findings for the close relationship between OPN and VEGF in our study agreed with the previous reports for the interaction of OPN and VEGF on endothelial cell migration and for the correlation of OPN staining and VEGF expression in head and neck cancers.^{24,33,34} In addition, it has been found that OPN expression correlated with tumor hypoxia.^{9,35} Taken together, the results of our previous and current studies suggest that OPN, as well as CD105 and VEGF, may participate in angiogenesis of early tongue cancers. It could result from a relatively hypoxic status that induces the expression of OPN and the activation of other angiogenesis factors including CD105 and VEGF during the early extension of the tumor.

In addition to clinical stage, many pathologic features, such as tumor thickness, tumor necrosis, perineurial invasion and tumor necrosis, and angiogenesis factors, such as CD105 and VEGF, have been reported to be significantly related to the prognosis in early tongue cancer.^{17,36} In the present study, the expression of OPN was also shown to be a prognostic predictor for early tongue cancers. This finding was consistent with other reports in a number of neoplasms involving breast, stomach, lung, and esophagus.^{4,5,18,37} Although the OPN expression was not an independent factor for prognosis in early tongue cancers by Cox regression analysis, this finding was similar to the previous report of OPN expression in esophageal squamous cell carcinoma.¹⁸ It implies that OPN expression could be influenced

by CD105 expression in angiogenesis of early tongue cancers.

Matsuzaki et al³⁸ previously reported a similar study for OPN expression in tongue cancer. The authors used the intensity of immunostaining to evaluate OPN expression in tongue cancer of T1 to T4. However, they failed to show any significance between the expression of OPN and lymphatic metastasis and survival. The present study adopted another method developed by Kita et al¹⁸ for the evaluation of OPN expression in esophageal squamous cell carcinoma. By this method, positive OPN expression significantly correlated with T classification (T1 vs T2), status of tumor necrosis, lymph node metastasis, and survival. To compare these 2 scoring systems, the method developed by Matsuzaki et al³⁸ was also used to reevaluate OPN expression in the present study (data not shown). The result showed that the expression of OPN still significantly correlated to the expressions of VEGF and CD105 (both $p < .001$), tumor invasion depth ($p = .001$), and regional nodal metastasis ($p < .001$) and these 2 evaluation systems correlated well with each other ($p < .001$). It suggests that both evaluation systems are valid for the scoring of OPN expression.

In conclusion, the present study demonstrated that OPN can be used as a helpful biomarker to predict the clinicopathologic features in T1 and T2 tongue cancers. Positive expression of OPN is associated with tumor progression and unfavorable prognosis among those patients with early tongue cancer. In these cases, a more aggressive treatment planning should be considered to pursue a better clinical outcome.

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