ORIGINIAL ARTICLE

EXPRESSION OF PLACENTA GROWTH FACTOR: AN INDEPENDENT FACTOR FOR PREDICTION OF PROGRESSION AND PROGNOSIS OF ORAL CANCER

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Abstract: Background. Expression of placenta growth factor (PlGF) is found to correlate with the progression and prognosis of several human cancers.

Methods. This study used an immunohistochemical technique to examine the expression of PlGF in 100 specimens of oral squamous cell carcinoma (OSCC).

Results. We found that the higher mean PlGF labeling index was significantly associated with OSCCs with positive lymph node metastasis (p = .014) or with more advanced clinical stages (p = .016). Positive lymph node metastasis (p = .008) and PlGF labeling index >40% (p = .010) were identified as independent unfavorable prognosis factors by multivariate analyses with Cox regression model. Moreover, a Kaplan–Meier curve showed that OSCC patients with a PlGF labeling index >40% had a significantly poorer cumulative survival than those with a PlGF labeling index ≤40% (log-rank test, p = .003).

Conclusions. The PlGF labeling index can predict the progression and prognosis of OSCCs in Taiwan. © 2010 Wiley Periodicals, Inc. Head Neck 32: 1363–1369, 2010

Keywords: placenta growth factor; oral cancer; cancer progression; prognosis

Oral squamous cell carcinoma (OSCC) is the most frequent malignant tumor of the oral cavity and the eighth most common cancer in the world.1 In Taiwan, oral cancers rank as the sixth most prevalent cancer in both sexes and account for the fourth most common cancers in males in 2006.2

Placenta growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family.3 It executes its function by binding to VEGF receptor 1 (VEGFR1, also known as FLT1).3 Overexpression of PlGF mRNA or protein has been reported in a variety of human...
Moreover, high PlGF mRNA or protein expression is known to be associated with pathological angiogenesis, tumor cell growth, positive lymph node metastasis, advanced clinical stage, recurrence, and poor prognosis of the cancer. However, the influence of PlGF expression on the progression and prognosis of areca quid chewing– and smoking-associated OSCCs in Taiwan has not yet been investigated.

In this study, we used an immunohistochemical technique to examine the expression of PlGF in 100 specimens of OSCC, 66 specimens of oral epithelial dysplasia (OED), and 36 specimens of normal oral mucosa. The PlGF labeling indices in OSCC, OED, and normal oral mucosa samples were calculated and compared between groups. The correlations between the PlGF labeling indices in OSCC samples and clinicopathological parameters or survival of OSCC patients were analyzed statistically to evaluate the possible influence of PlGF on the progression and prognosis of OSCC patients in Taiwan.

PATIENTS AND METHODS

Patients and Oral Cancers and Precancers. After approval by the Hospital Review Board, we obtained formalin-fixed, paraffin-embedded specimens from 100 patients (91 men and 9 women; mean age, 53 years; range, 30–81 years) with OSCC, 20 patients (19 men and 1 woman; mean age, 50 years; range, 36–63 years) with severe OED, 22 patients (22 men; mean age, 48 years; range, 23–77 years) with moderate OED, and 24 patients (24 men; mean age 48 years; range, 31–63 years) with mild OED. Diagnosis of OSCC and OED was based on histological examination of hematoxylin and eosin–stained tissue sections. All the patients received total surgical excision of their lesions of OSCC or OED at the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital, Taipei, Taiwan, during the period from 1995 to 2002. Specimens were obtained from total surgical excision of the lesion. Of the 100 cases of OSCC, 46 were buccal mucosa, 35 tongue, 11 gingiva, 5 palate, and 3 floor of the mouth cancers. Histological features of OSCCs were further classified into 3 different types (well-differentiated and moderately and poorly differentiated OSCC).

Patients’ Oral Habits. Details of patients’ oral habits, including daily/weekly consumption of areca quid, cigarettes, and alcohol, as well as the duration of these oral habits were recorded. Patients with OSCC were defined as areca quid chewers if they chewed 2 or more areca quids daily for at least 1 year, as cigarette smokers if they smoked every day for at least 1 year and consumed more than 50 packs of cigarettes per year, and as alcohol drinkers if they drank >4 days and consumed >20 g of pure alcohol per week for at least 1 year.

Normal, Positive, and Negative Controls. Thirty-six biopsy specimens of normal oral mucosa were obtained from 36 subjects (22 men and 14 women; mean age 30 years; range, 19–64 years) without oral habits and any oral mucosal disease during extraction of impacted permanent mandibular third molars after obtaining informed consent, and used as the controls. Sections of placental tissues that were previously shown to be positive for PlGF were used as positive controls, and Tris-buffered saline (TBS) instead of primary antibody was used for negative controls.

Immunohistochemistry. All the specimens for immunohistochemical staining were fixed in 10% neutral formalin, embedded in paraffin, and cut in serial sections of 4 μm. Immunohistochemical staining was performed using a peroxidase-labeled streptavidin–biotin technique. Briefly, tissue sections were deparaffinized and rehydrated. Then, sections were heated in a plastic slide holder (DAKO, Copenhagen, Denmark) containing 0.01 M citrate buffer in a microwave oven for 10 minutes to retrieve antigenicity, and treated with 3% H2O2 in methanol for 10 minutes to quench endogenous peroxidase activity. After washing in 10 mM TBS (pH 7.6), sections were incubated with 10% normal donkey serum for 10 minutes to block nonspecific binding. Sections were then incubated overnight at 4°C with goat anti-human PlGF polyclonal antibody (1:100; PlGF [C-20], Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing
in TBS, sections were treated with a 1:100 dilution of biotinylated donkey anti-goat immunoglobulins for 30 minutes and subsequently with a streptavidin–peroxidase conjugate for 30 minutes (DAKO). The 0.02% diaminobenzidine hydrochloride (DAB) containing 0.03% H₂O₂ was used as chromogen to visualize the peroxidase activity. The preparations were lightly counterstained with hematoxylin, mounted with Permount (Thermo Fisher Scientific, Waltham, MA), and examined by light microscopy.

Epithelial or cancer cells exhibiting a brown cytoplasmic staining were counted as positive for expression of PlGF in our samples. The sections were initially scanned at low power. For sections that showed heterogeneous staining, the predominant pattern was taken into account for scoring. At least 4 high-power fields were then chosen randomly, and 1000 cells were counted for each case. The PlGF labeling indices were counted as a ratio of immunostaining-positive cells to the total number of cells counted. An eyepiece graticule was used to ensure that all cells were evaluated once only. Each of these assessments was independently carried by 2 investigators. The sections with an interobserver variation of >10% were reassessed using a double-headed light microscope to achieve consensus. In this study, the interobserver reproducibility was 93%. The slides with discrepant assessments were reevaluated, and a consensus was reached in all cases.

Statistical Analysis. The mean PlGF labeling indices for OSCC, OED (severe, moderate, or mild), and normal oral mucosa samples were compared first among 5 groups by analysis of variance (ANOVA) and then between any 2 groups by Student’s t test. The correlation between PlGF labeling indices in OSCCs and clinicopathological parameters of patients with OSCC was analyzed by Student’s t test or ANOVA, where appropriate. Cumulative survival was analyzed with the Kaplan–Meier product-limit method. The duration of survival was measured from the beginning of treatment to the time of death or the last follow-up. Comparison of cumulative survival between groups was performed using the log-rank test with the Statisca program (StatSoft Inc., Tulsa, OK). Univariate and multivariate survival analyses were performed with the Cox proportional hazard regression model to assess additional prognostic values of the different variables using SAS 9.1 (SAS Institute Inc., Morrisville, NC). A value of p < .05 was considered statistically significant.

RESULTS

PIGF Expression in Oral Cancers and Precancers. Representative immunohistochemical staining photomicrographs for normal oral mucosa, OED, and OSCC are shown in Figure 1. In general, normal oral epithelium was negative for PIGF (Figure 1A) or showed very few PIGF-positive cells in the basal cell layer of the epithelium. The positive cytoplasmic PIGF staining was found predominantly in the lower one third of the epithelium in mild OED specimens, and it extended to the middle or upper one third of the epithelium in moderate or severe OED specimens (Figures 1B–1D). In OSCC samples, the stronger cytoplasmic PIGF staining was found in cells of OSCC tumor nests (Figures 1E and 1F). In addition, positive cytoplasmic PIGF staining could also be observed in endothelial cells and macrophages in the lamina propria of OED specimens or OSCC stromal tissues.

The mean PlGF labeling index increased significantly from normal oral mucosa (12 ± 4%) through mild OED (20 ± 9%), moderate OED (26 ± 11%), and severe OED (30 ± 13%) to OSCC samples (51 ± 19%, p = .000, Table 1). The detailed statistical data for comparison between any 2 groups are shown in Table 1.

Correlation Between PIGF Labeling Indices and Clinicopathological Parameters of OSCC. The higher mean PIGF labeling index was significantly associated with OSCCs with positive lymph node metastasis (p = .014) and with more advanced clinical stages (p = .016, Table 2). No significant association was found between PIGF labeling indices in OSCCs and patient age, cancer location, tumor size, recurrence, and histology of OSCC. In addition, there was no significant correlation between PIGF labeling indices in OSCCs and areca quid chewing, cigarette smoking, or alcohol drinking habits (Table 2).

Survival Analysis. Univariate analysis performed using the Cox proportional hazard regression model identified positive lymph node metastasis (p = .002), advanced clinical stage (p = .036), and PIGF labeling index >40% (p =
.006) as correlating with poor survival. However, only positive lymph node metastasis ($p = .008$) and PlGF labeling index $>40\%$ ($p = .010$) were identified as independent unfavorable prognosis factors by multivariate analyses with the Cox proportional hazard regression model (Table 3). A Kaplan–Meier curve showed that patients with OSCC, with a PlGF labeling index $>40\%$, had a significantly poorer cumulative survival than those with a PlGF labeling index $\leq 40\%$ ($p = .003$, log-rank test, Figure 2).

**DISCUSSION**

This study showed a stepwise and significant increase in the expression of PlGF from normal oral mucosa through mild, moderate, and severe OED to OSCC. This finding suggests that the expression of PlGF is an early event in oral carcinogenesis. Takahashi et al. found a significantly higher PlGF mRNA expression in hypervascular renal cell carcinoma tissues than that in adjacent normal kidney tissues. Chen et al. reported a significantly higher PlGF
protein level in gastric cancer tissues than in the corresponding noncancerous mucosal tissues. Parr et al\(^a\) also demonstrated a dramatic increase of PlGF protein expression in breast cancer tissues compared with that in normal breast tissues. In addition, this study showed a significantly higher PlGF protein expression in OSCCs than in normal oral mucosa samples. The significant increase in PlGF mRNA or protein expression in human carcinomas over that in normal counterpart tissues indicates that PlGF may be a good biomarker for certain types of human carcinomas. Actually, overexpression of PlGF mRNA or protein has been demonstrated in a variety of human carcinomas, including renal cell,\(^6\) lung,\(^7\) breast,\(^8\) uterine cervical,\(^9\) hepatocellular,\(^10\) gastric,\(^11\) and colorectal carcinomas.\(^12\)

An animal study showed that PlGF can increase melanoma growth and metastasis spreading in mice.\(^13\) In human gastric cancers, elevated PlGF protein level is significantly correlated with serosal invasion and positive lymph node metastasis.\(^14\) PlGF protein is overexpressed in human breast cancer tissues and is significantly related to nodal metastasis.\(^8\) This study also found a significantly higher expression of PlGF in OSCCs with lymph node metastases than in those without lymph node metastases. The reasons that PlGF increased cancer metastasis could be explained as follows. First, angiogenesis is a key factor for tumor growth and metastasis.\(^15\) PlGF can stimulate vessel growth and maturation directly by affecting endothelial and mural cells, as well as indirectly by recruiting pro-angiogenic cell types.\(^3\)

PlGF can also upregulate the expression of VEGFA, which is a potent angiogenic factor.\(^3\) Furthermore, PlGF displaces VEGFA from FLT1, which liberates VEGFA and allows it to activate FLK1 (also known as VEGFR2) and enhance VEGF-driven angiogenesis.\(^17\) PlGF also activates and attracts macrophages that release angiogenic and lymphangiogenic molecules.\(^16\) In addition, PlGF can promote tumor angiogenesis, lymphangiogenesis, and the formation of the premetastatic niche.\(^3\) Second, PlGF promotes the growth, survival, and migration of metastatic tumor cells.\(^3\) Third, PlGF

| Group                      | No. of samples | Mean PlGF LI ± SD, % |
|----------------------------|----------------|---------------------|
| Normal oral mucosa         | 36             | 12 ± 4              |
| Mild OED                   | 24             | 20 ± 9              |
| Moderate OED               | 22             | 26 ± 11             |
| Severe OED                 | 20             | 30 ± 13             |
| OSCC                       | 100            | 51 ± 19             |

Abbreviations: PlGF, placenta growth factor; LI, labeling index; OED, oral epithelial dysplasia; OSCC, oral squamous cell carcinoma.

Note: A significant difference in the mean PlGF labeling index was found among OSCC, severe OED, moderate OED, mild OED, and normal oral mucosa groups (p = .000). The following comparisons were statistically significant for the mean PlGF labeling index: OSCC vs severe OED, moderate OED, mild OED, or normal oral mucosa (all values of p = .000); severe OED vs mild OED (p = .004) or normal oral mucosa (p = .000); moderate OED vs mild OED (p = .048) or normal oral mucosa (p = .000); and mild OED vs normal oral mucosa (p = .000).

Table 1. Mean placenta growth factor labeling indices in normal oral mucosa, oral epithelial dysplasia, and oral squamous cell carcinoma samples.

| Factor                      | No. of patients | Mean PlGF LI ± SD, % | p value |
|-----------------------------|-----------------|----------------------|---------|
| Age, y                      |                 |                     | .550    |
| ≤50                         | 49              | 47 ± 36              |         |
| >50                         | 51              | 55 ± 33              |         |
| Sex                         |                 |                     | .034    |
| Male                        | 91              | 45 ± 22              |         |
| Female                      | 9               | 56 ± 8               |         |
| Cancer location             |                 |                     | .187    |
| Buccal mucosa               | 46              | 55 ± 34              |         |
| Tongue                      | 35              | 36 ± 32              |         |
| Other oral mucosal sites    | 19              | 38 ± 33              |         |
| T classification            |                 |                     | .205    |
| T1 + T2                     | 52              | 49 ± 29              |         |
| T3 + T4                     | 48              | 52 ± 28              |         |
| N classification            |                 |                     | .014    |
| N0                          | 69              | 37 ± 29              |         |
| N1 + N2 + N3                | 31              | 55 ± 24              |         |
| Clinical staging            |                 |                     | .016    |
| Stage 1 + Stage 2           | 37              | 32 ± 22              |         |
| Stage 3 + Stage 4           | 63              | 52 ± 24              |         |
| Recurrence                  |                 |                     | .609    |
| With                        | 17              | 47 ± 28              |         |
| Without                     | 83              | 52 ± 29              |         |
| Histology of OSCC           |                 |                     | .063    |
| Well-differentiated         | 79              | 49 ± 32              |         |
| Moderately differentiated   | 21              | 54 ± 26              |         |
| Oral habits                 |                 |                     | .527    |
| Areca quid chewing, smoking, and drinking | 69 | 53 ± 31 | |
| Areca quid chewing and smoking | 10 | 52 ± 28 | |
| Areca quid chewing and drinking | 3  | 50 ± 28 | |
| Smoking only                | 3               | 48 ± 32              |         |
| Drinking only               | 2               | 50 ± 29              |         |
| None                        | 7               | 46 ± 32              |         |

Abbreviations: PlGF, placenta growth factor; LI, labeling index; OSCC, oral squamous cell carcinoma.
increases the expression of matrix metalloproteinase 9 (MMP9), which facilitates the cancer cell invasion and metastasis. Fourth, PlGF directly regulates the motility of human non-small cell lung cancer cells and also stimulates in vitro motility and invasion of the human breast tumor cell lines. Moreover, an antagonistic PlGF/FLT1 peptide can inhibit the growth and metastasis of human breast cancer xenografts. Fifth, PlGF inhibits the differentiation of dendritic cells and in turn suppresses the antigen recognition and antitumor immune defense. In summary, PlGF may promote lymph node metastases through multiple mechanisms such as an increase in tumor angiogenesis and lymphangiogenesis; an increase in tumor cell survival, motility, migration, and invasion; an elevated expression of MMP9; and an inhibition of the immune surveillance by dendritic cells.

This study showed a positive association of PlGF overexpression with higher N classifications in OSCCs. Because higher N classifications always result in more advanced clinical stages of OSCC, it is not difficult to explain why OSCCs with higher PlGF expression are prone to have the more advanced clinical stages. Indeed, high expression of PlGF mRNA or protein is significantly associated with an advanced clinical stage of lung, gastric, and colorectal cancers. In the present study, we demonstrated a significant correlation between the high PlGF expression in OSCCs and the poor overall survival of patients with OSCC. In addition, a PlGF labeling index >40% was identified as an independent unfavorable prognosis factor by multivariate analyses. A previous study also showed a significant association of a higher level of PlGF mRNA with a shorter survival in patients with colorectal carcinoma. In addition, an increased level of PlGF protein is significantly related to poor prognosis in patients with breast or gastric carcinomas. The above-cited findings indicate that PlGF may be an important prognostic indicator for patients with certain types of human carcinomas, including OSCC.

Previous studies showed that a high PlGF protein or mRNA level is significantly related to recurrence of breast and hepatocellular carcinoma. However, this study did not show a significant association of PlGF protein expression with recurrence and histology of OSCC. We suggest that unclear section margins (4 of 17 recurrent OSCCs) or finding of tumor nests within 2 mm of section margins (5 of 17 recurrent OSCCs) may be the 2 major factors responsible for the recurrence of OSCC in this study. In addition, lack of poorly differentiated OSCCs in our oral cancer samples may partially explain why there is no association of PlGF protein expression with histology of OSCC.

### Table 3. Univariate and multivariate survival analyses of the placenta growth factor labeling index and clinicopathologic parameters in patients with OSCC by Cox proportional hazard regression model.

| Factor            | Hazard ratio (95% CI) | p value |
|-------------------|-----------------------|---------|
| **Univariate**    |                       |         |
| T classification  | 2.046 (0.802–2.246)   | .073    |
| (T1 + T2 vs T3 + T4) |                  |         |
| N classification  | 4.552 (1.715–12.082)  | .002    |
| (N0 vs N1 + N2 + N3) |                   |         |
| Clinical stage    | 3.359 (1.084–10.405)  | .036    |
| (Stages 1 + 2 vs Stages 3 + 4) |      |         |
| PlGF LI (LI ≤40% vs LI > 40%) | 4.304 (1.528–12.121) | .006    |
| **Multivariate**  |                       |         |
| N classification  | 3.254 (1.029–10.381)  | .008    |
| (N0 vs N1 + N2 + N3) |                  |         |
| PlGF LI (LI ≤40% vs LI > 40%) | 4.284 (1.516–20.564) | .010    |

**Abbreviations:** OSCC, oral squamous cell carcinoma; CI, confidence interval; PlGF, placenta growth factor; LI, labeling index.

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**FIGURE 2.** Kaplan–Meier survival curve showing relation between PlGF expression in primary tumors and survival in 100 patients with OSCCs. The duration of survival was measured from the beginning of treatment to the time of death (complete) or the last follow-up (censored). The cumulative survival for patients with OSCC, with PlGF labeling index ≤40% (<i>p</i> = .003, log-rank test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Our results showed a significant elevation in PlGF labeling indices from normal oral mucosa through mild and moderate OED to severe OED, suggesting that the expression of PlGF is an early event in oral carcinogenesis. We also found that the PlGF labeling index in OSCC samples was significantly correlated with N classification and clinical staging of OSCCs. Moreover, patients with OSCC with higher PlGF labeling indices had a poorer cumulative survival than patients with lower PlGF labeling indices. These results indicate that the PlGF may be a biomarker for prediction of the progression of OSCCs and the prognosis of OSCC patients in Taiwan.

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