Expression of Platelet-derived Endothelial Cell Growth Factor in Oral and Oropharyngeal Carcinoma

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Abstract
Platelet-derived endothelial cell growth factor (PD-ECGF) was isolated as an endothelial cell mitogen from platelets. In this study, we investigated the expression of PD-ECGF and counted microvessels in 58 oral and oropharyngeal squamous cell carcinoma (SCC) specimens by an immunohistochemical technique to examine their prognostic significance and performed tumor in vitro sensitivity to 5-fluorouracil (5-FU) and cisplatin as determined by a bioluminescence assay of the ATP values of tumor cells after continuous exposure.

The percentage of PD-ECGF-positive tumor cells (PD-ECGF score) was correlated with the frequency of the recurrence of disease ($P = 0.0043$) but not with sex, tumor size, metastasis, or clinical stage. Overall survival of the high PD-ECGF expression group (>40% PD-ECGF score) was shorter than the low expression (<40%) group ($P = 0.0365$). Vessel count was correlated with lymph node metastasis and clinical stage. The survival of patients with hypervascularity (more than the median of intratumor vessel counts, >82) was shorter than that of those with hypovascularity (vessel count <81, $P = 0.0446$). However, there was no association between PD-ECGF expression and vessel count. Cox proportional multivariate analysis showed that PD-ECGF expression was the most significant independent prognostic indicator for overall survival. The susceptibility to 5-FU cytotoxicity in the extremely high PD-ECGF expression groups (>70% of PD-ECGF score) was significantly higher than that in the low group, whereas there was no difference in their sensitivity to cisplatin.

These results showed that carcinoma cells with high PD-ECGF expression were sensitive to 5-FU in spite of poor prognosis. These data provide further information when deciding on adjuvant therapy for oral and oropharyngeal SCCs.

Introduction
The concept that "tumor growth is angiogenesis dependent" is supported by abundant evidence (1, 2). Most clinical research on angiogenesis has been conducted by two methods; one is the degree of angiogenesis as estimated by quantification of vascular density and, the other is the expression of angiogenic factors from cancer cells. An immunohistochemical approach using monoclonal antibodies for endothelial cells has been used to evaluate the degree of angiogenesis. Positively stained vessels within the tumor tissue in the section were counted directly under a microscope, suggesting that vessel count is correlated to the incidence of metastasis in invasive breast cancer (3-5), non-small cell lung carcinoma (6), prostate carcinoma (7), ovarian carcinoma (8), gastric carcinoma (9-11), colon carcinoma (12), and esophageal carcinoma (13). Tanigawa et al. (10-13) showed that tumor vascularization was closely associated with the overall survival of patients with cancer of the digestive tract and was identified as the most significant and independent prognostic variable.

Many angiogenic factors have been identified in the last decade. PD-ECGF (9) was originally isolated as an endothelial cell mitogen from platelets (14) and demonstrated to have angiogenic activity in vivo (15-18). Recently, a significant association between the expression of PD-ECGF and microvessel density was reported in breast cancer (19, 20) and colorectal cancer (21). In addition, positive expression of PD-ECGF showed a highly significant association with tumor size, the extent of invasion, lymph node metastasis, lymphatic invasion, venous invasion, and the poor prognosis of colorectal carcinoma (21). However, another group reported no correlation between PD-ECGF expression and vascularity, relapse-free survival, or overall survival in breast cancer (22) and bladder cancer (23). In gastric cancer, PD-ECGF expression was closely associated with the promotion of angiogenesis and hepatic metastasis but not the clinical outcome (24). Thus, it was speculated that the role of PD-ECGF is different among types of carcinoma and depends on the site of the disease.

As another functional activity, PD-ECGF has been demonstrated to be identical to TP, an enzyme involved in pyrimidine nucleoside metabolism (25, 26). TP activity of cancer cells was higher than that of normal cells (27, 28). PD-ECGF is of therapeutic interest, because TP converts 5'-DFUR and tegafur to 5-FU (29). Also, TP plays a role in metabolizing 5-FU to

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3 The abbreviations used are: PD-ECGF, platelet-derived endothelial cell growth factor; SCC, squamous cell carcinoma; 5-FU, 5-fluorouracil; TP, thymidine phosphorylase; 5'-DFUR, 5'-oxifluridine; TS, thymidylate synthase.
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FdUMP (30). Overexpression of PD-ECGF by transfection of PD-ECGF cDNA into cancer cells enhanced the sensitivity to 5'-DFUR and tegafur (31-33). Thus, the levels of PD-ECGF expression in cancer cells might indicate the susceptibility of tumor cells to 5-FU, 5'-DFUR, or tegafur. In general, first-line adjuvant chemotherapy using 5-FU has not improved the survival of patients with head and neck SCC (34). However, patients with poor prognostic factors may benefit by clinical trials using 5-FU or tegafur with cisplatin as an adjuvant chemotherapy or with postoperative irradiation therapy; this regimen could lead to an improvement in prognosis.

We investigated the expression of PD-ECGF and counted microvessels in oral and oropharyngeal SCCs by an immunohistochemical technique to examine their prognostic significance. We also performed an in vitro tumor chemosensitivity test for oral and oropharyngeal SCCs to evaluate the correlation between the expression of PD-ECGF and the sensitivity to 5-FU.

Materials and Methods

Patients and Sample Collection. We studied tumor specimens from 58 patients with primary oral and oropharyngeal squamous cell carcinoma who had undergone tumor curative resections at the Department of Otorhinolaryngology, Fukui Medical University, between 1984 and 1996. The mean age of the patients was 63.5 years (median, 65.0). According to the General Rules for Head and Neck Cancer (Tumor-Node-Metastasis classification), there were 7 cases of stage I (12%), 11 cases of stage II (19%), 13 cases of stage III (22%), and 27 cases of stage IV (47%). Seventy % of these patients (41 cases) were followed for 5 years. The other patients who underwent surgery between 1993 and 1996 were followed for at least 1 year. None of the 58 cases were lost to follow up. Ten patients died from other diseases. None of the patients received chemotherapy or irradiation therapy before surgery. The patients with oral and oropharyngeal SCCs had been treated with standard therapy, depending on the stage. Briefly, the patients with stages I and II underwent only tumor resection; stages III and IV patients underwent tumor resection, standard neck dissection, and irradiation (60 Gy) after the surgery. H. S. performed all of the surgeries. Surgically resected SCC tissues were quickly divided into two samples; one was fixed in 10% buffered formaldehyde for immunohistochemical analysis, and the other was suspended in culture medium for a chemosensitivity assay.

Immunohistochemical Staining. Immunohistochemical staining was performed to detect PD-ECGF and microvessels in oral and oropharyngeal SCC tissues using the avidin-biotin-peroxidase complex technique (35). Paraffin-embedded blocks were cut and were deparaffinized. After incubation of the blocks in 0.3% H2O2 solution dissolved in absolute methanol to inhibit endogenous peroxidase activity, all specimens were mounted with normal sheep serum (Dako LSAB kit; Dako, Carpinteria, CA) to block the background absorption of antiserum, followed by incubation with mouse anti-human PD-ECGF monoclonal antibodies (P-GF.44C; Refs. 22, 23, and 36) or mouse anti-CD34 monoclonal antibodies (QBEND-20; Novocastra laboratory, Newcastle, UK; Refs. 10-13 and 37). All specimens were reacted with biotinylated-anti-mouse IgG (Dako) for 1 h and allowed to react with the avidin-biotin-peroxidase complex for 40 min. After rinsing with PBS, peroxidase color visualization was carried out with a 3-3'-diamino benzidine tetrahydrochloride solution (Djin, Kumamoto, Japan; 30 mg dissolved in 150 ml of PBS, to which 10 μl of 30% H2O2 solution was added). Specimens that were stained by PD-ECGF were treated by microwave counterstaining for 10 min in distilled water. Nuclear counterstaining was carried out with Harris hematoxylin for 30 s before mounting.

Evaluation of the Specimen. For microscope analysis of PD-ECGF, we selected three staining hot spots that included more than 200 cells and estimated the percentage of cancer cells that stained positively (21). PD-ECGF staining was scored independently by two physicians (S. F. and H. T.) in a coded manner (without knowledge of the clinical parameters and outcomes). To determine the PD-ECGF score in this study, we calculated the average of six values of PD-ECGF-positive cells as a percentage.

Vessels were counted in the five most vascular areas within the tumor tissue under a ×200 field using anti-CD34 antibody (10). Vessel counts were also performed independently by two physicians (S. F. and H. T.) in a coded manner. The average of 10 vessel counts in a sample was calculated, and its value was shown as the vessel count of the sample in the text. Omission of the primary antibody was used as both negative controls.

Cemosensitivity Test in Vitro. Of 58 oral and oropharyngeal SCCs, 36 specimens were assessed to determine their sensitivities to chemotherapeutic drugs. Aseptic surgical specimens were obtained, and necrotic tumors and connective tissue were dissected. The remaining tumor tissues were minced mechanically and digested enzymatically (38, 39). Single-cell suspensions of cancer cells were purified by discontinuous gradient centrifugation with 70 and 100% Ficoll-Hypaque to remove the dead cells. The tumor-rich fraction consisted of >85% tumor cells, as determined by morphological examination Wright-Giemsa-stained smears. The viability of purified tumor cells was >80%, as determined by trypan blue dye exclusion. Purified tumor cells (5 × 106 cells/ml/well) were maintained at 37°C for 72 h with anticancer agents. ATP levels in the control well were monitored on days 1 and 3 to determine the progression of tumor cell viability and growth. Two anticancer agents, 5-FU and cisplatin were tested at peak plasma concentrations (5-FU, 10 μg/ml) and one-twentieth of the peak plasma concentration (CDDP, 0.1 μg/ml), respectively. 5-FU was kindly provided by Kyowa Hakko Co. Ltd. (Tokyo, Japan), and CDDP was obtained from Bristol-Myers Squibb Co. Ltd. (Tokyo, Japan).

After treating cancer cells with 5-FU or CDDP, a single-cell suspension was obtained by trypsinization and pipetting. We performed an ATP assay as the chemosensitivity test. The ATP assay has excellent potential as a simple, inexpensive, and rapid technique for determining the cytotoxic effect of a drug on cancer cells (40-42). Intracellular ATP levels were measured by the luciferin-luciferase method using an ATP monitoring kit (BioOrbit Co., Turku, Finland) under conditions recommended by the manufacturer. Briefly, the cell suspension was boiled and mixed with ATP-releasing reagent. The ATP level in the extract was then measured with a LKB Luminophotometer (LKB-Wallac, Turku, Finland), using standard techniques comparing a standard ATP (10⁻¹¹ to 10⁻⁶ mol; Ref. 40).
antibody recognizing PD-ECGF (22, 23, 32). The usual pattern
yses.
by a standard immunohistochemical technique, using PG-44C
embedded, oral and oropharyngeal SCC specimens were stained
with the Prognosis. Fifty-eight formalin-fixed, paraffin-
measured, and nuclear tumor cell staining was observed (×200). B, negative
staining of PD-ECGF (×200).

Fig. 1 Immunohistochemical staining of PD-ECGF in oral and oropharyngeal SCCs. A, positive staining of PD-ECGF. Positive cytoplasmic and nuclear tumor cell staining was observed (×200). B, negative staining of PD-ECGF (×200).

Values for in vitro chemosensitivity testing were calculated
by the following formula: \[ \frac{1.0 - (\text{mean ATP value of tumor cells treated with an anticancer drug/mean ATP value of tumor cells cultured with medium alone}) \times 100, \]
expressed as a percentage of sensitivity. Thus, a high percentage indicated a high susceptibility of the cancer cells to the chemotherapeutic agent treated.

Statistical Analyses. The clinical characteristics of pa-
patients in relation to PD-ECGF expression and vessel counts were
analyzed by the Mann-Whitney U test. The survival rate of the
patients was determined and plotted according to the Kaplan-
Meier test and analyzed by the log-rank (Mantel-Cox) tests.
Macintosh personal computers (Stat View software; Abacus
Concepts, Inc., Berkeley, CA) were used for all statistical anal-
yses.

Results
Expression of PD-ECGF Is Significantly Correlated
with the Prognosis. Fifty-eight formalin-fixed, paraffin-
embedded, oral and oropharyngeal SCC specimens were stained
by a standard immunohistochemical technique, using PG-44C
antibody recognizing PD-ECGF (22, 23, 32). The usual pattern
of positive staining for PD-ECGF in oral and oropharyngeal
SCCs was both cytoplasmic and nuclear, but occasionally only
one of these was present (Fig. 1), as seen with other carcinomas
described previously (22, 23). All PD-ECGF staining results
were reproducible in two successive paraffin sections from the
same blocks. Immunoreactivity of PD-ECGF was heterogene-
ous and occasionally focal within a section. The hotspots of
PD-ECGF staining could be found anywhere within an invasive
tumor but appeared most frequently at the margin of the carcin-
oma. A histogram of the PD-ECGF score is shown in Fig. 2.
Mainly, two peaks were found; one was a PD-ECGF score
<40%, and the other was >40%. The mean PD-ECGF score in
oral and oropharyngeal SCCs was 52.1 ± 27.7% (mean ± SD;
median, 54%), and there were only four negative cases with
fewer than 5% positive cells.

The association between clinical factors and the expression
of PD-ECGF in the oral and oropharyngeal SCCs is shown in
Table 1. There was no association between PD-ECGF expres-
sion and sex, tumor size, clinical stage, or metastasis. Differen-
tiation of oral and oropharyngeal SCCs did not correlate with
the PD-ECGF score (data not shown). The PD-ECGF score was
significantly correlated with the recurrence of disease \( P =
0.0043 \). Twenty-six of 58 patients failed primary cancer treat-
ment. In the patients with recurrent disease, the average PD-
ECGF score was 63.4 ± 24.2%, whereas in patients without
recurrence, it was 43.0 ± 27.4%. Also, a significant association
was found between PD-ECGF expression and death from dis-
ease. Nineteen patients died of disease in this study. The average
PD-ECGF score of the patients who died of oral and orophar-
yngeal SCCs was significantly higher than in well-controlled
patients \( 65.0 \pm 21.1\% \) versus \( 45.9 \pm 28.7\%, \) \( P =
0.0124 \).

We found that the PD-ECGF score was significantly corre-
lated with the overall survival of the patients, using the Cox
model for evaluation as a continuous variable \( P = 0.0400; \) odds
ratio, 1.019; 95% confidence interval, between 1.001 and
1.038). The odds of death increased with the elevation of the
PD-ECGF score. We determined that 40% PD-ECGF-positive

Fig. 2 Histogram of the PD-ECGF score. Percentage of cells expressing PD-ECGF (PD-ECGF score) in the 58 patients with oral and oropharyngeal SCCs in the study is shown on the transverse axis, and the number of patients is shown on the vertical axis.
Expression of PD-ECGF

Table 1 Association between the clinicopathological factors and the expression of PD-ECGF

<table>
<thead>
<tr>
<th>Gender</th>
<th>Number</th>
<th>PD-ECGF score (mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>38</td>
<td>52.1 ± 27.9</td>
<td>0.99</td>
</tr>
<tr>
<td>Female</td>
<td>20</td>
<td>52.1 ± 28.3</td>
<td></td>
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Tumor size
- T1: 8, 47.2 ± 30.2, NS*
- T2: 21, 54.4 ± 31.3
- T3: 15, 51.0 ± 27.0
- T4: 14, 52.7 ± 23.9

Lymph node metastasis
- Positive: 29, 49.3 ± 25.9, 0.44
- Negative: 29, 55.0 ± 29.7

Clinical stage
- I: 7, 45.3 ± 32.1, NS*
- II: 11, 60.1 ± 32.8
- III: 13, 53.7 ± 26.8
- IV: 27, 49.9 ± 25.7

Metastasis
- Yes: 36, 54.5 ± 28.7, 0.41
- No: 22, 48.2 ± 26.4

Recurrence
- Yes: 26, 63.4 ± 24.2, 0.0043
- No: 32, 43.0 ± 27.4

Death of disease
- Yes: 19, 65.0 ± 21.1, 0.0124
- No: 39, 45.9 ± 28.7

* NS, not significant.

Fig. 3 A survival curve for patients with oral and oropharyngeal SCCs.

<table>
<thead>
<tr>
<th>Tumor Stage</th>
<th>PD-ECGF Score</th>
<th>Overall Survival Rate</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>94.6 ± 27.6</td>
<td>80.3 ± 25.7</td>
</tr>
<tr>
<td>II</td>
<td>96.3 ± 25.9</td>
<td>64.3 ± 16.2</td>
</tr>
<tr>
<td>III</td>
<td>77.2 ± 25.1</td>
<td>0.035</td>
</tr>
<tr>
<td>IV</td>
<td>49.9 ± 25.7</td>
<td>0.035</td>
</tr>
</tbody>
</table>

PD-ECGF (<40% and >40%) were observed in the histogram, as shown in Fig. 2. As a result of this differentiation, 39 of 58 patients with oral and oropharyngeal SCCs were classified into the high PD-ECGF-expressing group (67%). The χ² test to examine the association between clinical factors and the expression level of PD-ECGF (high and low group) showed the same results as the PD-ECGF scores described above (data not shown). We calculated the survival rate of the two groups using the Kaplan-Meier method. Although 87.4% of the low PD-ECGF-expressing group reached an overall survival rate of 10 patients, 44.3% of high PD-ECGF patients survived for that length of time. There was a significant difference in overall survival between the low and high PD-ECGF groups (P = 0.0365; Fig. 3).

Correlation between Vessel Count and Clinicopathological Features. We selected anti-CD34 antibodies for staining of endothelial cells, because staining with anti-CD34 antibodies was most reproducible, specific, and clear among several antibodies to endothelial cells (10–13). Blood vessels within 10 microscopic fields at ×200 were counted, and the mean was calculated. Vessel counts ranged from 30 to 157, with a mean ± SD of 88.0 ± 27.9 (median, 82). Hotspots of CD34 staining were often present in tumor stroma. The clinicopathological features of the study population in relation to vessel counts is shown in Table 2. The average microvessel count in T1 (65.6 ± 15.5) was significantly lower than that in T2 (92.6 ± 32.0, P = 0.0318, compared with T1), T3 (86.9 ± 26.4, P = 0.0495), and T4 (92.9 ± 21.6, P = 0.0053), whereas no significant difference was found among other T (of Tumor-Node-Metastasis) classifications. Vessel counts of the patients with lymph node metastasis were significantly higher than in the patients without lymph node metastasis (94.6 ± 27.6 versus 80.3 ± 25.7, P = 0.0463). Vessel counts in stage IV were higher than those in stage I (96.3 ± 25.9 versus 64.3 ± 16.2, P = 0.0043) and stage III (versus 77.2 ± 25.1, P = 0.035). The tumors in stage II showed higher vascularity than those in stage 1 (92.6 ± 29.5, P = 0.0343). There were significant differences found among the four clinical stages by one way ANOVA (P = 0.0136). Also, the average vessel counts in the patients that died of oral and oropharyngeal SCCs were significantly higher than in the well-controlled patients (97.7 ± 25.6 versus 82.5 ± 27.1, P = 0.0468). Vessel counts were not associated with total metastasis (lymph node metastasis plus distant metastasis) nor recurrence of the disease.

The Cox model for evaluation of vessel counts as a continuous variable showed that correlation between microvessel counts and overall survival of the patients was on the boundary line of significance (P = 0.051; odds ratio, 1.015; 95% confidence interval, between 1.000 and 1.030). Also, the odds of death increased with increased microvessel counts. We determined the cutoff point for discrimination to be 82, because the median vessel count was 82. As shown in Fig. 4, there was a significant difference between the 2 groups (>82 and <82) in overall survival, as shown by Kaplan-Meier analysis (P = 0.0446). The survival rate after 5 years was 40.9 and 71.7% in the hyper/hypovascular group (30 cases) and hypovascular group (28 cases), respectively.

No Correlation between PD-ECGF Score and the Vessel Count. We examined the correlation between PD-ECGF expression and intratumor angiogenesis in oral and oropharyngeal SCCs. There is no significant correlation between the PD-ECGF score and intratumor vessel count by Spearman rank correlation coefficient test (r = 0.045, P = 0.7329). No association between the high/low PD-ECGF group and the hyper/hypovascular group was observed by the χ² test (P =...
The hotspots of PD-ECGF staining were mainly found at the invasive tumor edge, whereas those indicating vessel counts were present in the tumor stroma. There was no specific distance between hotspots of vessels and PD-ECGF expression.

**Relative Risks Contributing to Survival Time.** The prognostic values of PD-ECGF expression and angiogenesis in oral and oropharyngeal SCCs were examined by multivariate analysis using the Cox proportional hazard model. Among various clinicopathological variables, PD-ECGF expression was significant as an independent prognostic indicator for overall survival (Table 3). The risk ratio of death was 5.113 among patients who have a high percentage (>40%) of PD-ECGF-positive cells in the tumor versus those who showed low PD-ECGF expression (P = 0.031). Thus, the expression of PD-ECGF is superior to the vessel count as an indicator of prognosis for patients with oral and oropharyngeal SCCs.

**Carcinoma with a High PD-ECGF Score Was Sensitive to 5-FU Cytotoxicity.** Chemosensitivity testing in vitro by an ATP assay was performed in 36 specimens of oral and oropharyngeal SCC. The mean percentages of sensitivity to 5-FU and CDDP cytotoxicity in this study were 31.2 ± 28.9% and 34.6 ± 30.0%, respectively (mean ± SD). The susceptibility to 5-Fu cytotoxicity in the patients with high PD-ECGF expression (26 patients) was higher than in those with low PD-ECGF (10 patients) but was not significant (35.1 ± 29.8% versus 24.4 ± 24.7%). Therefore, we divided the high PD-ECGF expression group (26 patients) into two more groups according to PD-ECGF score (cutoff point, 70%) and designated then the medium (40–70% of PD-ECGF score) and extremely high PD-ECGF expression groups (>70% of PD-ECGF score). Significant differences in the sensitivity to 5-FU among the three groups were demonstrated by one-way ANOVA (P = 0.0128). As shown in Fig. 5, the degree of sensitivity to 5-FU cytotoxicity in the extremely high PD-ECGF expression group had significantly higher susceptibilities to 5-FU cytotoxicity than the 10 patients in the low PD-ECGF group (54.0 ± 24.3% versus 23.2 ± 24.7%, P = 0.0057). The mean percentage of sensitivity to 5-FU cytotoxicity in the medium PD-ECGF group (40–70% of PD-ECGF score) was 32.9 ± 27.4%. However, there was no difference in the sensitivity to CDDP in vitro among the three groups (28.4 ± 22.7% in 10 patients with extremely high PD-ECGF expression, 25.1 ± 30.1% in 16 patients with medium PD-ECGF expression, and 31.6 ± 30.8% in 10 patients with low PD-ECGF expression). The degree of sensitivity to 5-FU did not correlate with gender, tumor size, lymph node metastasis, clinical stage, differentiation of tumor, or vessel counts (data not shown).

**Discussion**

In this study, we demonstrated that not only the expression of PD-ECGF but also the vessel count were significantly correlated with the clinical outcome in oral and oropharyngeal SCCs. Vessel count was significantly associated with the clinical outcome in oral and oropharyngeal SCCs. Vessel count was significantly associated with the clinical outcome in oral and oropharyngeal SCCs. Vessel count was significantly associated with the clinical outcome in oral and oropharyngeal SCCs. Vessel count was significantly associated with the clinical outcome in oral and oropharyngeal SCCs.
Expression of PD-ECGF provides important information concerning the risk of recurrence and the prognosis after primary therapy, although it is unclear why oral and oropharyngeal SCCs are different from other carcinomas and why PD-ECGF expression is correlated with clinical outcome. It was reported recently that the expression of PD-ECGF \textit{in vitro} in cancer cells was dependent on both the pH and oxygen concentration of the media and that acidic conditions (from pH 6.3 to pH 6.7) and hypoxic exposure (0.3% of O2 concentration) were the optimal conditions for the production of PD-ECGF (43). The presence of hypoxia is associated with poor response to radiation (44). The patients with stages III and IV received postirradiation treatment in this study. Stages III and IV patients in the high PD-ECGF group showed poor outcomes compared with those in the low PD-ECGF group. Also, among the early cancer patients who did not receive postirradiation therapy, the association between the expression of PD-ECGF and prognosis was similar to that in the advanced cases. Thus, outcomes in the subgroup were not differentially affected by PD-ECGF expression levels.

Numerous studies showed significantly more acidic pH in tumorous tissue than that in normal tissue (45). Tumor-induced alteration of microenvironmental pH provides a simple but complete mechanism for cancer invasion (46, 47). Acidic medium activated invasion of the cancer cells through greater elevation of type IV collagenase released from cancer cells compared with that of those in a neutral medium (48). Thus, PD-ECGF expression might be a marker of not only hypoxia but also acidic condition. Additional studies are required to clarify the affect of PD-ECGF expression levels on tumor invasion and response to irradiation.

Although there are several reports that the vessel count is associated with PD-ECGF expression in clinical samples (19–21), there was no significant correlation between the PD-ECGF expression score and vessel count in this study, suggesting that \textit{in vivo} angiogenesis of oral and oropharyngeal SCCs might be mediated by another, separate contributor in addition to PD-ECGF activity. O’Brien et al. (49) reported that there are at least two distinct angiogenic pathways involved in different stages of bladder cancer; one is the PD-ECGF pathway in invasive cancer, and the other is the vascular endothelial growth factor pathway in early superficial cancer (49). When we omitted eight patients with superficial oral cancer (clinical stage I) and analyzed the association between PD-ECGF expression and vessel counts, there was no significant correlation with either (data not shown).

Because many angiogenic factors such as acidic and basic fibroblast growth factors (50, 51), pleiotrophin (51, 52), transforming growth factor β (51, 53), and vascular endothelial growth factor (51, 54, 55) were established, we speculated that angiogenesis in oral and oropharyngeal SCCs was more complicated than in other carcinomas (19–21).

PD-ECGF has received considerable attention as a TP that converts the prodrug 5′-DFUR and tegafur to activate 5-FU (29). Additionally, TP converts 5-FU toFdUMP, which is the first step in one pathway for the metabolic activation of the cancer chemotherapeutic agent to deoxyribonucleotides (30). In both human KB epidermal carcinoma cells and human MCF-7 breast cancer cells transfected with the cDNA of PD-ECGF, the activity of TP was increased and the susceptibility to 5′-DFUR and tegafur cytotoxicity, but not to 5-FU, was significantly enhanced (31, 32); however, human PC-9 lung adenocarcinoma cells, which also overexpressed TP activity by the same transfection approach, were more sensitive than the original untransfected cells, not only to 5′-DFUR and tegafur but also to 5-FU (33). Also, Schwartz et al. (56) found a significant correlation between the relative increase in sensitivity to 5-FU cytotoxicity and the increase in both TP mRNA levels and TP activity in HT-29 human colon carcinoma transfected with PD-ECGF cDNA. As other biochemical determinants of the response to 5-FU, TS is a critical therapeutic target. Johnston et al. reported that tumors with high levels of TS protein and TS gene expression were refractory to clinical 5-FU therapy, suggesting that low levels of TS protein and gene expression in tumors appear to be highly susceptible to 5-FU cytotoxicity (57). Thus, susceptibility of tumor cells to 5-FU cytotoxicity usually depends on the balance of the activity of at least TP, TS, and other unidentified factors (28). Because extremely high PD-ECGF-expressing tumors are not greatly influenced by the level of TS protein or others with regards to their sensitivity to 5-FU, oral and oropharyngeal SCCs with extremely high PD-ECGF expression showed significantly higher susceptibility to 5-FU.

\[\text{Table 3} \quad \text{Multivariate Cox proportional hazard analysis}\]

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>Hazards ratio</th>
<th>95% CI*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (female or male)</td>
<td>1.334</td>
<td>0.49–3.60</td>
<td>0.570</td>
</tr>
<tr>
<td>Tumor size (T1 or T2 or T3 or T4)</td>
<td>1.812</td>
<td>0.64–5.11</td>
<td>0.261</td>
</tr>
<tr>
<td>PD-ECGF (low or high)*</td>
<td>5.113</td>
<td>1.16–22.60</td>
<td>0.031</td>
</tr>
<tr>
<td>Vessel count (hypo or hyper)*</td>
<td>2.103</td>
<td>0.74–5.98</td>
<td>0.163</td>
</tr>
</tbody>
</table>

* CI, confidence interval.
* Low, <40% of PD-ECGF score; high, >40% of score.
* Hypo, vessel counts were <82; Hyper, vessel counts were >82 in the field under a microscope (×200).

**Fig. 5** Sensitivity \textit{in vitro} of oral and oropharyngeal SCCs to the cytotoxicity of 5-FU or CDDP. The sensitivity to 5-FU in the extremely high PD-ECGF group (>70% of PD-ECGF, 10 patients) was significantly higher than in the low PD-ECGF group (<40% of PD-ECGF, 10 patients). There was no difference in the sensitivity to CDDP among the three groups. Results are expressed as means; bars, 1 SE.
cytotoxicity than those with low PD-ECGF expression in this study. However, the medium PD-ECGF-expressing tumors were relatively affected by other factors. The sensitivity to 5-FU cytotoxicity in the medium PD-ECGF expression tumors was slightly higher than in the low PD-ECGF expression group.

Finally, evidence that carcinoma cells with high PD-ECGF expression were sensitive to 5-FU cytotoxicity in spite of poor prognosis brings us to the benefit of treating oral and oropharyngeal SCCs. A tumor in vitro chemosensitivity assay had been developed to improve patient survival by chemotherapy; however, chemosensitivity testing has not been accepted because of technical feasibility issues (58, 59). By the immunohistochemical approach, we can evaluate PD-ECGF expression of cancer tissue without any difficulty and have the chance to decide whether to use adjuvant chemotherapy using tegafur or 5-FU for patients with oral and oropharyngeal SCCs, according to their PD-ECGF expression levels. Subsequently a prospective clinical trial is now being directed to determine the high priority of PD-ECGF expression in chemotherapy for head and neck cancer.

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References


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Expression of platelet-derived endothelial cell growth factor in oral and oropharyngeal carcinoma.

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