Reliability of Platelet-Derived Endothelial Cell Growth Factor as a Prognostic Factor for Oral and Oropharyngeal Carcinomas

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Objective: To identify a strong prognostic biological marker for patients with oral and oropharyngeal squamous cell carcinomas.

Design: We evaluated the protein expressions of 26 tumor-associated factors, including cytokines and cytokine receptors (granulocyte colony-stimulating factor [G-CSF], interleukin 10 [IL-10], G-CSF receptor [G-CSFR], and IL-12 receptor); angiogenic factors (platelet-derived endothelial cell growth factor [PD-ECGF] and vessel count); cell cycle–related proteins (p27, cyclin D1, and cyclin E); apoptosis-related factors (wild-type p53, Bax, Bcl-2, apoptotic index, Fas, and Fas ligand); oncogene proteins (c-fos and c-Myc); cell-surface proteins (P-glycoprotein, multidrug resistance–associated protein, nm23, and CD40); intracellular proteins (aryl hydrocarbon receptor nuclear translocator, aryl hydrocarbon receptor, and heat shock protein 27); and DNA mismatch-repair genes (protein encoded by human mutL homologue 1 [hMLH1] and the human mutS homologue of the chromosome 2p gene) by means of immunohistochemical analysis.

Setting: Department of Otorhinolaryngology—Head and Neck Surgery, University of Fukui, Fukui, Japan.

Patients: Fifty-eight patients who underwent surgical resections of oral and oropharyngeal squamous cell carcinomas.

Results: A low-level PD-ECGF expression, a hypovascular count, or a low-level G-CSFR expression was associated with a favorable clinical outcome using the Kaplan-Meier method. Univariate analysis showed that PD-ECGF expression (odds, 4.19; \(P = .02\)), G-CSFR expression (odds, 4.10; \(P = .01\)), and vessel count (odds, 2.80; \(P = .04\)) had significant hazard rates. When multivariate analysis was performed on 6 factors, including sex, tumor size, lymph node metastasis, PD-ECGF expression, G-CSFR expression, and vessel count, patients with a positive expression of PD-ECGF had the highest relative risk value for death due to the disease (odds, 4.94; \(P = .01\)). Also, G-CSFR was an independent prognostic indicator in the model (odds, 3.29; \(P = .04\)). No correlations between other factors and prognoses were detected.

Conclusion: Expression of PD-ECGF was the most effective marker for making prognoses for oral and oropharyngeal squamous cell carcinomas, and G-CSFR expression was the second most effective among 26 tumor-associated factors.


Despite recent developments in the understanding of cancer at the level of regulatory protein expression, the direct clinical relevance of squamous cell carcinomas of the head and neck (HNSCCs) still remains unclear. To find an independent prognostic factor for patients with HNSCCs, we investigated the expression of tumor-associated factors from all angles, including cytokines and cytokine receptors (granulocyte colony-stimulating factor [G-CSF], interleukin 10 [IL-10], G-CSF receptor [G-CSFR], and IL-12 receptor [IL-12R]) \(^1\)-\(^3\); angiogenic factors (platelet-derived endothelial cell growth factor [PD-ECGF] and endothelial cell marker for a blood microvessel [CD34]) \(^4\); cell cycle–related proteins (p27, cyclin D1, and cyclin E) \(^5\)-\(^7\); apoptosis-related factors (wild-type p53 [wt-p53], Bax, Bcl-2, apoptotic index, Fas, and Fas ligand [FasL]) \(^8\),\(^9\); oncogene proteins (c-fos and c-Myc); cell-surface proteins (P-glycoprotein [P-gp], multidrug resistance–associated protein [MRP], nm23, and CD40) \(^10\)-\(^12\); intracellular proteins (aryl hydrocarbon receptor nuclear translocator [Arnt], aryl hydrocarbon receptor, and heat shock protein 27); and DNA mismatch-repair protein (protein encoded by human mutL homologue 1 [hMLH1] and the human mutS homologue of the chromosome 2p gene [hMSH2]) \(^13\).

Granulocyte colony-stimulating factor regulates the proliferation and differentiation of granulocytic progenitor cells and ac-
activated mature neutrophils through G-CSFR. Functional receptors for G-CSF were identified in hematopoietic cells and several cancer cells. The prognostic marker G-CSFR in oral cancer was confirmed by Kato et al. In the case of nasopharyngeal carcinoma, there was a significant difference in overall survival rates between the negative and positive expressions of IL-10. Interleukin 12 plays an important role in host defense against several tumors by an increased ability to induce apoptosis in cancer cells through IL-12R.

Platelet-derived endothelial cell growth factor is a potent factor in the stimulation of endothelial cell migration and proliferation. Because PD-ECGF has an effect on angiogenesis, a good correlation between the level of PD-ECGF and increased intratumor microvessel density has been identified in several carcinomas. Blood microvessels are clearly visualized with expression of the endothelial cell marker CD34 antigen using immuno-histochemical techniques.

Cyclin D1 has been demonstrated to promote cell cycle transitions from the G1 to S phases by binding and activating cyclin-dependent kinase 4, with cyclin E–cyclin-dependent kinase 2 and cyclin A–cyclin-dependent kinase 2 complexes. Inhibition of progression from the G1 to S phases is caused by p27 binding to the cyclin D–cyclin-dependent kinase 4 complex to arrest the cell cycle. We found that patients with laryngeal SCCs expressing p27 had good prognoses compared with those not expressing p27.

Wild-type p53 is induced in response to DNA injury. Unless DNA replication is successful, cells with DNA damage are induced to undergo apoptosis via a p53-dependent pathway. Bax promotes apoptosis, and Bcl-2 serves to prolong cell survival. The balance between Bax and Bcl-2 levels helps to determine the susceptibility of a cell to apoptotic stimulation. Fas is a 48-kDa cell-surface glycoprotein that is a member of the tumor necrosis factor/nerve growth factor receptor family, and the Fas antigen is expressed on a broad range of lymphoid cell lines and some cancer cell lines. Fas ligand is a member of the tumor necrosis factor family, which includes tumor necrosis factor α, α and β chains of lymphotxin, and the CD40 ligand. When Fas cross-links with FasL, Fas mediates the apoptosis of tumor cells.

The oncoprotein genes c-fos and c-Myc have various functions implicated in cell growth, differentiation, and development and are concerned with developing tumorigenicity. P-glycoprotein and MRP, which are present in cellular transmembranes, function in the adenosine triphosphate–dependent efflux pump of multidrugs. Multidrug resistance–associated protein has partial structural homology with P-gp, but differs substantially from P-gp. Tumors expressing P-gp and/or MRP are resistant to many anticancer drugs.

Stimulation of CD40 has been shown to inhibit Fas-mediated apoptosis in thyroid cancer cell lines. Arnt is a basic helix-loop-helix transcription factor that forms heterodimers with the aryl hydrocarbon receptor or hypoxia-inducible factor 1α to activate transcription via a xenobiotic response element or a hypoxia response element, respectively. Thus, Arnt plays a major role in 2 key biochemical pathways involved in tumor growth, and can therefore be used in the prognosis of patients with Arnt-expressing breast cancer. Heat shock protein 27 belongs to the heat shock protein family and prevents stress-induced cellular damage under physiological conditions. Heat shock protein 27 has been suggested to be a useful prognostic marker in oral SCCs.

A common feature in tumor initiation or progression is DNA mismatch-repair. Human mutL homologue 1 (and its homologue hMSH2) is 1 of at least 4 genes encoding proteins involved in the repair of mismatched nucleotides after DNA replication or repair and is a prognostic marker for several malignant tumors.

What is a strong prognostic factor for oral and oropharyngeal SCCs? After a long period of translational research, we determined a reliable marker from a total of 26 factors in patients with oral and oropharyngeal SCCs in this study.

METHODS

We included 58 patients with oral and oropharyngeal SCCs who were treated initially at the Department of Otorhinolaryngology–Head and Neck Surgery, University of Fukui, Fukui, Japan, from May 13, 1984, through September 13, 1996, and were completely followed up for 5 years. The mean age of the patients was 63.5 years (median age, 65.0 years). The classification of oral and oropharyngeal SCCs, including primary tumors, regional lymph nodes, distant metastasis, and stage grouping, was determined according to the International Union Against Cancer rules for head and neck cancer (TNM classification). The entire International Union Against Cancer classification criteria, notation, and stage grouping are identical to those published by the American Joint Committee on Cancer. There were 7 stage I tumors (12%), 11 stage II tumors (19%), 12 stage III tumors (21%), and 28 stage IV tumors (48%) (Table 1). None of the patients had distant metastases. The primary sites included the tongue (24 cases), oral floor (7), soft palate (9), tonsil (2), tonsillar pillar or fossa (6), base of tongue (2), buccal mucosa (4), and gingiva (4). Twenty patients died of the oral and oropharyngeal carcinomas. Ten patients died of causes unrelated to the oral and oropharyngeal carcinomas, including septicemia (2 patients), heart failure (3), and pneumonia (5). All samples were obtained by surgery before radiation therapy and chemotherapy. The patients with oral and oropharyngeal SCCs had been treated with standard therapies appropriate for the stages of the SCCs. The patients with stages I and II tumors underwent only tumor resection, whereas those with stages III and IV tumors underwent tumor resection, standard neck dissection, and irradiation (60 Gy) after the surgery. Surgically resected SCC tissues were quickly put into 10% buffered formaldehyde for fixation.
IMMUNOHISTOCHEMICAL STAINING

Paraffin-embedded blocks were sliced into sections 4 µm thick. The procedure for staining has been described previously. Briefly, the sections were deparaffinized with xylene and dehydrated in ethanol, and then put into methanol containing 0.3% hydrogen peroxide for 15 minutes to block the endogenous peroxidase activity. The sections were incubated with normal bovine serum for 15 minutes at room temperature, and then treated overnight at 4°C with specific antibody. The primary antibody was replaced by phosphate-buffered saline solution in negative control samples. The sections were then washed with phosphate-buffered saline solution and incubated with biotinated goat antimouse IgG antibody, goat antirabbit IgG antibody, or rabbit antigoat IgG antibody at room temperature for 1 hour. After washing, a combination of streptavidin, biotin, and peroxidase was applied for 1 hour. The sections were then incubated in phosphate-buffered saline solution containing 0.03% diaminobenzadine and 0.01% hydrogen peroxide. The slides were lightly counterstained with Mayer hematoxylin. The stained factors were as follows: G-CSF, IL-10, G-CSFR, IL-12R, PD-ECGF, CD34, p27, cyclin D1, cyclin E, wt-p53, Bax, Bcl-2, Fas, Fasl, c-fos, c-Myc, P-gp, MRP, nm23, CD40, Arnt, aryl hydrocarbon receptor, heat shock protein 27, hMLH1, and hMSH2.

DETERMINATION OF RESULTS FOR THE IMMUNOHISTOCHEMICAL STAINING

The staining was scored independently by 2 physicians (H.S. and T.I.) without knowledge of the clinical variables and outcomes. For microscopic analysis of the staining, the 2 physicians each randomly selected 5 high-power fields, with each field containing more than 200 tumor cells, and counted the positive and negative cancer cells. We regarded tumor cells stained identically with control cells as positive. For example, infiltrating neutrophils in the sections were used as a positive control of the staining for the G-CSFR monoclonal antibody, and mouse IgG or rabbit serum was used as a negative control instead of the primary antibody for G-CSFR. In total, at least 1000 tumor cells were counted. To determine staining scores in this study, we calculated the average of 10 readings of positive cells as a percentage. We counted microvessels in the 5 most vascular areas within the tumor tissue under a ×200 magnification field using anti-CD34 antibody. We calculated the average of 10 vessel counts in a sample, and its value was shown as the vessel count of the sample in the text.

We judged the expression of each factor as positive according to a histogram of the staining score. For factors with a histogram showing 2 peaks, the bottom value separating the 2 peaks was considered the cutoff point. For other factors with a histogram showing 1 peak, the median and/or mean staining score was considered the cutoff point. As a result of this judging system, 20% of the staining score was the cutoff point for almost all factors. Exceptions included 40% for Fas, 40% for Fasl, 40% for PD-ECGF, and 10% for hMSH2 as cutoff values. A vessel count of 82 (CD34+ cells) was the cutoff value.

STAINING FOR APOPTOSIS DETECTION AND THE DETERMINATION OF APOPTOTIC INDEX

We performed ApopTag in situ detection kit procedures (Oncon Inc, Gaithersburg, Md) under conditions recommended by the manufacturer to visualize apoptotic cells and bodies. The labeling targets of the ApopTag kit were the multitude of new 3'-OH DNA ends generated by DNA fragmentation, and are typically localized in morphologically identifiable nuclei and apoptotic bodies using the TdT enzyme. The apoptotic index, calculated as the number of positively staining apoptotic tumor cells per 1000 tumor cells, was evaluated by 2 investigators (N.N. and C.S.) in a blinded manner.

STATISTICAL ANALYSIS

The survival rates of the patients were estimated according to the Kaplan-Meier method. The cumulative incidence curve explicitly accounted for other causes of cancer death and was computed according to the method of Gooley et al.* These curves were compared using the log-rank test. We used Macintosh personal computers (Apple Computers, Cupertino, Calif) with StatView software (Abacus Concepts, Inc, Berkeley, Calif) for all statistical analyses.

RESULTS

STAINING RESULTS AND OVERALL SURVIVAL RATES FOR 5 YEARS

According to the staining status, the overall survival rates for 5 years classified by 26 tumor-associated factors were calculated using the Kaplan-Meier method (Table 3). Of the 26 factors, PD-ECGF, G-CSFR, vessel count (CD34-positive blood vessels), and Bax had significant P values in overall 5-year survival rates, when comparing patients with positive and negative staining results. Overall survival curves stratified by PD-ECGF or G-CSFR are shown in Figure 1, and immunohistochemical stainings of PD-ECGF and G-CSFR are shown.
without cervical lymph node metastases had better survival rate than the large-tumor group (T3 and T4) vs 75.1%; 
hand, patients with Bax-negative SCCs had lower survival rates than those with Bax-positive SCCs (50.1% vs 67.2%; P = .02). Furthermore, patients without cervical lymph node metastases had better prognoses than those with cervical lymph node metastases (75.2% vs 48.3%; P = .02). Histological grades were not associated with prognoses.

**RELATIVE RISKS CONTRIBUTING TO SURVIVAL TIME (SIMPLE VARIATE ANALYSIS)**

We examined the prognostic value of each factor expression by simple variate analysis using the Cox proportional hazards model (Table 4). Expression of PD-ECGF and G-CSFR and vessel count were significant as independent prognostic indicators for overall survival. The highest risk ratio of death was 4.19 among the PD-ECGF–positive group vs those in the PD-ECGF–negative group (P = .02). Bax expression was not an independent prognostic factor. The existence of lymph node metastasis was also an independent prognostic factor for...
overall survival ($P = .03$). However, tumor size (>$4$ vs $\leq 4$ cm) was not significant as an independent prognostic factor ($P = .05$).

**RELATIVE RISKS CONTRIBUTING TO SURVIVAL TIME (MULTIVARIATE ANALYSIS)**

In addition, we determined prognostic values by multivariate analysis using the Cox proportional hazards model. When we analyzed 4 factors, including sex, tumor size, metastasis of lymph nodes, and each of the 3 factors of G-CSFR and PD-ECGF expression and vessel count, patients exhibiting G-CSFR expression had the highest relative risk of death (hazard ratio, 4.28; $P = .01$). The vessel count was not significant as an independent prognostic factor ($Table 5$).

Finally, we combined and analyzed 6 factors: sex, tumor size, metastasis of lymph nodes, G-CSFR expression, PD-ECGF expression, and vessel count. Expression of PD-ECGF was the most powerful prognostic factor in oral and oropharyngeal SCCs among these 6 factors ($Table 6$). The relative risk of death for patients with oral and oropharyngeal SCC exhibiting high-level PD-ECGF expressions was 4.94-fold higher than the risk for those with low-level PD-ECGF expressions ($P = .01$). The relative risk of death for patients with oral and oropharyngeal SCC exhibiting G-CSFR expression was also significant (hazard ratio, 3.29; $P = .04$). The vessel count was not an independent indicator in this model.

**COMMENT**

Various biological markers have been reported to be prognostic factors in oral and oropharyngeal SCCs.$^{45-47}$ As a result of this study, in which we investigated 26 tumor-associated factors, we determined that PD-ECGF expression was the strongest independent prognostic factor for oral and oropharyngeal SCCs, and G-CSFR expression was the second most important prognostic marker. Although 2 factors, vessel count and the expression of Bax, significantly correlated with overall 5-year survival, we could not find a correlation between these factors and relative risk contributing to survival time in simple and/or multivariate Cox proportional hazards analyses. Because Bax works as a tumor suppressor owing to inactivation of rapid tumor growth, leading cancer cells to apoptosis, the patients with SCCs expressing Bax had good prognoses compared with those with SCCs not expressing Bax.$^{48}$ However, Bax expression was inferior to PD-ECGF and G-CSFR expression as an independent prognostic factor in this study. Another 22 factors, including cytokines and cytokine receptors (G-CSF, IL-10, and IL-12R), cell cycle–related proteins (p27, cyclin D, and cyclin E), apoptosis-related factors (wt-p53, Bcl-2, apoptotic index, Fas, and FasL), oncogene proteins (c-fos and c-Myc), cell-surface proteins (P-gp, MRP, nm23, and CD40), intracellular proteins (Arnt, aryl hydrocarbon receptor, and heat shock pro-
that of parental cell lines in vitro. Strong overexpression in cell lines owing to gene transfer was about 2-fold faster than controls. Hypoxia has a major influence on the production of PD-ECGF, hypoxia was the optimal condition for the production of PD-ECGF. For remaining abbreviations, see Table 2.

Platelet-derived endothelial cell growth factor is an important molecule associated with angiogenesis. Hypoxia has a major influence on the recruitment of endothelium and the establishment of a vascular supply via the up-regulation of angiogenic factors, including PD-ECGF, in cancer tissues. Then, the overexpression of PD-ECGF enhanced tumor growth. Under hypoxic conditions, the growth rate of PD-ECGF–overexpressing cell lines owing to gene transfer was about 2-fold faster than that of parental cell lines in vitro. Strong overexpression of PD-ECGF by stable transfection in an HNSCC cell line showed rapid cell growth through the regulation of p27 and cyclin D1 expression. Hypoxia also induces apoptosis in cancer cells. The induction of apoptosis is a crucial factor in the treatment of cancer. Because PD-ECGF conferred resistance to the apoptosis induced by hypoxia in cancer cells, the expression of PD-ECGF promotes tumor growth and proliferation. Furthermore, the presence of hypoxia is associated with poor response to radiation in cancer models in vitro and in vivo. Resistance to radiotherapy and chemotherapy is seen in HNSCC-expressed PD-ECGF and highly vascularized HNSCC, which leads to poorer survival rates. These results support our data, which showed poor prognoses for patients with oral and oropharyngeal SCCs expressing PD-ECGF.

In our results, there was no significant correlation between PD-ECGF expression and vessel count. Fukuiwa et al reported that PD-ECGF had effects on tumor growth other than angiogenic activity in HNSCC because PD-ECGF expression was significantly higher in squamous cell carcinomas expressing PD-ECGF and highly vascularized HNSCC, which leads to poorer survival rates. These results support our data, which showed poor prognoses for patients with oral and oropharyngeal SCCs expressing PD-ECGF.
correlated with proliferating cell nuclear antigens but not with vessel count in HN SCC. We also suggested that the in vivo angiogenesis of oral and oropharyngeal SCCS might be mediated by another, separate contributor in addition to PD-ECGF activity, such as vascular endothelial growth factor activity. Furthermore, PD-ECGF may also be closely associated with many events in cancer cells, including angiogenesis, apoptosis, and the cell cycle. Further studies are required to clarify the importance of PD-ECGF expression in oncology.

The most important independent prognostic factor is G-CSFR. It is thought that the signals of G-CSF via G-CSFR in cancer cells have 2 functions, including cell proliferation and cell invasion. Carcinoma cells positive for expression of G-CSFR have been shown to proliferate in vitro by means of spontaneous G-CSF production by cancer cells or by the addition of exogenous G-CSF, and this proliferation was blocked by anti-G-CSF antibody. Granulocyte colony-stimulating factor and its receptor are considered to act as a paracrine and/or an autocrine loop mechanism for the proliferation of tumor cells. On the other hand, in our basic study, recombinant G-CSF significantly augmented the invasion potential of HNSCCs in vitro through the elevation of type IV collagenase activity, but exogenous recombinant G-CSF did not enhance tumor growth of HNSCCs in vitro. In our previous clinical study, patients with oral and oropharyngeal SCCS expressing G-CSFR had significantly higher local recurrence rates than those not expressing G-CSFR (P = .007), suggesting that the signal through G-CSFR enhanced tumor invasion. However, no correlation between tumor size and G-CSFR expression was found in oral and oropharyngeal SCCS, suggesting that the expression of G-CSFR is not associated with tumor proliferation. From these results, we speculate that the main function of the signal via G-CSFR is to enhance cell invasion in oral and oropharyngeal SCCS, and this function may give patients with SCCS expressing G-CSFR poor prognoses. Granulocyte colony-stimulating factor produced by SCCS was not associated with prognosis in this study, and no correlation was detected between G-CSF production and G-CSFR expression in oral and oropharyngeal SCCS. Cancer cells do not necessarily produce G-CSF by themselves, because G-CSF is usually produced by normal fibroblasts and tissues adjacent to the cancers. In one interesting report, G-CSF via G-CSFR induced endothelial vascular cells to express activation/differentiation. Thus, G-CSF via G-CSFR also functions as an angiogenic factor.

In conclusion, in oral and oropharyngeal SCCS, PD-ECGF may be the most reliable prognostic factor and G-CSFR the second most effective factor among 26 tumor-associated factors. Because PD-ECGF and G-CSFR participate in angiogenesis, the expression of the angiogenic factors may be important to predict the prognosis of patients with oral and oropharyngeal SCCS among cytokines, cell cycle–related proteins, apoptosis-related factors, oncogene-related proteins, cell-surface proteins, intracellular proteins, and DNA repair proteins. A prospective study in a larger cohort would support our conclusion.

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