Expression of epiregulin, a novel epidermal growth factor ligand associated with prognosis in human oral squamous cell carcinomas

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Abstract. We examined the expression of *epiregulin* and *amphiregulin* mRNA in 39 oral SCCs, 2 epithelial dysplasias and 7 normal gingivae by real-time RT-PCR. The mean expression level of *epiregulin* mRNA was higher in oral SCCs (0.29±0.50) than normal gingivae (0.01±0.007) and epithelial dysplasias (0.01±0.001). The expression level of *epiregulin* mRNA was significantly higher in oral SCCs than normal gingivae (Mann-Whitney U test, P=0.023). *Epiregulin* mRNA was higher in stage III/IV than in stage I/II oral SCCs. However, a significant association was not found. The mean expression level of *amphiregulin* mRNA was higher in oral SCCs (0.18±0.24) than normal gingivae (0.002±0.003) and epithelial dysplasias (0.01±0.001). *Amphiregulin* mRNA was significantly higher in oral SCCs than normal gingivae (Mann-Whitney U test, P=0.001). We then examined the expression of four *EGF receptor* mRNA in oral SCCs. The expression levels of *HER1*, *HER2*, *HER3* and *HER4* mRNA in oral oral SCCs were increased compared to those in normal gingivae. A significant correlation was found between the mRNA expression levels of *epiregulin* and *HER2*, *HER3* and *HER4* (Spearman's correlation coefficient by rank test, P=0.031, P=0.004 and P=0.027, respectively). Patients with oral SCC that have a high expression of epiregulin had a significantly shorter survival than those with low a expression (log-rank test, P<0.05). These results indicate that human epiregulin is closely linked to the increased or abnormal cell proliferation in human oral SCC.

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Introduction

The epidermal growth factor (EGF) system is involved in embryogenesis, development, proliferation and differentiation (1,2). There are several EGF family ligands that can bind the EGF receptors including heparin-binding epidermal growth factor (HB-EGF), transforming growth factor-α (TGF-α) amphiregulin, epiregulin, betacellulin and neuregulin (3-10). The four types of EGF receptors include: ErbB-1 (HER1), ErbB-2 (Neu/HER2), ErbB-3 (HER3) and ErbB-4 (HER4) (11-14). These receptors have a similar molecular structure consisting of transmembrane glycoproteins with an extra-cellular ligand-binding domain, a transmembrane region and an intracellular domain (14). EGF family ligands can be divided into two groups. One group of these ligands binds to HER1, and includes HB-EGF, TGF-α amphiregulin, epiregulin and betacellulin (3-9). The other group includes neuregulin, which is a ligand of HER3 and HER4 (10).

Epiregulin is a new member of the EGF family, purified from the conditioned medium of the mouse fibroblast-derived tumor cell line (15). Epiregulin shows a dual biological activity; stimulating the proliferation of fibroblasts, hepatocytes, smooth muscle cells and keratinocytes but inhibiting the growth of several tumor-derived cell lines (15-17). It binds directly to HER4 as well as HER1 and induces tyrosine phosphorylation of HER2, HER3 and HER4 (18).

Amphiregulin was originally purified from a serum-free conditioned medium of the MCF-7 breast cancer epithelial cells treated with the phorbol 12-myristate-13-acetate (6). The carboxyl-terminal domain of amphiregulin binds to the EGF receptor (HER1) (19). Amphiregulin induces tyrosine phosphorylation of the EGF receptor and downstream activation of the extra-cellular regulated kinase signaling cascade (19). It promotes the growth of fibroblasts, tumor cells and human epidermal keratinocytes but inhibits the growth of some normal and neoplastic cell lines (20). Studies have clarified the evidence that an amphiregulin-mediated autocrine loop exists in human cancers (21,22).

In human carcinomas, the EGF family ligands participate in tumor proliferation, migration, invasion and angiogenesis (23). However, attempts to examine the expression of the epiregulin in human oral squamous cell carcinomas have yet to be made. In the present study, we examined the expression

of the human epiregulin gene in oral squamous cell carcinomas to clarify the correlation between epiregulin expression and clinicopathological factors. We also examined the expression of *amphiregulin* mRNA in human oral squamous cell carcinomas. The correlation between the expression of EGF receptors and *epiregulin/amphiregulin* was investigated. Furthermore, the correlation between the mRNA expression levels of *epiregulin/amphiregulin* and survival rates of OSCC patients were examined.

Materials and methods

Tissue samples. We examined 39 oral squamous cell carcinomas (SCCs), 2 epithelial dysplasias and 7 normal gingivae. Tumor tissues and specimens of normal gingivae were obtained with informed consent and approval from the institutional review board at Hiroshima University Dental Hospital (Japan) between 1995 and 2006. The oral SCC samples were derived from the tongue, upper gingiva, lower gingiva and buccal mucosa. The clinical staging was determined according to the International Union Against Cancer TNM classification (24). The primary tumors were classified histopathologically as well- or moderately-differentiated in the World Health Organization (WHO) classification (25). For molecular analyses, tissue samples obtained at the time of surgery were frozen immediately in liquid nitrogen and stored at -80˚C.

RNA extraction and quantitative RT-PCR analysis. RNA was extracted with RNAeasy mini kit (Qiagen, Hilden, Germany). Total RNA (1 μg) was subjected to a reverse-transcriptase reaction using the first strand cDNA synthesis kit (Amersham Biosciences, Uppsala, Sweden). The quantification of mRNA levels was carried out using a real-time fluorescence detection method according to the method of Eads *et al* (26). The fluorescence was detected by the laser detector of the Line Gene Fluorecent Quantitative Detection System (Bio Flux, Tokyo, Japan) and the detection was carried out by measuring the binding of a fluorescence dye, SYBR-Green I, to double-stranded DNA. The PCR was run in microtubes in a volume of 20 μl. The reaction mixture contained 1.0 μg of cDNA, 10 μl of SYBR-Green PCR master mix (Toyobo, Osaka, Japan) and 10 pmol of each pair of oligonucleotide primers. The primer sequences were: *epiregulin*, 5'-CAAAGTGTA GCTCTGACATG-3' (sense) and 5'-CTGTACCATCTGC AGAAATA-3' (antisense); *amphiregulin*, 5'-CGGGAGCG ACTATGACTACTC-3' (sense) and 5'-GGGCTTAACTA CCTGTCAACTGG-3' (antisense); *HER1*, 5'-GAGAGGA GAACTGCCAGAA-3' (sense) and 5'-GTAGCATTTAT GGAGAGTG-3' (antisense); *HER2*, 5'-CCAGGACCTGCT GAACTGGT-3' (sense) and 5'-TGTACGAGCCGCAC ATCC-3' (antisense); *HER3*, 5'-GGTGCTGGGCTTGC TTTT-3' (sense) and 5'-CGTGGCTGGAGTTGGTGTTA-3' (antisense); *HER4*, 5'-TGTGAGAAGATGGAAGATGGC-3' (sense) and 5'-GTTGTGGTAAAGTGGAATGGC-3' (antisense), and *G3PDH*, 5'-ACCACAGTCCATGCCAT CAC-3' (sense) and 5'-TCCACCACCCTGTGGCTGTA-3' (antisense). The PCR program was as follows: initial melting at 95˚C for 30 sec followed by 40 cycles at 95˚C for 15 sec, 57˚C for 10 sec and 72˚C for 15 sec. The threshold cycle (CT) of each PCR product was defined as the cycle number at the point where the fluorescence signal had passed the fixed threshold. The relative quantification was calculated as 2^{-ct} and normalized to an internal control (G3PDH).

Table I. Expression of *epiregulin* mRNA in oral SCCs and its correlation with clinicopathological parameters.

	Case no.	Expression level of epiregulin	
		Mean ± SD	P-value[b]
Sex			
Male	20	0.37±0.52	0.57
Female	19	0.21±0.48	
Site			
Tongue	12	0.21±0.36	0.94
Upper gingiva	3	0.42±0.70	
Lower gingiva	19	0.32±0.58	
Buccal mucosa	4	0.34±0.53	
Histology[a]			
Well	24	0.23±0.48	0.94
Moderate	15	0.40±0.53	
Tumor size[a]			
T1	5	0.054±0.05	0.96
T2	16	0.23±0.36	
T3	6	0.43±0.77	
T4	12	0.41±0.60	
Clinical stage[a]			
I/II	18	0.09±0.01	0.29
III/IV	21	0.45±0.02	
Lymph node metastasis			
Positive	10	0.18±0.25	0.88
Negative	29	0.33±0.56	

[a]According to the American Joint Committee on Cancer Staging Manual, 5th edition. [b]P-value, the correlation was analyzed using the Mann-Whitney U test or Kruskal-Wallis test and the P-values are shown. P<0.05 was regarded as statistically significant.

Immunohistochemistry. Avidin-biotin-peroxidase complex immunostaining was performed as previously described (27). Epiregulin staining using an anti-epiregulin polyclonal antibody (R&D Systems, Minneapolis, USA) (diluted 1:100) was graded as positive (at least 10% of tumor cells showed moderate to intense immunoreactivity) or negative (<10% of tumor cells showed weak or no immunoreactivity).

Statistical methods. The results of quantitative RT-PCR analysis were compared with the patient clinicopathological information using the Mann-Whitney U test and Spearman's correlation coefficient by rank test. The overall survival rates were calculated by the Kaplan-Meier method and analyzed

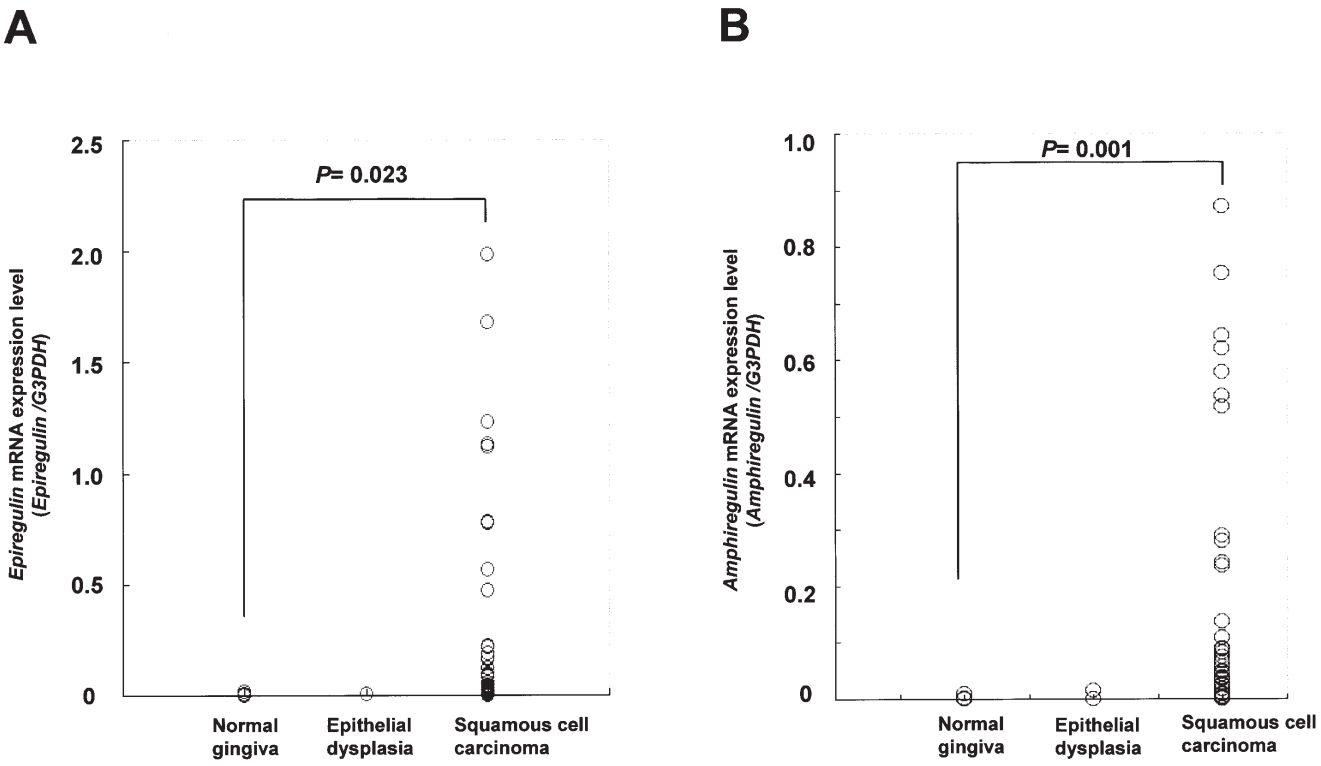

Figure 1. (A) Levels of *epiregulin* mRNA expression in normal gingivae, epithelial dysplasias and oral SCCs. Each point is the *epiregulin* mRNA expression level. *Epiregulin* mRNA expression levels were significantly higher in oral SCCs than normal gingivae (Mann-Whitney U test, P=0.023). (B) Levels of *amphiregulin* mRNA expression in normal gingivae, epithelial dysplasias and oral SCCs. Each point is the *amphiregulin* mRNA expression level. *Amphiregulin* mRNA expression levels were significantly higher in oral SCCs than normal gingivae (Mann-Whitney U test, P=0.001).

by means of the log-rank test. P<0.05 was regarded as statistically significant.

Results

Expression of epiregulin and amphiregulin mRNA in oral SCCs. We examined the expression of *epiregulin* and *amphiregulin* mRNA in 39 oral SCCs, 2 epithelial dysplasias and 7 normal gingivae by real-time RT-PCR. The mean expression level of *epiregulin* mRNA was higher in oral SCCs (0.29±0.50) than normal gingivae (0.01±0.007) and epithelial dysplasias (0.01±0.001) as shown in Fig. 1A. The expression level of *epiregulin* mRNA was significantly higher in oral SCCs than normal gingivae (Mann-Whitney U test, P=0.023). In addition, oral SCCs showed high levels of *epiregulin* mRNA expression compared to epithelial dysplasias, although the difference was not significant (Mann-Whitney U test, P=0.17). The expression of *epiregulin* mRNA was not correlated to clinicopathological factors such as age, gender, tumor type and location. Data on *epiregulin* mRNA expression, tumor size, clinical stage and lymph node metastasis are summarized in Table I. The expression level of *epiregulin* mRNA was higher in stage III/IV than in stage I/II oral SCCs. However, a significant association was not found. The mean expression level of *amphiregulin* mRNA was higher in oral SCCs (0.18±0.24) than normal gingivae (0.002±0.003) and epithelial dysplasias (0.01±0.001) as shown in Fig. 1B. The expression level of *amphiregulin* mRNA was significantly higher in oral SCCs than normal gingivae (Mann-Whitney U test, P=0.001). Data on *amphiregulin* mRNA expression, tumor size, clinical stage and lymph node metastasis are summarized in Table II. We could not find any correlation between the amphiregulin expression and clinicopathological parameters.

Immunohistochemistry for epiregulin in oral SCCs. We then studied the expression of epiregulin protein in 13 oral SCCs with higher levels of *epiregulin* mRNA(expression level >0.1) immunohistochemically. Normal gingivae and epithelial dysplasias showed weak or no epiregulin staining (data not shown). Most of the oral SCCs with higher levels of *epiregulin* mRNA showed positive staining. Epiregulin expression was observed in the cytoplasm of the cancer cells (Fig. 2).

Expression of EGF receptor mRNA in oral SCCs. The mRNA expression of four *EGF receptors* (*HER1*, *HER2*, *HER3* and *HER4*) was examined in oral SCCs and normal ginigivae. The mean expression level of *HER1* mRNA was higher in oral SCCs (0.73±1.33) than normal gingivae (0.042±0.056). However, a significant correlation was not found between oral SCCs and normal gingivae (Mann-Whitney U test, P=0.15). The expression levels of *HER2*, *HER3* and *HER4* mRNA in oral SCCs (0.49±0.27, 0.20±0.23 and 0.15±0.40, respectively) were also increased compared to those in normal gingivae (0.39±0.36, 0.07±0.057 and 0.11±0.11, respectively). Statistical correlations were not found (Mann-Whitney U test, P=0.088, P=0.063 and P=0.81, respectively). The expression levels of *HER3* and *HER4* mRNA were higher in

Table II. Expression of *amphiregulin* mRNA in oral SCCs and its correlation with clinicopathological parameters.

		Expression level of amphiregulin	
	Case no.	Mean ± SD	P-value[b]
Sex			
Male	20	0.21±0.26	0.72
Female	19	0.15±0.23	
Site			
Tongue	13	0.16±0.27	0.71
Upper gingiva	3	0.84±0.13	
Lower gingiva	19	0.20±0.24	
Buccal mucosa	4	0.18±0.24	
Histology[a]			
Well	24	0.20±0.24	0.91
Moderate	15	0.17±0.24	
Tumor size[a]			
T1	5	0.068±0.031	0.50
T2	16	0.26±0.30	
T3	6	0.18±0.23	
T4	12	0.11±0.16	
Clinical stage[a]			
I/II	18	0.22±0.28	0.14
III/IV	21	0.14±0.20	
Lymph node metastasis			
Positive	10	0.18±0.23	0.75
Negative	29	0.18±0.24	

[a]According to the American Joint Committee on Cancer Staging Manual, 5th edition. [b]P-value, the correlation was analyzed using the Mann-Whitney U test or Kruskal-Wallis test and the P-values are shown. P<0.05 was regarded as statistically significant.

stage III/IV (0.26±0.29 and 0.21±0.54) than in stage I/II oral SCCs (0.13±0.12 and 0.082±0.065). However, a significant association was not found (Mann-Whitney U test, P=0.20 and P=0.19).

Correlation of the mRNA expression levels between epiregulin/amphiregulin and EGF receptors. The co-expression of the epiregulin/amphiregulin and four EGF receptors was also examined. A significant correlation was found between the mRNA expression levels of *epiregulin* and *HER2*, *HER3* and *HER4* (Spearman's correlation coefficient by rank test, P=0.031, P=0.004 and P=0.027, respectively) (Fig. 3). However, a significant correlation between *epiregulin* and *HER1* could not be found (Spearman's correlation coefficient by rank test, P=0.24). The significant correlation between the mRNA expression levels of *amphiregulin* and *HER4* was found (Spearman's correlation coefficient by rank test, P=0.011) (Fig. 4). We could not find a significant correlation between *amphiregulin* and *HER1*, *HER2* and *HER3* (Spearman's correlation coefficient by rank test, P=0.059, P=0.35 and P=0.77, respectively) (Fig. 4).

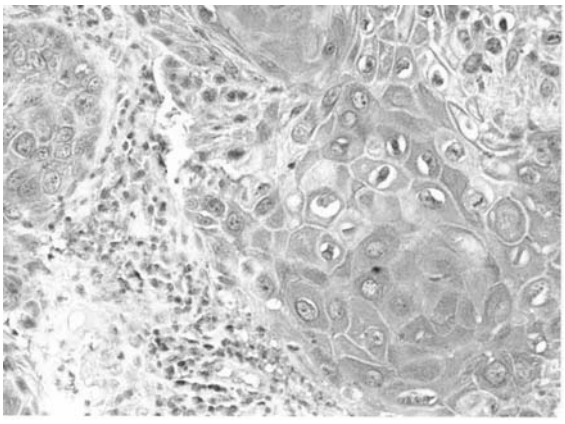

Figure 2. Immunohistochemistry for epiregulin in oral SCC. Epiregulin expression was observed in the cytoplasm of the cancer cells.

Correlation between the mRNA expression levels of epiregulin/amphiregulin and survival rates of OSCC patients. The 13 patients whose tumors expressed increased levels of *epiregulin* mRNA (expression level >0.1) showed a poor survival compared to the 17 expressing *epiregulin* mRNA at lower levels (expression level <0.1); the difference being statistically significant (log-rank test, P<0.05) (Fig. 5A). On the other hand, the 15 patients whose tumors expressed increased levels of *amphiregulin* (expression level >0.05) showed a poor survival compared to the 15 expressing *amphiregulin* at lower levels (expression level <0.05) (Fig. 5B). However, a significant difference was not found (log-rank test, P>0.05). These results suggest that epiregulin has a correlation with the prognosis of oral SCC patients.

Discussion

Head and neck cancer is one of the most common cancers worldwide. Oral cancers account for 40% of head and neck cancers (28). Despite advances in diagnostic and therapeutic possibilities, many oral SCC patients have a poor prognosis. Early diagnosis is the most important factor in determining the survival of the oral SCC patients (29). Prevention with involves reducing the exposure of tobacco and alcohol has also been shown to be effective in reducing oral SCC (29,30).

EGF and EGF receptors participate in cell proliferation, invasion, differentiation and angiogenesis in several carcinomas (23). Epiregulin is a new member of the EGF family (15). The human epiregulin gene encodes a 163-residue putative transmembrane precursor containing an EGF-like domain and the structural organization is similar to that of other EGF family ligands (16). It binds to epidermal growth factor (HER1) and HER4, and induces the tyrosine phosphorylation of HER2, HER3 and HER4 (18). Lee *et al* have reported that epiregulin acts as a dual biological activity, by stimulating the proliferation of fibroblasts, hepatocytes, smooth muscle cells and keratinocytes but also inhibiting the

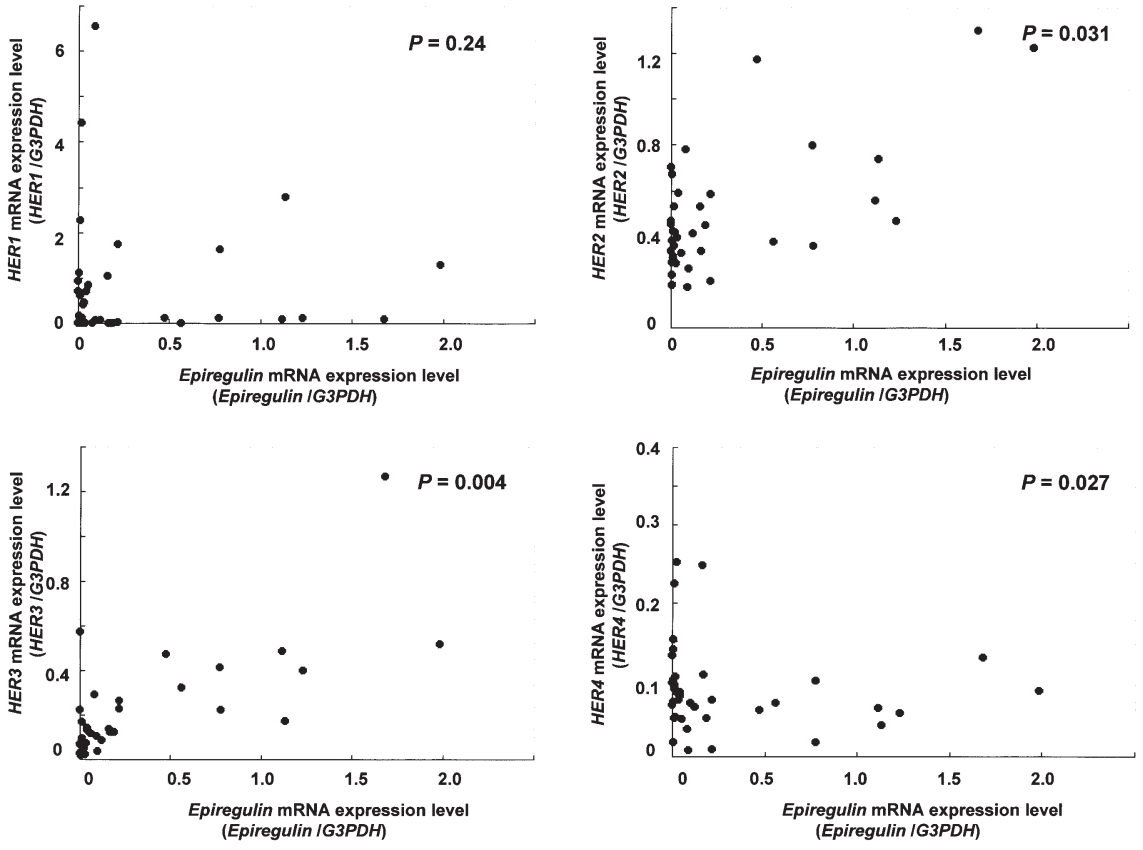

Figure 3. Correlation of the mRNA expression levels between *epiregulin* and *EGF receptors*. Spearman's correlation coefficient by rank test. P<0.05 was regarded as statistically significant.

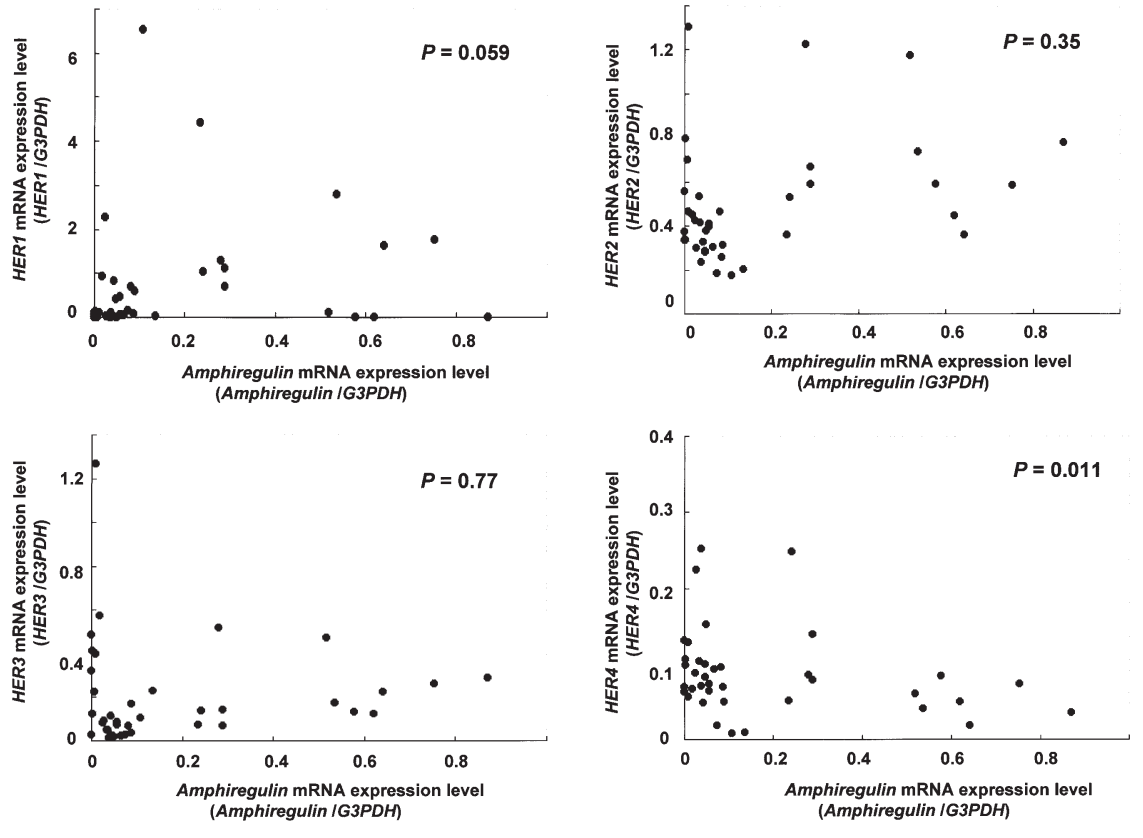

Figure 4. Correlation of the mRNA expression levels between *amphiregulin* and *EGF receptors*. Spearman's correlation coefficient by rank test. P<0.05 was regarded as statistically significant.

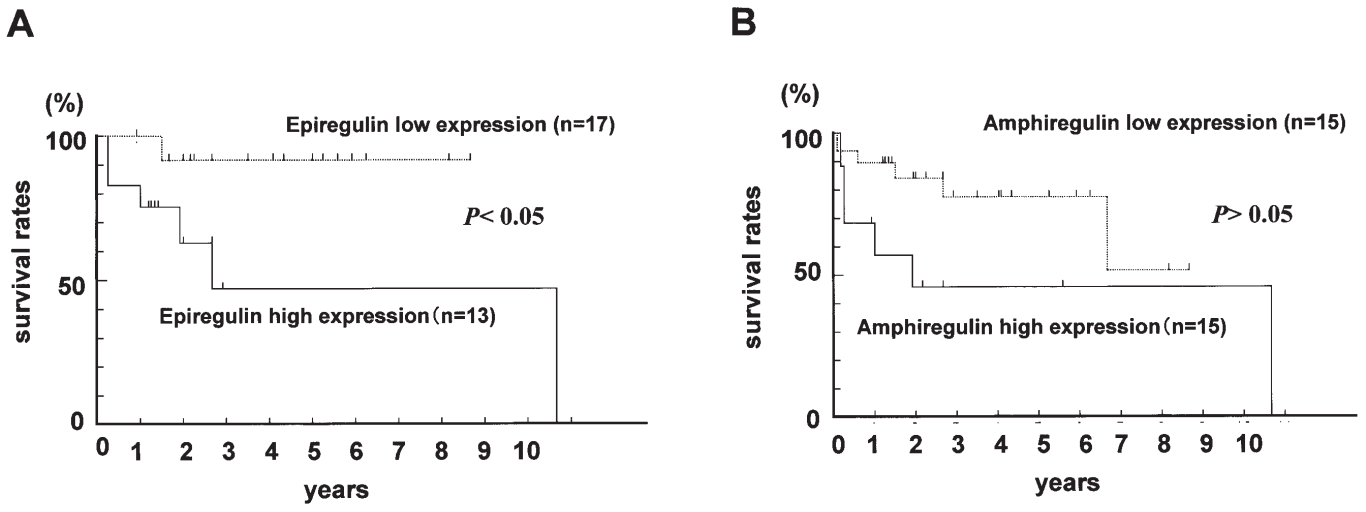

Figure 5. (A) Correlation between mRNA expression levels of *epiregulin* and survival rates of OSCC patients. The overall survival rates were calculated by the Kaplan-Meier method and analyzed by means of the log-rank test. P<0.05 was regarded as statistically significant. A significant correlation was observed (P<0.05). (B) Correlation between mRNA expression levels of *amphiregulin* and survival rates of OSCC patients. A significant correlation was not observed (P>0.05).

growth of several cancer cell lines (17). The epiregulin gene has different characteristics from those of other EGF family ligands.

Toyoda *et al* have reported that *epiregulin* mRNA expression was not observed in human normal tissues except the placenta and peripheral blood leukocytes, but was detected in various types of human cancer cell lines (16). A high expression of *epiregulin* mRNA was recently reported in human bladder and colorectal cancers (31,32). In the present study, we showed that *epiregulin* mRNA expression was significantly higher in oral SCC than normal gingivae. Most of the oral SCCs with higher levels of *epiregulin* mRNA showed the expression of epiregulin protein. Such observations suggest that the up-regulation of human epiregulin correlates with the malignant condition. We have shown the correlation between the expression of *epiregulin* mRNA and patient survival. The OSCC patients whose tumors expressed increased levels of epiregulin showed a poor survival. These results suggest that human epiregulin may be a useful prognostic factor of oral SCC patients.

Amphiregulin is a heparin-binding growth factor and acts as a ligand EGF receptor (HER1) (7). It is the predominant autocrine growth factor produced by keratinocytes (33). Overexpression of amphiregulin results in the rapid growth of keratinocytic tumors (34). Bostwick *et al* have shown the increased expression of amphiregulin protein in prostatic adenocarcinomas by immunohistochemical analysis (35). Castillo *et al* recently reported that amphiregulin was expressed in human hepatocellular carcinomas and cell lines and behaved as a growth factor for hepatocarcinoma cells (21). They have shown the existence of the amphiregulin-mediated autocrine loop that contributes to the transformed phenotype in hepatocellular carcinoma cell lines. In this study, we showed the increased expression of *amphiregulin* mRNA in oral SCC. Furthermore, we found the increased expression of *amphiregulin* mRNA in salivary gland carcinomas as compared with pleomorphic adenomas and submandibular glands (Shigeishi *et al*, unpublished observations). These results indicate that amphiregulin is involved in the cell proliferation of human malignant tumors.

Overexpression of the EGF receptor has been reported in several human neoplasms such as breast, lung, colon, prostate and ovarian carcinomas (36,37). In our data, the mRNA expression of *EGF receptors* (*HER1*, *HER2*, *HER3* and *HER4*) was increased in oral SCCs. The increased expression of EGF receptors is associated with the up-regulation of EGF ligands (37-39). Todd *et al* reported that *TGF-α* and *EGF receptor* mRNA were overexpressed in oral cancer cell lines (40). In the present study, the mRNA expression levels of *epiregulin* and *EGF receptor* are increased in oral SCCs as compared with normal gingivae. A significant correlation was found between the mRNA expression levels of *epiregulin* and *HER1*, *HER3* and *HER4*. Epiregulin directly binds to HER1 and HER4. Activation of the EGF receptor (HER1 and HER4) may contribute to the biological response such as tumor proliferation in oral SCC. High incidences of DNA amplification and overexpression of the EGF receptor in human oral SCCs have been reported (34,36,41). However, the growth of SCC cells was inhibited by EGF *in vitro* (42). Further investigations are needed to clarify the molecular mechanism leading to the inhibitory or stimulatory effects of EGF in the growth of SCC cells.

This is the first report on the *epiregulin* mRNA expression in oral SCC patients. Patients with tumors that have a low expression of epiregulin have a significantly longer survival than patients with a high expression. Our results indicate that there is a strong correlation between the expression of epiregulin and survival. The identification of a specific factor for predicting clinical outcome in cases of oral SCC would be helpful for selecting effective treatments. Our results suggest that epiregulin may be a potential marker in patients with oral SCC.

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