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Papers

The Significance of Epidermal Growth Factor Receptor and Matrix Metalloproteinase-3 in Squamous Cell Carcinoma of the Oral Cavity

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Surgical specimens from 65 patients with squamous cell carcinoma (SCC) of the oral cavity were examined immunohistochemically. The clinicopathological significance of the expression of epidermal growth factor receptor (EGFR) and matrix metalloproteinase-3 (MMP-3) was assessed. Among the 65 tumours, 20 (30.8%) and 37 (56.9%) tested positively for EGFR and MMP-3, respectively. A positive correlation between the expression of EGFR and MMP-3 was found. The expression of EGFR in oral SCCs was associated with an advanced T stage of the primary tumour, an advanced pathological stage, and a high incidence of neck metastasis. In addition, MMP-3 was primarily expressed at the advancing front of cancer with a diffuse invasive mode. Thus, overexpression of MMP-3 was associated with an advanced pathological stage, a diffuse invasive mode, and a high incidence of neck metastasis. The analysis of MMP-3 expression is useful to evaluate the pathological status of tumours. Because EGFR-overexpressed tumour should produce larger amounts of MMP-3 *in vivo*, a close examination of oral SCC for expression of EGFR and MMP-3 should be helpful to predict their malignant potential. Copyright © 1996 Elsevier Science Ltd

Keywords: matrix metalloproteinase-3, epidermal growth factor receptor, squamous cell carcinoma, oral cavity, lymph node metastasis

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INTRODUCTION

Squamous cell carcinomas of the oral cavity are characterised by a high potential for local invasiveness and a great propensity to metastasis to cervical lymph nodes. Because the degradation of extracellular matrix macromolecules is a critical step for tumour invasion and metastasis, the ability of carcinomas to produce proteolytic enzymes is an important factor affecting their malignant potential. Among these proteolytic enzymes, matrix metalloproteinases (MMP) are believed to play a key role in their malignant behaviours [1].

Our previous *in vitro* study has also shown that oral squamous carcinoma cell lines can produce at least two MMPs under normal culture condition, namely MMP-2 (gelatinase A) and MMP-3 (stromelysin-1) [2]. The production of MMP-3 is stimulated co-ordinately with MMP-1 by EGF and TPA [3]. EGF also stimulates the *in vitro* production of MMP-9 (gelatinase B) by oesophageal squamous cell carcinomas [4]. Interestingly, the overexpression of the EGF receptor (EGFR) is a remarkable property of squamous cell carcinoma. It is not clear, however, whether EGFR overexpression is implicated in the increased production of MMP-3 by oral squamous carcinoma.

In this paper, the *in vivo* relationship between the expression of EGFR and MMP-3 in oral squamous cell carcinoma was examined using immunohistochemical staining and the clinicopathological significance of EGFR and MMP-3 in oral squamous carcinoma is also studied.

MATERIALS AND METHODS

Materials

Sixty-five surgical specimens were obtained from previously untreated patients who underwent surgery for squamous cell carcinoma of the oral cavity at the Department of

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Table 1. Summary of age, sex, tumour sites, and stage grouping of 65 patients with oral squamous carcinoma

Mean age (range) (year)		60.2 ± 15.2 (27–89)
Men: women		40:25
Tumour sites	Tonue	36
	Gum and alveolus	16
	Floor of the mouth	11
	Lip	1
	Jaw (intraosseous carcinoma)	1
Clinical stage*	I and II	34
	III and IV	31
Pathological stage†	I and II	35
	III and IV	30
Neck metastasis	Absent	38
	Present	27

*Clinical stage grouping was based on TNM classification in UICC (1987).
†Pathological stage grouping was based on the pathological status in primary site and neck.

Oral and Maxillofacial Surgery, Kurume University School of Medicine, Kurume, Japan. A summary of the patients is shown in Table 1. The following clinicopathological data were available: T-category of primary tumour, clinical stage grouping, pathological stage grouping, degree of histological differentiation, mode of invasion at the tumour–host borderline, absence or presence of neck metastasis, and absence or presence of extranodal spreading. The T-category of the primary tumour and the clinical stage grouping were based on UICC classification in 1987. Pathological stage was determined according to the histologically evaluated extent of the tumour. The mode of invasion at the tumour–host borderline was classified into three types (W, M and D) as described previously [5]. Briefly, type W had a well-defined borderline, type M exhibited groups of tumour cells with no distinct borderline, and type D invade diffusely without forming tumour nests or in small aggregates with finger-like projections.

Antibodies

Anti-human MMP-3 serum was a kind gift from Dr H. Nagase (University of Kansas Medical Center, Kansas City, Kansas, U.S.A.) [2, 6]. The rabbit anti-human EGFR polyclonal antibody, Ab-1, was purchased from Oncogene Science (New York, U.S.A.).

Immunohistochemical staining procedure

The immunohistochemical staining was carried out by means of the avidin–biotin–peroxidase complex (ABC) staining technique as described previously [7–10]. Briefly, 5-μm formalin fixed and paraffin embedded sections were deparaffinised and rehydrated. Endogenous peroxidase activity was eliminated by immersing the slides in absolute methanol containing 0.3% hydrogen peroxide for 30 min. The tissue sections were stained by the ABC method using the Vectastain ABC kit (Vector Laboratories, Burlingame, California, U.S.A.). The sections were treated for 12 h in a 4°C moist chamber with rabbit anti-human EGFR antibody (1:400 dilution) or sheep anti-human MMP-3 serum (1:500 dilution). Immunohistochemical reactions were developed in 20 mg freshly prepared 3,3′-diaminobenzoidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) in 50 ml of 0.05 mol/l Tris-buffered saline containing 0.015% hydrogen peroxide. Specimens were counterstained with haematoxylin, and then observed using a light microscope. Human placental tissue and human rheumatoid synovium were used as positive controls for EGFR and MMP-3, respectively, and sections omitting the first antibodies were used as negative controls.

Immunoreactivities for EGFR and MMP-3 in the cancer nests were evaluated by a comparison with those in normal oral epithelia as described previously [7–10]. According to the intensity of the positive reaction for EGFR, the tumours were divided into two groups: EGFR(−), tumours tested negative or faintly positive (i.e. the same as that in normal oral epithelia); EGFR(+), tumours with a moderately or markedly stronger intensity stain than that of normal oral epithelia. In the same way, the tumours were divided into two groups of MMP-3(−) and MMP-3(+).

The data obtained were analysed using the chi-square test or Fisher's exact test.

RESULTS

Immunoreactivity for the EGF receptor was observed in the cytoplasmic membrane and cytoplasm of cancer cells in the entire cancerous tissue except the keratinised portion, whereas such immunoreactivity was negligible in normal oral epithelium. On the other hand, immunoreactivity for MMP-3 was detected primarily in small cancer nests of the advancing front or marginal portion of cancerous tissue. However, such immunoreactivity for MMP-3 was not observed or observed only faintly in normal oral epithelium. These immunostainings of cancerous tissue and normal oral epithelium are shown in Fig. 1.

Of the 65 patients investigated, 20 (30.8%) were EGF receptor positive and 37 (56.9%) were MMP-3 positive. We found a positive correlation between the expression of EGF receptor and MMP-3 ($P=0.01$) (Table 2).

The expression of the EGF receptor had a significant correlation to the T-category of the primary tumour ($P=0.038$), pathological stage ($P=0.042$), and neck metastasis ($P=0.010$) (Table 3). On the other hand, we found a significant correlation between the expression of MMP-3 and pathological stage ($P=0.048$), mode of invasion ($P=0.002$), and neck metastasis ($P<0.001$) (Table 4).

DISCUSSION

MMP-3 can degrade a wide range of substrates and type IV collagen [6] and may be a potent activator of proMMP-1 (interstitial collagenase) [11]. In addition, several studies have shown that the production of MMP-3 is induced by EGF, platelet-derived growth factor (PDGF), interleukin-1 (IL-1), oncogenes such as *v-src* and *ras*, and tumour-promoting phorbol esters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) [12–15]. In oral squamous cell carcinoma cell lines, our previous study has shown that the production of MMP-3 is stimulated co-ordinately with that of MMP-1 by EGF and TPA [3]. EGF also stimulates the *in vitro* expression of MMP-9 (92 kDa gelatinase/type IV collagenase) in oesophageal squamous cell carcinoma [4]. Therefore, the expression level of EGFR in oral squamous carcinoma should be

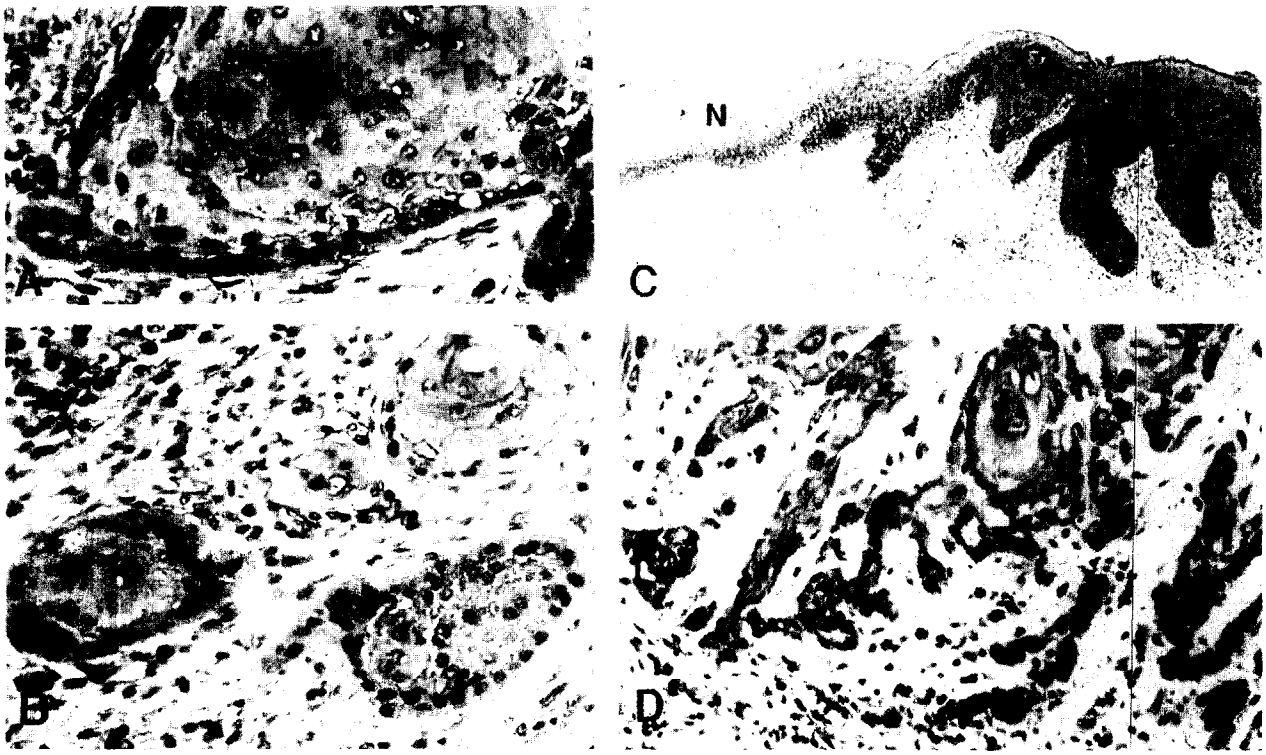

Fig. 1. Expression of EGFR (A and B) and MMP-3 (C and D) in oral squamous cell carcinomas. Surgical specimens from the patients with squamous cell carcinoma of the oral cavity were fixed in formalin and embedded in paraffin, and the sections were stained with monospecific antibodies against EGFR or MMP-3 by the avidin–biotin–peroxidase complex method. Immunoreactivity for EGFR was primarily detected in the cell surface and cytoplasm of the cancer cells. MMP-3 immunoreactivity was detected in the invasive or peripheral nests of the tumour (P) but not in the normal squamous epithelium (N).

Table 2. Correlation between the expression of EGF receptor and MMP-3

Expression of MMP-3	Expression of EGF receptor		
	Negative	Positive	*P*-value
Negative	25	3	0.002
Positive	20	17	

an important factor affecting the production of these proteolytic enzymes, which should be directly associated with the potential of tumour invasion and metastasis.

Interestingly, the overexpression of EGFR is frequently observed in squamous cell carcinoma [16–19]. The expression of EGF and transforming growth factor-α (TGF-α), which are assumed to bind to EGFR, have been identified in oral cancer cells and stromal cells. Todd *et al.* demonstrated the consistent presence of TGF-α mRNA in oral squamous carcinoma cells [20]. Additionally, Shirasuna *et al.* reported the immunohistochemical localisation of EGF in stromal tissue of invasive oral carcinoma [21]. In the present study, we found a positive correlation between the expression of EGFR and MMP-3 by using the immunohistochemical staining method. These findings strongly sugggest that oral squamous cell carcinomas with overexpression of EGFR may produce larger amounts of MMP-3, which is associated with tumour invasion, not only by direct degradation of extracellular matrix components, but also by activation of proMMP-1, through paracrine and/or autocrine EGF or TGF-α-EGFR system.

Table 3. Correlation between expression of EGF receptor and clinicopathological parameters

		Expression of EGF receptor		
		Positive	Negative	*P*-value
T-category*	T1 and T2	11	36	0.038
	T3 and T4	9	9	
Clinical stage*	I and II	8	26	NS
	III and IV	12	19	
Pathological stage†	I and II	7	28	0.042
	III and IV	13	17	
Histological type‡	Well	14	34	NS
	Moderate	5	11	
	Poor	1	0	
Mode of invasion	Type W	2	8	NS
	Type M	8	22	
	Type D	10	15	
Neck metastasis	Absent	7	31	0.010
	Present	13	14	
Extranodal spreading§	Absent	8	9	NS
	Present	5	5	

*T-category and clinical stage grouping were based on classification of TNM in UICC (1987).

†Pathological stage grouping was determined based on the pathological status in the primary site and neck.

‡Well = well-differentiated squamous cell carcinoma (SCC); moderate = moderately differentiated SCC; poor = poorly differentiated SCC.

§Extranodal spreading evaluated only in patients with histologically proven cervical node metastasis ($n = 27$).

Table 4. Correlation between expression of MMP-3 and clinicopathological parameters

		Expression of MMP-3		
		Positive	Negative	*P*-value
T-category*	T1 and T2	25	22	NS
	T3 and T4	12	6	
Clinical stage*	I and II	18	16	NS
	III and IV	19	12	
Pathological stage†	I and II	16	19	0.048
	III and IV	21	9	
Histological type‡	Well	24	24	NS
	Moderate	12	4	
	Poor	1	0	
Mode of invasion	Type W	3	7	0.002
	Type M	13	17	
	Type D	21	4	
Neck metastasis	Absent	15	23	<0.001
	Present	22	5	
Extranodal spreading§	Absent	12	5	NS
	Present	10	0	

*T-category and clinical stage grouping were based on classification of TNM in UICC (1987).

†Pathological stage grouping was determined based on the pathological status in the primary site and neck.

‡Well = well-differentiated squamous cell carcinoma (SCC); moderate = moderately differentiated SCC; poor = poorly differentiated SCC.

§Extranodal spreading evaluated only in patients with histologically proven cervical node metastasis ($n = 27$).

The significance of the overexpression of EGFR in squamous cell carcinoma is not fully understood. However, several studies have reported that the overexpression of EGFR in squamous cell carcinoma is associated with advanced tumour stage, a high incidence of neck metastasis, and a worse prognosis [9, 18, 22]. We also found a significant correlation between the expression of EGFR and T-category of the primary tumour, pathological stage, and neck metastasis. These aggressivities of squamous cell carcinoma associated with the overexpression of EGFR may be due to the overexpression of MMP-3.

Our present study shows a significant correlation between the expression of MMP-3 and clinicopathological parameters, including pathological stage, mode of invasion, and neck metastasis. Several studies have also shown a positive correlation between mode of invasion and lymph node metastasis [5, 23, 24]. From these observations, the more oral carcinoma cells produce MMP-3, the more diffusely and aggressively tumours invade to adjacent normal tissue, including lymphatic and blood vessels. Thus, the diffuse invasive mode in the tumour–host borderline is a noteworthy morphological characteristic of tumour producing MMP-3.

However, 85% (17/20) of EGFR-positive tumours were also tested positive for MMP-3 expression; whereas 44.4% (20/45) of EGFR-negative tumours were tested positive for MMP-3 expression. This discrepancy between the expression of MMP-3 and EGFR may be due to other factors taking part in the regulation of MMP-3, such as IL-1. In fact, Ahn *et al.* demonstrated the expression of IL-1 in squamous cell carcinoma of the head and neck [25].

In conclusion, the expression level of EGFR in oral squamous cell carcinoma is a key factor affecting MMP-3 production, and MMP-3 produced by tumour cells will play a direct role in tumour invasion and metastasis. Therefore, the analysis of the expression of EGFR and that of MMP-3 is useful in predicting the malignant potential in individual squamous cell carcinoma of the oral cavity.

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