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(Short Communication)

Expression of Epidermal Growth Factor in Salivary Adenoid Cystic Carcinoma

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ABSTRACT

This study used an immunohistochemical technique to assess the expression of epidermal growth factor (EGF) in 40 specimens of salivary adenoid cystic carcinoma (ACC), 7 specimens of labial glands adjacent to mucocele, and 5 specimens of normal submandibular glands. In normal submandibular glands, immunohistochemically detectable EGF was demonstrated in all ductal segments, including intercalated, striated, and excretory duct cells. No EGF positive staining was found in acinar compartments, including serous and mucous acinar cells. In degenerated labial glands adjacent to mucocele, no EGF staining was detected in the remaining acinar and ductal cells. In salivary ACCs, positive EGF immunostaining was observed in one of the 5 (20%) ACCs with a solid pattern and in 13 of the 35 (37.1%) ACCs with a tubular-cribriform pattern. The overall EGF expression rate in 40 salivary ACCs was 35%. Positive EGF staining was predominantly found in tubular structures in the tubular ACCs and in duct-like structures in large cribriform patterns or in the stroma of the cribriform ACCs. There was no significant correlation between EGF expression in salivary ACCs and any of the clinicopathological parameters including patient age and sex, cancer location, TNM status, clinical stage, histologic type, perivascular or perineural invasion, focal necrosis of tumor, and cellular atypia. We conclude that the duct segments of the normal submandibular gland are the sites of EGF synthesis and secretion. In degenerated labial glands adjacent to mucocele, EGF synthesis is completely inhibited. Furthermore, EGF is mainly biosynthesized in cells forming tubular or duct-like structures in tubular or cribriform salivary ACCs, and EGF may play a biologic role, particularly as a mitogen in salivary ACC growth.

Key Words: epidermal growth factor, salivary adenoid cystic carcinoma, mucocele, submandibular gland

I. Introduction

Salivary gland tumors are relatively infrequent in the head and neck region. Adenoid cystic carcinoma (ACC) is an infiltrative malignant salivary gland tumor with three different histological patterns: cribriform, tubular or solid. All ACCs, regardless of their histological patterns, are biologically aggressive, can cause insidious destruction of surrounding tissues and give rise to metastasis many years after excision of the primary tumor. The clinical behavior of salivary ACC has been associated with the histological pattern of the tumor (Szanto *et al*., 1984). Tumors with a cribriform or tubular pattern have a better prognosis with regard to length of survival of the patient, whereas the solid pattern seems to have the worst prognosis (Wen *et al*., 1992).

Epidermal growth factor (EGF), a single-chain polypeptide consisting of 53 amino acids, was first isolated from the male mouse submandibular gland (Cohen, 1962). Mouse EGF is confined to the granular convoluted tubule cells in the submandibular gland, and its level is affected by male hormones and thyroxine (Mori *et al*., 1983; Walker *et al*., 1981). Human EGF, subsequently isolated from human urine, is considered to be identical to human β-urogastrone, a potent inhibitor of stimulated gastric acid secretion (Cohen and Carpenter, 1975). Human EGF has been found in human extracellular fluids, urine, saliva and milk (Starkey and Orth,

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1977; Dailey *et al*., 1978; Hirata and Orth, 1979). EGF is a potent mitogen and initiates cellular response by binding to EGF receptor (EGFR) on the surface of target cells. Ligand-receptor binding activates a tyrosine-specific protein kinase which is part of the intracellular domain of the EGFR (Ushiro and Cohen, 1980). This, in turn, leads to intracellular changes that prepare the cell for DNA synthesis and cell division (Barrandon and Green, 1987). EGF can stimulate the growth and differentiation of a variety of mammalian cells, including epithelial cells (Cohen, 1965; Carpenter and Cohen, 1979). Immunohistochemical studies suggest widespread distribution of EGF in human tissues and organs, such as the salivary gland, Brunner's gland, liver, kidney, prostate gland, mammary gland and a variety of endocrine organs (Elder *et al*., 1978; Heitz *et al*., 1978; Kasselberg *et al*., 1985; Poulsen *et al*., 1986; Tsukitani *et al*., 1987; Tatemoto *et al*., 1988; Mori *et al*., 1989; Kajikawa *et al*., 1991). The expression of EGF in oral squamous cell carcinoma (Shirasuna *et al*., 1991; Christensen *et al*., 1993), in gastric carcinoma (Tahara *et al*., 1986), and in salivary gland tumors (Mori *et al*., 1987; Tsukitani *et al*., 1987) has been investigated. In only one paper has the expression of EGF in salivary ACC been reported (Mori *et al*., 1987).

Tahara *et al*. (1986) reported that no EGF-immunoreactivity was found in 52 early gastric carcinomas, while EGF-positive tumor cells were detected in 24 (21.2%) of the 113 advanced gastric carcinomas. They also found that patients with EGF-positive gastric carcinomas had much worse prognosis than did those with EGF-negative gastric carcinomas. Therefore, they suggested that EGF produced by tumor cells plays an important role in the invasive growth of gastric carcinomas and also serves as a biologic marker of high malignancy in patients with gastric cancers. In this study, we used an imunohistochemical technique to investigate the expression of EGF in 40 specimens of ACC from major and minor salivary glands, 5 specimens of normal submandibular glands, and 7 specimens of degenerated labial glands adjacent to mucocele. The immunostaining results of 40 salivary ACC cases were further correlated with the clinicopathological parameters of the tumors and with overall patient survival to determine the possible influence of EGF on the progression and prognosis of salivary ACC.

II. Materials and Methods

Formalin-fixed, paraffin-embedded specimens were obtained from 40 patients with ACC of the salivary glands and 7 patients with mucocele. Of the 40 ACC patients, 20 were men, and 20 were women. Their ages ranged from 23 to 75 (mean, 45.4) years; 29 (72.5%) of them were from 41 to 70 years of age. The diagnosis of each salivary ACC or mucocele was confirmed by histologic examination of hematoxylin and eosin-stained slides.

All patients were treated at National Taiwan University Hospital, Taipei, during the period from 1978 to 1996. The 40 ACC specimens were taken either from the three major salivary glands (parotid, 10; sublingual, 4; submandibular, 2) or from minor salivary glands in the oral cavity (palate, 13; tongue, 5; floor of the mouth, 4; alveolar mucosa, 1; anterior faucial pillar, 1). The seven mucocele specimens were excised from the lower labial mucosa and used as disease controls. Five specimens of normal submandibular gland were obtained from 5 oral cancer patients who underwent radical neck dissection and were used as normal controls. None of the patients had received any treatment before the initial biopsies or surgery, and all surgical procedures were performed with the patients' informed consent and with the approval of the Ethics Committee for Scientific Research on Human Beings of this institution.

The TNM status and clinical stages of the 40 salivary ACC at initial presentation were determined according to the UICC convention (Hermanek and Sobin, 1987). Histologically, ACCs were divided into two groups: those composed of tubular and cribriform without the solid pattern, and those with the predominantly solid pattern. Histopathological parameters, including perivascular or perineural invasion, focal necrosis of the tumor and cellular atypia, were also checked and recorded.

Immunohistochemical staining was performed using a peroxidase-labeled streptavidin-biotin technique, as previously described (Kuo *et al*., 1995). Briefly, 4 μm-thick tissue sections were deparaffinized, rehydrated, and treated with 3% H_2O_2 for 10 minutes to quench endogenous peroxidase activity. After washing in 10 mmol/L Tris-buffered saline (TBS), pH 7.6, sections were incubated with 10% normal goat serum (Zymed Laboratories, San Francisco, CA, U.S.A.) for 10 minutes to block nonspecific binding. Sections were then incubated overnight at 4°C with a 1:50 dilution of rabbit polyclonal anti-EGF antibody (Ab-3, Oncogene Science, Uniondale, NY, U.S.A.). Bound antibodies were detected using biotinylated goat anti-rabbit immunoglobulin (IgG) and, subsequently, using a streptavidin-peroxidase conjugate (Zymed Laboratories). Then, 0.02% diaminobenzidine hydrochloride containing 0.03% H_2O_2 was used as the chromogen to visualize the peroxidase activity. A brown precipitate seen within the cytoplasm confirmed the presence of EGF. The sections were lightly counterstained with hematoxylin and mounted with Permount (Merck, Darmstadt, Germany). Positive control for EGF antibodies was a specimen of oral squamous cell carcinoma known to express high levels of EGF. Negative control was obtained by omission of the primary antibodies. Both positive and negative controls were included in each staining process.

The EGF immunoreactivity in sections of salivary ACC was assessed by scanning the entire section and recorded as follows: negative (–); low grade expression (+) positive staining of 10% or less of cancer cells; medium grade expression (++), positive staining of 11% – 50% of cancer cells; high

grade expression (+++), positive staining of more than 50% of cancer cells. Each of these assessments was carried out by two independent investigators. In case of interobserver variation in the grade of EGF expression, the two oral pathologists reassessed the specimen by using a double-headed light microscope to achieve consensus. The EGF immunoreactions in sections of normal submandibular glands and degenerated labial glands adjacent to mucocele were judged as positive or negative based on the ductal and acinar cells by the same investigators.

The correlation between EGF expression in salivary ACCs and the clinicopathological parameters of the ACC patients was analyzed using Fisher's exact test. Cumulative survival of the ACC patients was analyzed using the Kaplan-Meier product-limit method. The duration of survival was measured from the beginning of treatment to the time of death or the last follow-up. Comparison of cumulative survival between EGF-positive and EGF-negative ACC patients was performed using the log-rank test. A p value of less than 0.05 was considered statistically significant.

III. Results

Positive control of oral squamous cell carcinoma showed strong EGF staining in the cytoplasm of the cancer cells. Methodological control with omission of the primary antibodies gave negative staining. In normal submandibular glands, immunohistochemically detectable EGF was demonstrated in all ductal segments, including intercalated, striated, and excretory duct cells. The positive staining for EGF was weak and evenly distributed in the cytoplasm of the duct cells. No EGF positive staining was found in acinar compartments, including serous and mucous acinar cells (Fig. 1).

In labial glands adjacent to mucocele, obstructive alterations, including acinar atrophy, ductal dilation and degeneration, and a mild to moderate lymphoplasma cell infiltrate in the interstitial connective tissue of the glands were found. No EGF staining was detected in the remaining acinar and ductal cells in the seven degenerated labial gland specimens adjacent to mucocele (Fig. 2).

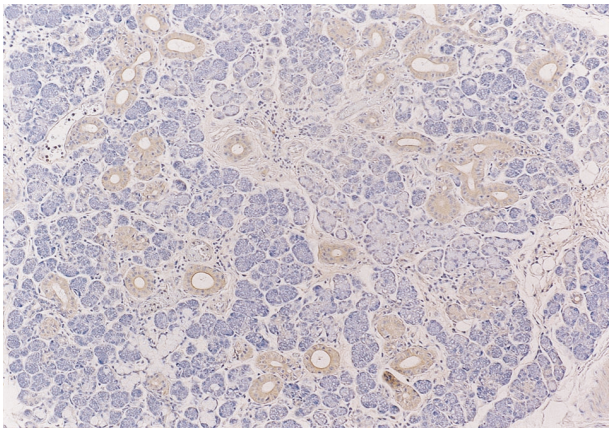

Fig. 1. All intercalated and striated duct cells show weak and diffuse EGF cytoplasmic staining, while the acinar compartments are negative in a normal submandibular gland (original magnification, ×90).

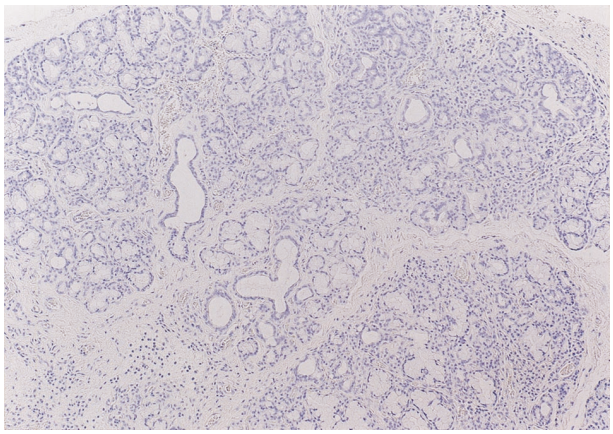

Fig. 2. No EGF staining in the remaining acinar and ductal cells of a degenerated labial gland adjacent to a mucocele (original magnification, ×90).

In this study, 40 salivary ACCs were divided into two groups: those with tubular and cribriform without the solid pattern and those with the predominantly solid pattern. Positive EGF immunostaining was observed in one of the 5 (20%) ACCs with the solid pattern and in 13 of the 35 (37.1%) ACCs with the tubular-cribriform pattern. The overall EGF expression rate in the 40 salivary ACCs was 35%. In the only EGF-positive ACC with the solid pattern, immunohistochemical detectable EGF reaction with weak to moderate intensity was found in some cells in the solid tumor nests. In the EGF-positive ACCs with the tubular pattern, the majority of the tubular structures showed cytoplasmic staining for EGF with various intensities from the strongest to the weakest (Fig. 3). In the EGF-positive ACCs with the cribriform pattern, small tubular structures in a large cribriform pattern or in the stroma showed strong cytoplasmic EGF staining. In addition, some of the tumor cells that formed pseudocysts in large cribriform patterns displayed a weak EGF reaction in their cytoplasm (Fig. 4).

The relationships between EGF expression in salivary ACCs and the clinicopathological parameters of the 40 ACC patients are shown in Table 1. Of the 40 patients with ACC, one had a tumor smaller than 2 cm (T1), six had tumors between 2 and 4 cm (T2), three had tumors larger than or equal to 4 cm (T3), and 26 had tumors that invaded adjacent structures (T4). There were six ACCs with regional lymph node metastases (N1 + N2) and 33 ACCs without regional lymph node metastases (N0). Distant metastases were found in six ACC patients (M1), while the remaining 34 patients did not have distant metastases (M0). Of the 40 ACC patients, there was one with stage I cancer, five with stage II cancer, four

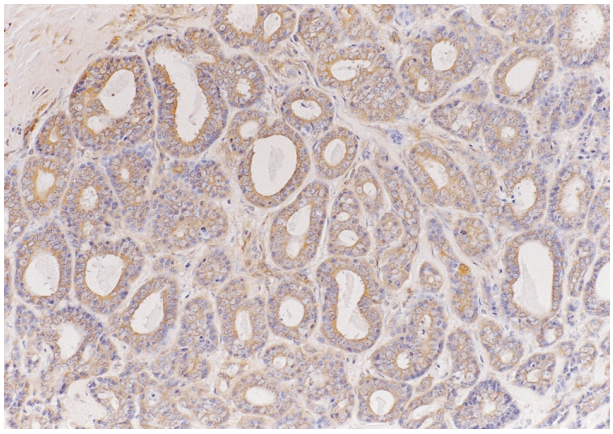

Fig. 3. Moderate cytoplasmic EGF staining in the majority of the ACC tubular structures (original magnification, ×180).

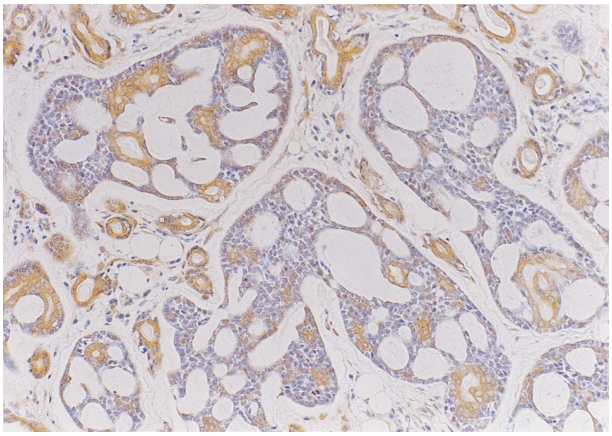

Fig. 4. Strong cytoplasmic EGF staining in small tubular structures both in the cribriform pattern and in the stroma of ACC. Slight cytoplasmic staining in some of the tumor cells forming pseudocysts (original magnification, ×180).

with stage III cancer, and 28 with stage IV cancer. Perivascular invasion was discovered in 32 (80%) ACCs, perineural invasion in 27 (67.5%) ACCs, focal necrosis of the tumor in 20 (50%) ACCs, and cellular atypia in 5 (12.5%) ACCs. Based on Fisher's exact test, no significant association was found between EGF expression in salivary ACCs and any of the clinicopathological parameters, including patient age and sex, cancer location, TNM status, clinical stage, histologic type, perivascular or perineural invasion, focal necrosis of tumor, and cellular atypia. In addition, Kaplan-Meier analysis showed that there was no significant difference in cumulative survival between the EGF-positive and EGF-negative ACC patients ($p > 0.05$).

Table 1. Association of EGF Expression in Salivary ACCs with Various Clinicopathological Parameters in 40 ACC Patients

	EGF staining in ACCs					
	–	+	++	+++	Total	*p* value[a]
Patient age (yr)						0.408
21 – 30	1	2	1	0	4	
31 – 40	3	0	1	1	5	
41 – 50	10	2	0	0	12	
51 – 60	5	1	2	1	9	
61 – 70	5	2	1	0	8	
71 – 80	2	0	0	0	2	
Patient sex						0.685
Male	14	4	1	1	20	
Female	12	3	4	1	20	
Cancer location						0.449
Major salivary glands	10	2	2	2	16	
Minor salivary glands	16	5	3	0	24	
T status[b]						0.496
T1	0	1	0	0	1	
T2	6	0	0	0	6	
T3	2	1	0	0	3	
T4	14	5	5	2	26	
N status[b]						0.631
N0	22	6	3	2	33	
N1	2	1	1	0	4	
N2	1	0	1	0	2	
M status						1.000
M0	22	6	4	2	34	
M1	4	1	1	0	6	
Clinical stage						0.703
Stage I	0	1	0	0	1	
Stage II	5	0	0	0	5	
Stage III	3	1	0	0	4	
Stage IV	16	5	5	2	28	
Histologic type						0.489
Solid	4	0	1	0	5	
Tubular-cribriform	22	7	4	2	35	
Perivascular invasion						0.478
With	22	5	4	1	32	
Without	4	2	1	1	8	
Perineural invasion						0.571
With	18	5	2	2	27	
Without	8	2	3	0	13	
Focal necrosis of tumor						0.349
With	14	2	2	2	20	
Without	12	5	3	0	20	
Cellular atypia						0.841
With	3	1	1	0	5	
Without	23	6	4	2	35	

Nomenclature: – : negative; + : low grade expression, positive staining of 10% or less of cancer cells; ++ : medium grade expression, positive staining of 11% – 50% of cancer cells; +++ : high grade expression, positive staining of more than 50% of cancer cells.

[a] Fisher's exact test

[b] The T status was unknown in four ACCs, and the N status was unknown in one ACC.

IV. Discussion

In this study, EGF positive staining was confined to intercalated, striated and excretory duct cells of the normal submandibular glands. Although EGF has been immunohistochemically identified in submandibular glands in previous

studies, the results are controversial; i.e., it has sometimes been found in duct cells (Elder *et al*., 1978; Mori *et al*., 1987; Tsukitani *et al*., 1987; Tatemoto *et al*., 1988; Kajikawa *et al*., 1991), while others have reported that it to be restricted to serous acinar cells (Heitz *et al*., 1978; Kasselberg *et al*., 1985; Poulson *et al*., 1986). The discrepancies between the results of these investigations may be related to differences in tissue fixation or to different antibodies used in these studies, as previously suggested by Tatemoto *et al*. (1988). Immunohistochemical localization of EGF in ductal segments or serous acinar compartments of the submandibular gland as well as the detection of EGF in the saliva (Starkey and Orth, 1977; Dailey *et al*., 1978; Hirata and Orth, 1979) suggest that the submandibular gland is a site of EGF synthesis and secretion. In this study, we could not detect any EGF positive staining in either acinar or duct cells in degenerated labial glands adjacent to mucocele. Mori *et al*. (1987) found that staining was reduced or absent for EGF in degenerated duct segments of salivary glands adjacent to and compressed by pleomorphic adenoma. Tatemoto *et al*. (1988) also demonstrated reduced levels or the total disappearance of immunohistochemically detectable EGF in degenerated duct cells of submandibular glands with obstructive sialadenitis; the decrease in staining intensity paralleled the degree of histological degeneration. Furthermore, duct ligation of mouse submandibular glands has been found to result in a marked reduction in mouse EGF staining intensity in granular convoluted tubular cells, even when male hormone is administered (Takai *et al*., 1985). The results of the present and previous studies suggest that the presence of EGF in the ductal segments of salivary glands may be related to the secretory phase in terminal acinar cells; thus, if the main excretory duct is obstructed, EGF synthesis may be reduced or completely absent.

Salivary ACC has been described as one of the most biologically destructive and unpredictable tumors of the head and neck (Conley and Dingman, 1974). In the present study, about 70% of ACC patients had stage IV cancer and T4 tumors. In addition, six of the 40 ACCs had regional lymph node metastases, and six of the 40 ACCs had distant metastases. Therefore, we suggest that although the cells of ACCs are innocent in appearance and have slow growth rates, local invasion of adjacent structures occurs frequently, and regional or distant metastases are not uncommonly found in salivary ACCs. In addition, the majority of the ACC patients had terminal stage (stage IV) cancer at the initial presentation to our clinic; this suggests delayed treatment of salivary ACC in our cases.

In the present study, we found an overall EGF expression rate of 35% in salivary ACCs. There was no significant difference in the EGF expression rate between solid and tubular-cribriform ACCs or between ACCs from major salivary glands and those from minor salivary glands. EGF positive staining was found predominantly in tubular structures in the tubular ACCs and in duct-like structures in the stroma or in large cribriform patterns of the cribriform ACCs. The tumor cells that formed pseudocysts in large cribriform patterns had only weak EGF staining. Immunolocalization of EGF in 32 cases of salivary ACC was studied by Mori *et al*. (1987). Although no quantitative data were provided, they reported a low incidence of EGF expression in their ACCs. Similar to our finding, the positive EGF staining was mainly noted in tubular structures in tubular-cribriform ACCs. The histogenesis of ACC has been strongly suggested to involve intercalated duct cells and myoepithelial cells (Kleinsasser *et al*., 1969) as is the case with pleomorphic adenoma. In normal salivary glands, immunohistochemical detectable EGF is predominantly demonstrated in duct cells. In pleomorphic adenoma, luminal surface cells of tubular and duct-like structures demonstrate a comparatively high concentration of EGF, and outer myoepithelial cells show either a weak EGF reaction or none (Mori *et al*., 1987; Tsukitani *et al*., 1987). Taking these findings together, we suggest that tubulo-ductal cells in salivary ACC may be of ductal origin, whereas weak or negative staining tumor cells may arise from myoepithelial cells. In addition, EGF is mainly biosynthesized in cells forming tubular or duct-like structures in tubular or cribriform salivary ACCs, and it may play a biologic role, particularly as a mitogen, in salivary ACC growth.

EGF initiates cellular response by binding to EGFR on the surface of target cells. To address whether salivary ACC uses a paracrine or autocrine mechanism to stimulate tumor growth, we need to further assess whether the tumor cells producing EGF also express EGFR on their cell surfaces and further study the distribution of EGFR on those EGF-negative tumor cells in salivary ACC. Such experiments are now being carried out in our laboratory to further clarify the interaction between EGF production and EGFR expression in salivary ACCs.

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