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Original

Expression of HER2, EGFR, CD44, PPARγ and AR in Salivary Cancer — Immunohistochemical Analysis Focusing on the Possibility of Specialized Molecular-targeted and Hormonal Therapy for Different Histological Subtypes —

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Abstract : The aim of this study was to determine the expression of human epidermal growth factor receptor type 2 (HER2), epidermal growth factor receptor (EGFR), peroxisome proliferator-activated receptor γ (PPARγ), CD44 and androgen receptor (AR) in adenoid cystic carcinomas (ACC), carcinoma ex pleomorphic adenomas (CXPA) and mucoepidermoid carcinomas (MEC) of the salivary glands, to investigate their molecular difference and to estimate the availability of molecular-targeted and hormonal therapy in salivary-gland tumors. Forthy patients with a salivary gland tumor, diagnosed and treated at our hospital, were studied. On the basis of histopathology, 10, 19 and 11 patients were identified with ACC, CXPA and MEC, respectively. The associations between histological types were evaluated by the chi-square test. Differences were considered statistically significant at $P < 0.05$. HER2-positive expression was observed in 10% of ACC, 84% of CXPA and 18% of MEC. EGFR-positive expression was observed in 40% of ACC, 68% of CXPA and 91% of MEC. CD44-positive expression was observed in 40% of ACC, 47% of CXPA and 91% of MEC. PPARγ-positive expression was observed in 10% of ACC, 53% of CXPA and 18% of MEC. AR-positive expression was observed in 20% of ACC, 32% of CXPA and 9% of MEC. Compared with other histological types, CXPA demonstrated significant HER2 and PPARγ staining and MEC demonstrated significant EGFR and CD44 staining. The differences in expression of markers between histological types in our study suggests the possibility that HER2- and PPARγ-targeted therapy may be effective in CXPA, and that EGFR-target therapy may be effective in MEC of the salivary glands.

Key words : salivary gland tumor, molecular-targeted therapy, hormonal therapy, immunohistology

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Introduction

Tumors of the salivary glands account for 5% of all neoplasms of the head and neck[1]. These tumors arise primarily in the parotid gland, occasionally in the submandibular and intraoral glands, and rarely in the sublingual glands[1]. Approximately 25% of tumors in the parotid gland and 50% of tumors in the other salivary glands are malignant[2]. There are various histological types of the malignant neoplasm of the salivary glands. These include adenoid cystic carcinomas (ACC), carcinoma ex pleomorphic adenomas (CXPA) and mucoepidermoid carcinomas (MEC), which account for approximately 5% of salivary gland neoplasms. Other histological types are uncommon.

Surgical resection is the primary treatment of neoplasms of the salivary gland at initial diagnosis. Radiation therapy is usually administered to patients with advanced disease, inadequate margins and those who exhibit poor prognostic features such as perineural invasion or anaplastic histology[3]. Chemotherapy has generally been reserved for patients with incurable salivary neoplasms. The typical treatment-response rate of patients with salivary gland tumors is 15－30%, and patients usually have a short survival period. The most active single agents include cisplatin, cyclophosphamide, doxorubicin and 5-fluorouracil. There is a definite need for additional therapeutic strategies to improve the survival and quality of life for these patients.

Molecular-targeted therapy and hormonal therapy are impacting positively on the daily practice of clinical oncology and are potential treatment strategies for this patient group. Tumor biomarker overexpression also has therapeutic implications. Several studies have demonstrated the *in vitro* and *in vivo* efficacy of some antibodies on human cancers. Those that are efficacious include human epidermal growth factor receptor type 2 (HER2) in breast cancer[4], epidermal growth factor receptor (EGFR) in colon cancer[5], CD44 in head and neck squamous cell carcinoma cells[6], peroxisome proliferator activated receptor γ (PPARγ) in bladder tumor cells[7], and androgen receptor (AR) in prostate cancer[8].

The purpose of the present study was to determine the expression of HER2, EGFR, PPARγ, CD44 and AR in ACC, CXPA and MEC of the salivary glands, to investigate the difference in their expression and to estimate the efficacy of molecular-targeted and hormonal therapy in salivary gland tumors.

Materials and Methods

Patients and samples

This study included 40 patients diagnosed and treated for a primary malignant salivary gland tumor at our hospital since 1990. All patients underwent surgical resection of the tumor. On the basis of histopathology, 10, 19 and 11 patients were identified with ACC, CXPA and MEC, respectively.

Immunohistological staining

For the immunohistochemical staining for HER2 (clone CB11; Ventana, Tucson, AZ, USA, diluted 1:100), EGFR (clone 3C6; Ventana, Tucson, USA, diluted 1:100), CD44 (clone G44-26; BD Bioscience, San Jose, California, USA, diluted 1:100), PPARγ (clone E-8; Santa Cruz Biotechnology, Santa Cruz, CA, USA, diluted 1:50) and AR (clone AR27; invitrogen, Flynn RD, Camarillo, CA, USA, diluted 1:200). 5-μm-thick sections from representative blocks of each tumor were used.

The labeled streptavidin-biotin-peroxidase technique was used. Sections were deparaffinized in xylene and dehydrated in descending grades (100-50%) of ethanol. For HER2, they were incubated with 1% hydrogen peroxide in ethanol for 30 minutes to quench endogenous peroxidase activity. For EGFR, CD44, PPARγ and AR, sections were incubated with Tris-EDTA buffer (pH 9.0) for 30 minutes. In each case, nonspecific immunoreactivity was blocked by incubation with normal donkey serum for 30 min. The sections were then incubated with primary antibody for 30 minutes. After washing with phosphate-buffered saline (PBS) three times, each for 5 min, the sections were incubated for 60 min with the multilink biotinylated anti-immunoglobulin. They were then washed with PBS three times, each for 5 min, before and after being treated with streptavidin-peroxidase reagent for 30 min. The reactions were visualized with diaminobenzidine (Dako) as a chromogen. All steps were followed by adequate washes in PBS. Finally, sections were counterstained with hematoxylin, dehydrated and mounted.

Scoring

The intensity of the immunoreactions (negative, positive) was assessed for each case. For HER2, EGFR and CD44, expression was considered positive only if distinct membranous immunoreactivity was present. The tumors in which immunoreactive tumor cells constituted >10% of the tumor were graded as positive[9, 10]. PPARγ expression was considered positive only if distinct cytoplasmic immunoreactivity was present. The tumors in which immunoreactive tumor cells constituted >20% of the tumor were graded as positive[11]. AR expression was considered positive only if distinct nuclear immunoreactivity was present. The tumors in which immunoreactive tumor cells constituted >10% of the tumor were graded as positive[12].

All slides were evaluated independently by at least two investigators.

Analysis

The associations between histological types were evaluated by the chi-square test. Differences were considered statistically significant at $P < 0.05$.

Results

HER2-positive expression was observed in 10% (1/10) of ACC, 84% (16/19) of CXPA

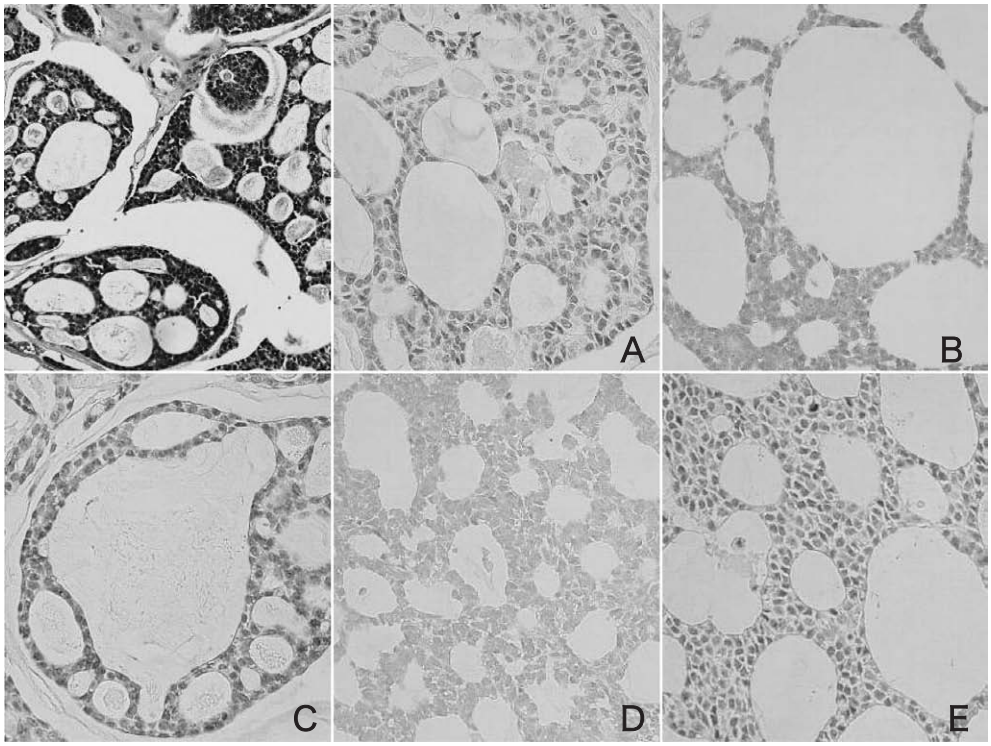

Fig. 1. Immunohistochemical expression of ACC
A : Membrane staining for HER2 was not expressed in ACC.
B : Membrane staining for EGFR was not expressed in ACC.
C : Membrane staining for CD44 was not expressed in ACC.
D : Cytoplasmic staining for PPARγ was not expressed in ACC.
E : Nuclei staining for AR was not expressed in ACC.

and 18% (2/11) of MEC (Figs. 1, 2, 3). There was a significant difference in HER2 expression between each histological type ($P < 0.05$) (Table 1). Moreover significant differences in HER2 expression between ACC and CXPA, and between CXPA and MEC were observed ($P < 0.05$) (Fig. 4).

EGFR-positive expression was observed in 40% (4/10) of ACC, 68% (13/19) of CXPA and 91 (10/11) of MEC (Figs 1, 2, 3). A significant difference in EGFR expression between each histological type was observed ($P < 0.05$) (Table 1). A significant difference in EGFR expression between ACC and MEC was also observed ($P < 0.05$) (Fig. 4).

CD44-positive expression was observed in 40% (4/10) of ACC, 47% (9/19) of CXPA and 91% (10/11) of MEC (Figs. 1, 2, 3). A significant difference in CD44 expression between each histological type was observed ($P < 0.05$) (Table 1). CD44 expression also differed between ACC and MEC, and between CXPA and MEC ($P < 0.05$) (Fig. 5).

PPARγ-positive expression was observed in 10% (1/10) of ACC, 53% (10/19) of CXPA and 18% (2/11) of MEC (Figs. 1, 2, 3). Significant differences in PPARγ expression

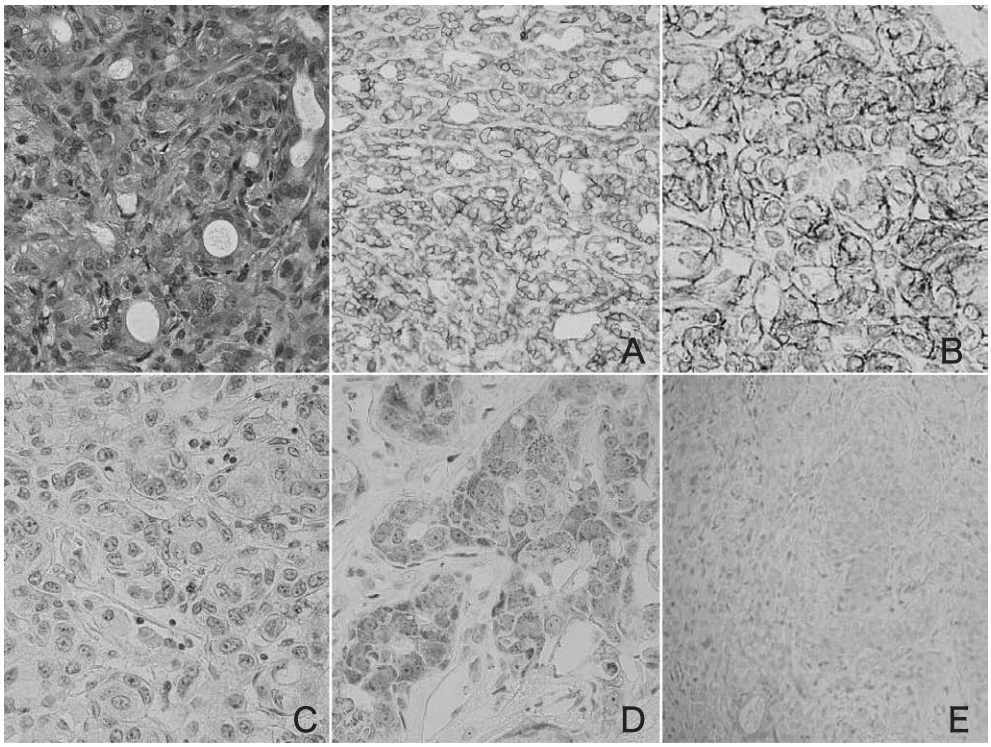

Fig. 2. Immunohistochemical expression of CXPA
A : Membrane staining for HER2 was expressed in CXPA.
B : Membrane staining for EGFR was expressed in CXPA.
C : Membrane staining for CD44 was not expressed in CXPA.
D : Cytoplasmic staining for PPAR γ was expressed in CXPA.
E : Nuclei staining for AR was not expressed in CXPA.

between each histological type ($P < 0.05$) (Table 1), and between ACC and CXPA were observed ($P < 0.05$) (Fig. 5).

AR-positive expression was observed in 20% (2/10) of ACC, 32% (6/19) of CXPA and 9% (1/11) of MEC (Figs. 1, 2, 3). No significant differences between each histological type were observed (Fig. 6, Table 1).

Discussion

Additional strategies are required to improve the survival and quality of life of patients with salivary gland tumors. In several other tumors, molecular targeted therapy with antibodies or antagonists and hormonal therapy are promising therapeutic strategies. Some reports indicate that the appearance rates of these markers are reflected in the therapeutic gain *in vitro* and *in vivo*.

In breast cancer patients, HER2-overexpressing tumors are responsive to trastuzumab both as a single agent and in combination with other chemotherapeutic agents[4]. Matsui *et*

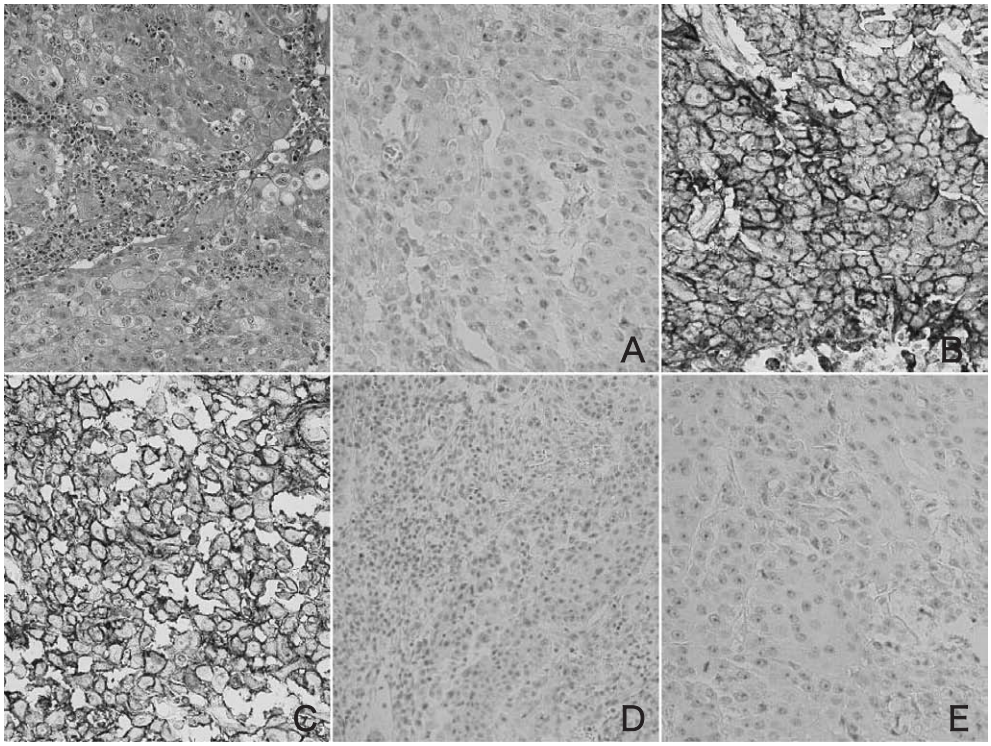

Fig. 3. Immunohistochemical expression of MEC
A : Membrane staining for HER2 was not expressed in MEC.
B : Membrane staining for EGFR was expressed in MEC.
C : Membrane staining for CD44 was expressed in MEC.
D : Cytoplasmic staining for PPARγ was not expressed in MEC.
E : Nuclei staining for AR was not expressed in MEC.

Table 1. Immunohistochemical expression of HER2, EGFR, CD44, PPARγ, AR and the difference between these expressions in each histological type

	HER2		EGFR		CD 44		PPARγ		AR	
	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*
ACC	1/10 (10)		4/10 (40)		4/10 (40)		1/10 (10)		2/10 (20)	
CXPA	16/19 (84)		13/19 (68)		9/19 (47)		10/19 (53)		6/19 (32)	
MEC	2/11 (18)	*	10/11 (91)	*	10/11 (91)	*	2/11 (18)	*	1/11 (9)	0.356

al[13] used four human gastric cancer cell lines with various expression levels of the HER2 protein to study the association between the expression of HER2 protein and sensitivity to trastuzumab. They concluded that trastuzumab suppressed the growth of human gastric cancer with HER2 overexpression *in vitro* and *in vivo* and improved survival of mice with peritoneal dissemination and gastric cancer ascites. In the present study, expression of

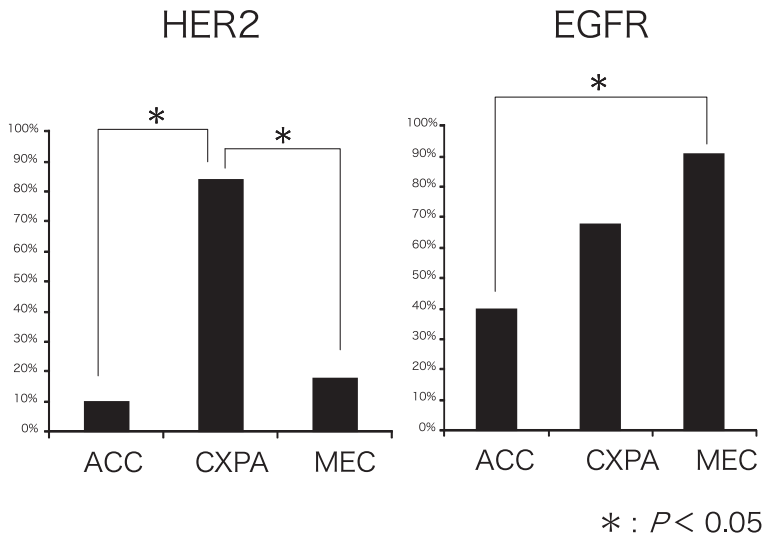

Fig. 4. HER2 expression : A significant difference between each histological type (P < 0.05) was observed, and significant differences between ACC and CXPA and between CXPA and MEC were observed (*P < 0.05).
EGFR expression : There was a significant difference between ACC and MEC (*P < 0.05).

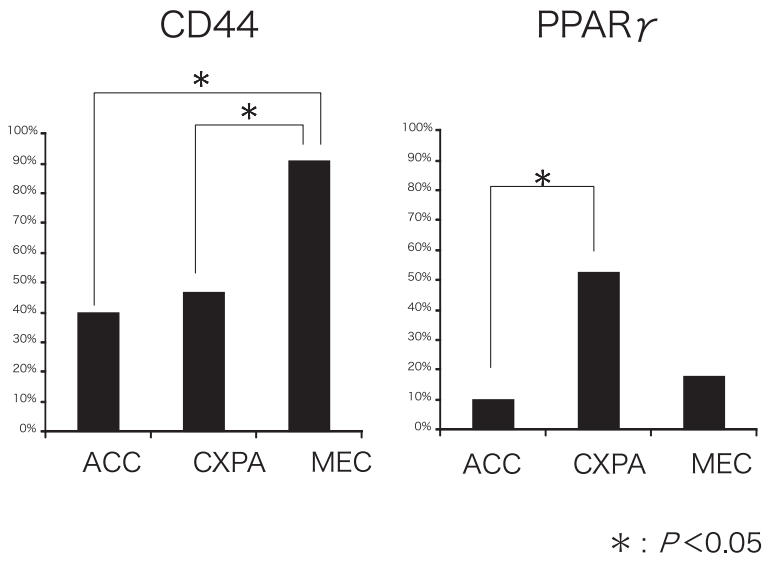

Fig. 5. CD44 expression : A significant difference between each histological type (P < 0.05) was observed, and significant differences between ACC and MEC and between CXPA and MEC (*P < 0.05) were observed.
PPARγ : Significant differences between each histological type (P < 0.05) and between ACC and CXPA (*P < 0.05) were observed.

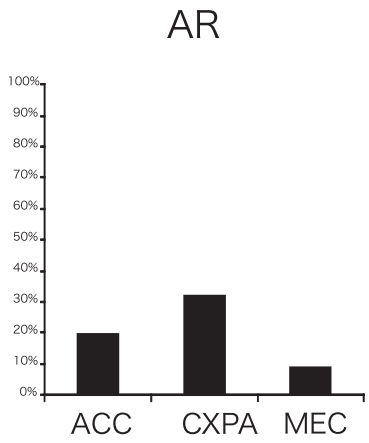

Fig. 6. AR expression: No significant difference between each histological type was observed.

HER2 was higher than the expression of other markers in CXPA suggesting that the HER2 receptor should be specifically targeted in the treatment of patients with CXPA.

A monoclonal antibody against EGFR (Cetuximab) has demonstrated clinical efficacy when used alone or in combination with other chemotherapeutic agents in patients harboring EGFR-positive tumors, including chemotherapy-refractory head and neck, and colorectal, cancer[14]. In addition, Bellone *et al*[15] reported that EGFR is highly expressed in primary and recurrent cervical tumors, and cetuximab appears to be a novel and attractive therapeutic strategy in patients harboring chemotherapy-resistant, recurrent or metastatic cervical cancers. In the present study, EGFR expression in MEC was higher than expression of other receptors. This suggests that specifically targeting this receptor would be effective in the treatment of patients with MEC.

CD44 is expressed in several tumor types. It is involved in several processes including tumor proliferation, adhesion and invasion, and its role in mediating tumor progression in a variety of solid tumors including head and neck squamous cell carcinoma (HNSCC) has been studied[16]. Wang *et al*[6] reported that CD44 contributes to HNSCC progression behaviors *in vitro* including tumor cell migration, proliferation and cisplatin response. Marangoni *et al*[18] reported that CD44-positive breast cancer cells inhibited tumor growth and tumor recurrence following chemotherapy. They demonstrated that CD44-positive cells play a key role in chemotherapy-resistant breast cancer recurrences, suggesting an innovative strategy to improve breast cancer treatment. In the present study, CD44 expression in MEC was higher than the expression of other receptors. Cisplatin is often used to treat patients with MEC. Thus, the expression of CD44 in MEC suggested that specifically targeting this receptor will be effective in the treatment of patients with MEC.

PPARγ is a member of a nuclear receptor family with diverse biological functions including mediation of adipocyte differentiation, regulation of monocyte–macrophage anti-

inflammatory activity and inhibition of cancer cell growth *in vitro* and *in vivo*[19]. PPARγ is upregulated in malignant tissues, and PPARγ ligands induce terminal differentiation in human breast, colon and lung cancer cells, and inhibit the growth of human breast, prostate, gastric and lung cancer cells[20, 21]. Chang *et al*[21] demonstrated that two distinct PPARγ ligands induce differentiation and apoptosis in nonsmall-cell lung cancer *in vitro*. Moreover, Inoue *et al*[22] found that the PPARγ agonist thiazolidinedione compounds inhibit the growth of renal cell carcinoma. In the present study, PPARγ expression in CXPA was higher than the expression of other receptors suggesting the possibility of specifically targeting this receptor in the treatment of patients with CXPA.

Androgens are required for the development and maintenance of normal prostate tissue and are also associated with carcinogenesis, tumor growth and progression in human prostate cancer (PCa)[23]. Barnes *et al*[8] demonstrated that patients with prostate carcinoma, which has a high frequency of AR expression, are successfully managed by anti-androgen hormonal treatment. In breast cancer, Agrawal *et al*[24] found that the therapeutic efficacy of adjuvant hormone therapy was higher in AR-positive patients than in AR-negative patients. The prognosis for AR-positive patients who underwent adjuvant hormone therapy was better than for those AR-positive patients who did not receive hormone therapy after primary radical surgery for breast cancer. In the present study, AR-expression levels were low in all three histological types; therefore, the efficacy of AR-targeted therapy for these tumors is yet to be established.

Recent studies have reported that the markers evaluated in our study are associated with carcinogenesis and tumor proliferation in salivary gland tumors, and the efficacy of targeted therapy for salivary gland tumors has been suggested[9, 11, 25, 26]. Haddad *et al*[25] reported the results of a phase II trial of trastuzumab in a cohort of patients with salivary gland tumors overexpressing HER2. One of the three patients with MEC had a partial response, which is still ongoing at 45 months. Locati *et al*[26] reported the results of a phase II trial using cetuximab in a cohort of patients with recurrent and/or metastatic salivary gland carcinomas (RMSGCs). EGFR overexpression was not statistically correlated with clinical benefit, but high EGFR expression was correlated with a prolonged disease stabilization in half of the cases. Shang *et al*[9] reported the results of HER2 and EGFR expression in 46 MEC and demonstrated that the frequency of HER2 overexpression in MEC was low, suggesting a limited role for HER2 in the targeted treatment of MEC. The high frequency of EGFT expression in MEC gave good reason to use EGFR-targeted agents for the treatment of these patients. The results of the present study and of others examining HER2 and EGFR expression consistently demonstrate high efficacy of EGFR and low efficacy of HER2 in MEC.

Franchi *et al*[27] examined the expression of CD44 in parotid-gland tumors. They found that the malignant area of CXPA exhibited a markedly decreased CD44 expression, suggesting a loss of CD44 expression associated with CXPA onset. Consistent with the findings of

Franchi *et al*, expression of CD44 in CXPA was low in the present study; therefore CD44 expression appears to correlate well with CXPA carcinogenesis.

The present study is the first to evaluate PPARγ expression in ACC, CXPA and MEC. Mukunyadzi *et al*[11] reported expression levels of PPARγ in 15 salivary duct carcinomas (SDC). They found no correlation between PPARγ expression and tumor stage, recurrence or survival, but that PPARγ is a potential target site for therapeutic manipulation.

Sugut *et al*[28] reported the expression levels of AR in four acinic cell carcinomas, seven adenocarcinomas not otherwise specified (NOS), 13 ACC, two CXPA, seven MEC and three SDC. They found that AR was expressed in three of four SDCs, two of seven adenocarcinomas NOS and one of two CXPA, but no immunoreactivity was observed in 13 ACC, seven MEC, or four acinic-cell carcinomas. Although there were only two CXPA in the present series, the results of our report were consistent with previous reports and suggest that the expression of AR in high-grade salivary gland tumors may be used in the clinical management of these neoplasms. However, Fan *et al*[29] examined the expression of AR in 13 SDC and observed strong immunostaining for AR in all cases. They suggested that antiandrogen therapy used in the treatment of prostatic carcinomas may also be beneficial in patients with SDC.

Previous reports have suggested the potential effectiveness of HER2-targeted therapy in patients with CXPA and of EGFR-targeted therapy in patients with MEC. Furthermore, the effectiveness of PPARγ in CXPA and CD44 in MEC has been speculated. By examining the expression of these markers it is possible to select those patients who would best respond to targeted therapies, and to identify markers associated with effective therapy. Concurrent therapy using combined targeted therapies and improved treatment regimens would result in the effective treatment of patients with salivary gland tumors.

In summary, we investigated the expression of HER2, EGFR, CD44, PPARγ and AR in salivary gland tumors. Differences in the expression of markers between the various histological types indicate the efficacy of targeted therapy of salivary gland tumors.

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