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Original contribution

Expression of amphiregulin in mucoepidermoid carcinoma of the major salivary glands: a molecular and clinicopathological study☆,☆☆

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Summary In mucoepidermoid carcinoma (MEC), *CRTC1-MAML2* fusion indicates a favorable prognosis. Amphiregulin (AREG), an epidermal growth factor receptor (EGFR) ligand, has been shown to be a downstream target of *CRTC1-MAML2* fusion, and to play a role in tumor growth and survival in *CRTC1-MAML2*–positive MEC cell lines. The aim of this study was to characterize the AREG and EGFR expression in the fusion-positive and fusion-negative MEC of the major salivary gland. The AREG and EGFR expression were studied by immunochemistry in 33 MEC cases of the major salivary glands. *CRTC1-MAML2* fusion was tested by reverse-transcription polymerase chain reaction (23 *CRTC1-MAML2* fusion-positive, 10 fusion-negative). Of 23 fusion-positive cases, AREG and EGFR overexpression were detected in 17 (73.9%) and 14 (60.9%) cases, respectively. Of 10 fusion-negative cases, AREG and EGFR overexpression were detected in 1 (10%) and 3 (30.0%) cases, respectively. There was a positive correlation between *CRTC1-MAML2* fusion and AREG overexpression ($P < .01$), but not between *CRTC1-MAML2* fusion and EGFR overexpression. The AREG overexpression was associated with a longer disease-free survival of the MEC patients ($P = .042$), but EGFR overexpression was not. In this study, we showed that AREG overexpression was detected more frequently in the *CRTC1-MAML2* fusion-positive tumors than in fusion-negative tumors. Detection of AREG expression may be useful for identifying *CRTC1-MAML2*–positive MECs and as a marker for favorable prognosis.

Abbreviations SGC, salivary gland carcinoma; MEC, mucoepidermoid carcinoma; CREB, cAMP-response element binding protein; RT–PCR, reverse-transcription polymerase chain reaction; AREG, amphiregulin; IHC, immunohistochemistry; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

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1. Introduction

Salivary gland carcinomas (SGCs) are rare neoplasms, which make up 6% of head and neck cancers [1], and among all SGCs, mucoepidermoid carcinoma (MEC) is the most common histological subtype [2]. The MEC is generally an indolent tumor, but some MEC cases present a poor prognosis. The pathological grade of the tumor may be a useful marker for tumor prognosis, but, unfortunately, a universally accepted grading system for MEC has not been fully established [3]. There are some grading systems available, for example, Armed Forces Institute of Pathology (AFIP) [4] and Brandwein [5] systems, and the tumor grade can differ depending on the grading system used. In addition, pathological grade does not always indicate a good prognosis in MEC [6], which suggests that a more objective prognostic marker is desirable.

Earlier studies indicated that the t(11;19) (q21;p13) gene fusion called *CRTC1-MAML2* is a characteristic and specific abnormality of MEC, found in 34% to 81% of all MEC cases [6-8]. *CRTC1-MAML2* fusion consists of a cAMP-response element binding protein (CREB)–regulated transcription coactivator 1 (*CRTC1*) fused to the Notch receptor coactivator encoded in *MAML2* [9-11]. The mechanism of tumorigenesis in fusion gene–positive MECs is considered to be either the Notch signaling originating from the *MAML2* part [12] or the CREB pathway originating from the *CRTC1* part [9,13].

The fusions are associated with good clinical course [7,8,14], indicating that they are a good prognostic factor, as reported by many studies. But it is difficult, from the standpoint of both technique and environment, to examine the fusion gene in general hospitals, because the reverse-transcription polymerase chain reaction (RT-PCR) or fluorescence in situ hybridization method is an essential part of the process. This once again suggests a need for a convenient prognostic marker.

In vitro, HeLa cells induced with *CRTC1-MAML2* showed statistically higher expressions of 156 genes. Among these, 3 of the top 5 genes were cAMP/CREB target genes: *PEPCK1/PCK1*, *AREG*, and *NR4A3/NOR1* [10]. Chen et al [15] reported that amphiregulin (AREG) is a target marker for *CRTC1-MAML2* because it is directly induced by ectopic *CRTC1-MAML2* expression and suppressed by its depletion in vivo and in vitro. Furthermore, AREG is also the ligand of epidermal growth factor receptor (EGFR), which might be a main tumorigenetic pathway of the fusion gene.

Although *CRTC1-MAML2* is a good prognostic factor, there have been no surrogate markers to date. From past reports, we assumed that AREG expression may relate to the fusion status. In this study we examined (1) the relationship of AREG expression with the fusion status, (2) the clinicopathological significance of AREG expression, and (3) the relationship of AREG expression and its receptor; EGFR expression. In this study, we examined 33 cases of MEC of salivary gland about expression of *CRTC1-MAML2*, AREG, and EGFR.

2. Materials and methods

2.1. Patients and tissue specimens

A total of 36 cases surgically treated and diagnosed as MEC of the salivary glands treated at the Department of Otolaryngology–Head and Neck Surgery, Kobe University Graduate School of Medicine and Hyogo Cancer Center between 1994 and 2013 were reviewed. Three patients were excluded from the present study, because those tumor specimens were absent of mucin production, leaving 33 patients. All these cases were examined for p63 and androgen receptor expression by immunohistochemistry (IHC) and were finally diagnosed as MEC. All tumors originated in the parotid glands or submandibular glands, and all tumor samples were formalin-fixed and paraffin-embedded. The slides from the cases included in the study were reviewed by dedicated pathologists (Y.I., H.I.). Pathological grades of the MECs were classified according to the AFIP [4] and Brandwein [5] grading systems. This study was approved by the Institutional Review Board of Kobe University Graduate School of Medicine (No.1531), Nagoya City University, and Hyogo Cancer Center. This study was also conducted in accordance with the Declaration of Helsinki. Clinical and pathological data were collected from patients' medical records.

2.2. Reverse-transcription polymerase chain reaction analysis for the *CRTC1/3-MAML2* fusion transcripts

CRTC1-MAML2 and *CRTC3-MAML2* fusion transcripts were detected using a method consisting of one-tube RT-PCR and nested PCR [16]. Total RNA was extracted from formalin-fixed, paraffin-embedded specimens as previously described. Tissue sections were deparaffinized and incubated at 56°C overnight in a protease K digestion buffer. The RNA was extracted using concentrated phenol/guanidine isothiocyanate (Trizol LS; Gibco BRL, Friendswood, TX) followed by DNase I treatment (Takara, Otsu, Japan). Five microliters of the extracted RNA were heated to 70°C and placed on ice. Then, an RT-PCR mixture including outer primers was added. The thermocycler was programmed for an initial incubation of 30 minutes at 42°C and 10 minutes at 95°C to inactivate reverse transcriptase as well as to activate DNA polymerase. This was followed by 50 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. The products were diluted 1:20 with water, and subjected to a nested PCR with inner primers consisting of 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Primers used in the study are shown in Table 1. The PCR fragments were directly sequenced. As an internal control for RNA quality, the ubiquitously expressed ACTB mRNA fragment (190 bp) was amplified. The MECs known to possess the gene fusions and normal salivary gland tissue were used as positive and negative controls.