# Establishment of a patient-derived mucoepidermoid carcinoma cell line with the *CRTC1-MAML2* fusion gene

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**Abstract.** Mucoepidermoid carcinoma (MEC) is the most common malignant tumor of the major and minor salivary glands. Surgical resection is the only curative treatment and there is no effective post-operative therapy for MEC. The present study reports an Institutional Review Board-approved case of a 45-year-old Japanese female diagnosed with low-grade MEC in the hard palate. Radical resection, supraomohyoid neck dissection and antero-lateral thigh flap reconstruction was performed. A MEC cell line was then established from the resected tumor tissue. Short tandem repeat profiling confirmed the origin and authenticity of the cell line, that harbors a *CRTC1-MAML2* translocation, which is frequently observed in MEC. Amphiregulin (AREG), identified as one of the targets of the *CRTC1-MAML2* fusion gene, was expressed in the cell line. The AREG receptor, epidermal growth factor receptor (EGFR) was also highly phosphorylated. The results predicted that AREG-EGFR signaling, which is required for tumor growth and survival, might be activated in the cell line in a cell-autonomous manner. As AREG expression is associated with EGFR-targeted drug resistance, this cell line might assist with the identification of novel strategies for MEC treatment.

## Introduction

Mucoepidermoid carcinoma (MEC), representing 5% of all salivary gland tumors and 26% of malignant salivary gland tumors registered for the last 39 years in Hiroshima, Japan, is the most common malignant tumor of the major and minor salivary glands (1,2). MEC is characterized by its cellular heterogeneity and consists of mucin-producing, epidermoid and intermediate cells. Clinical and pathological parameters (age, tumor size, presence of cervical lymphadenopathy, distant spread, perineural invasion and histological grade) of MEC have been associated with tumor biological behavior and patient management (3). Pathological classification of MEC is graded as low-, intermediate- or high-grade based on adverse features, such as perineural invasion, angiolymphatic invasion, coagulative necrosis, infiltrative growth, high mitotic rate, anaplasia and cystic components of <20% (4).

An important genetic abnormality in MEC is the translocation between chromosomes 11q and 19p, which has been hypothesized to be an early event in the pathogenesis of MEC (5,6), and has been reported in >50% of MEC tumors (7). Low-grade tumors have a higher incidence rate of this fusion compared with that in high-grade tumors (8) and patients with fusion-positive cancer tend to have improved survival time, with significantly lower risks of recurrence, metastases or cancer-related mortality (9). The majority of fusion genes in MEC are associated with a specific chromosomal t(11;19)(q14-21;p12-13) translocation that joins exon 1 of the cAMP response element-binding (CREB) protein-binding domain of CREB-regulated transcription coactivator 1 (*CRTC1)* gene to exons 2-5 of the Notch coactivator mastermind-like gene 2 (*MAML2)* gene, resulting in the expression of a new *CRTC1-MAML2* fusion gene (10). This translocation generates a fusion protein comprised of *CRTC1* (also called *MECT1*, *TORC1* or *WAMP1*) at 19q21 and the C-terminal transcriptional activation domain of *MAML2* at 11q21 (11-14). Previous analysis suggested that another member of the *CRTC* family, at 15q26, *CRTC3*, also fused with *MAML2* (15). Okabe *et al* (16) and Nakayama *et al* (17) showed that *CRTC1-MAML2* or *CRTC3-MAML2* fusions occurred in 40-80% of primary salivary gland MECs, and was associated with a distinct tumor subset that had favorable clinicopathological features and an indolent clinical course.

Previously, amphiregulin (AREG), a member of the epidermal growth factor (EGF) family, was identified as a target of the *CRTC1-MAML2* fusion gene and secreted AREG was shown to activate EGF receptor (EGFR) signaling in an

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*Abbreviations:* MEC, mucoepidermoid carcinoma; AREG, amphiregulin; EGFR, epidermal growth factor receptor

*Key words:* patient-derived cell line, mucoepidermoid carcinoma, salivary gland carcinomas, *CRTC1-MAML2*, fusion gene

autocrine manner (18). Furthermore, mutations in *EGFR* itself are rare in salivary gland carcinomas (19), while copy number alternations in *EGFR* are frequently found in high-grade MEC, regardless of fusion gene positivity (20). The molecular pathology and oncology of MEC are still poorly understood. Established authentic cell lines are essential to determine the biological characteristics of MEC, and a number of cell cultures and models have emerged; however, the cell line usability is limited (21). The present study reports the establishment of a MEC cell line (HCM-MEC010) carrying the *CRTC1-MAML2* fusion gene and activated EGFR. The potential uses for this cell line will also be discussed to understand the biological characteristics of MEC.

## Materials and methods

*Cell line generation and cell culture*. A patient with MEC provided consent in accordance with Hyogo College of Medicine (Hyogo, Japan) institutional policies. Tumor samples were obtained according to an approved Institutional Review Board protocol of Hyogo College of Medicine (approval no. 276; Hyogo, Japan). The present study was also conducted in accordance with the Declaration of Helsinki. Clinical and pathological data were collected from the medical records of the patient. Tumor tissues were minced into 1-2-mm pieces with a disposable scalpel and placed in primary culture. To separate the stromal cells from the mass culture, a magnetic-activated cell sorting (MACS) system was used. Briefly, MACS buffer, containing 1X PBS, 0.5% BSA, 2 mM EDTA (pH 7.2) (cat. no. 130-042-901; Miltenyi Biotec Inc.), was pre-cooled to 4˚C. To remove the fibroblasts, the single cell suspension was centrifuged at 300 x g for 10 min at room temperature. and positive selection was performed using CD326 (EpCAM) MicroBeads and a MidiMACSTM Separator (Miltenyi Biotic GmbH), according to the manufacturer's instructions. The obtained primary human MEC cells were seeded in F-medium (22) with 10 $\mu$M Y-27632 (FUJIFILM Wako Pure Chemical Corporation). After 1 week, the culture medium was replaced with fresh medium, which was changed every 4 days thereafter. At the same time, the fibroblasts derived from the tumor tissue of the same patient, were obtained and grown in F-medium. Once cells reached confluence (80%), they were washed with PBS ($Mg^{2+}$ and $Ca^{2+}$ free) (23) and detached with 0.05% EDTA/trypsin for 5 min at 38˚C (24). After centrifugation at 167 x g for 5 min at 4˚C, the MEC cells were resuspended in F-medium, containing Y-27632 and seeded ($0.3x10^6$ cells) in 60 mm dishes. An epithelial cell line was successfully established from the sample of the patient and was termed HCM-MEC010. The morphology of the exponentially proliferating cells in a monolayer was reviewed and documented using inverted phase contrast microscopy. The cells were also tested for mycoplasma infection using the MycoAlert® Assay (Lonza Group, Ltd.) and the cell culture growth medium and with fluorescent microscopy using the Mycoplasma Hoechst Stain Assay (MP Biomedicals, LLC).

*Short tandem repeat (STR) authentication of the MEC cell line*. To verify the identity of the cell line, genomic DNA was extracted from the blood of the patient, whose tumor sample was used to generate the HCM-MEC010 cell line, as well as from the cell line using the QIAamp DNA Mini kit (Qiagen, Inc.) according to the manufacturer's protocol. DNA genotyping using STR profiling was performed using the GenePrint 10 System (Promega Corporation) and the Applied Biosystems 3130x1 Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) and analyzed by BEX Co., Ltd. The evaluation value (EV) was determined using the following equation: EV=(number of coincidental peaks) x 2/total number of peaks in cell A and total number of peaks in cell B.

*Reverse transcription (RT)-PCR of the CRTC1-MAML2 fusion oncogene*. The HCM-MEC010 cell line was plated in 100-mm dishes and cultured to 90% confluence. RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and RT-PCR was performed using the PrimeScript RT-PCR kit (Takara Bio, Inc.) according to the manufacturer's instructions. The following primers were used: CRTC1 forward 1, 5'-TTC GAGGAGGTCATGAAGGA-3' and 2, 5'-ATGGCGACT TCGAACAATCCGCGGAA-3'; MAML2 reverse 1, 5'-TTG CTGTTGGCAGGAGATAG-3' and 2, 5'-GGGTCGCTTGCT GTTGGCAGGAG-3' (18), which amplified 101 and 194 bp fragments, respectively. Amplification of the *GAPDH* gene (forward, 5'-CAATGACCCCTTCATTGACC-3' and reverse, 5'-GACAAGCTTCCCGTTCTCAG-3') was performed as a control. Successfully amplified RT-PCR products of the *CRTC1-MAML2* fusion gene were purified and sequenced (24) using BigDye™ Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Inc.) and 2% agarose gel electrophoresis.

*Western blot analysis*. The culture medium was removed and the cells were washed with PBS ($Mg^{2+}$ and $Ca^{2+}$ free). RIPA buffer was added (cat. no. sc-24948; Santa Cruz, Inc.) and the cells were incubated at 4˚C for 60 min, then centrifuged at 12,000 x g for 20 min 4˚C. The supernatant was the total cell lysate. Proteins were extracted from the HCM-MEC010 and human tongue squamous cell carcinoma (SAS; purchased from the Japanese Collection of Research Bioresources Cell Bank) cell lines as previously described (25). Protein concentration was measured using a Bradford assay (26) Western blot analysis was performed as previously described (25). The primary and secondary antibodies are listed in Table I. The protein expression ratio, compared with that in SAS cells, was measured using ImageJ v1.53e software (National Institutes of Health). The data are presented as the mean ± SD. The experiment was repeated three times.

*Immunofluorescence staining*. The cultured HCM-MEC010 and SAS cell lines were fixed in 3.7% formaldehyde for 20 min at room temperature. After permeabilization with 0.2% Triton-X/PBS for 5 min at room temperature, the cells were blocked with 2% (w/v) BSA (Nacalai Tesque, Inc.)/PBS, then washed with PBS ($Mg^{2+}$ and $Ca^{2+}$ free) and incubated with the primary antibodies overnight at 4˚C. The cells were washed with PBS ($Mg^{2+}$ and $Ca^{2+}$ free), then incubated with the secondary antibody and Rhodamine phalloidin (Cytoskeleton, Inc.) for 2 h at room temperature. The samples were mounted in Vecta shield containing DAPI (Vector Laboratories). Fluorescent images were captured using a

Table I. Primary and secondary antibodies used for western blot analysis and immunofluorescence.

A, Primary antibodies

| Name | Cat. no. | Dilution | | Supplier |
|---|---|---|---|---|
| | | Western blot | Immunofluorescence | |
| Rabbit monoclonal anti-EGFR | 4267 | 1/1000 | 1/60 | CST |
| Rabbit monoclonal anti-p-EGFR | 3777 | 1/1000 | | CST |
| Rabbit monoclonal anti-AKT | 4691 | 1/1000 | | CST |
| Rabbit monoclonal anti-p-AKT | 4060 | 1/1000 | | CST |
| Rabbit monoclonal anti-AREG | 16036-1-AP | 1/1000 | | ProteinTech Group, Inc. |
| Rabbit monoclonal anti-E-cadherin | 3195 | 1/1000 | 1/100 | CST |
| Rabbit monoclonal anti-N-cadherin | 13116 | 1/1000 | 1/100 | CST |
| Rabbit monoclonal anti-vimentin | 5741 | 1/1000 | 1/100 | CST |
| Rabbit monoclonal anti-tubulin | 2148 | 1/1000 | | CST |
| Mouse monoclonal anti-actin | 47778 | 1/1000 | | |

B, Secondary antibodies

| Name | Cat. no. | Dilution | | Supplier |
|---|---|---|---|---|
| | | Western blot | Immunofluorescence | |
| Alexa Flur 488 goat anti-rabbit IgG (H+L) | A-11008 | | 1/400 | Molecular Probes; Thermo Fisher Scientific, Inc. |
| Anti-mouse IgG, HRP-linked | 7076 | 1/1000 | | CST |
| Anti-IgG (H+L chain) rabbit pAb-HRP | 458 | 1/10000 | | Molecular and Biological Laboratories Co., Ltd. |
| Goat anti-mouse HRP | ab97023 | 1/1000 | | Abcam |

CST, Cell Signaling Technology, Inc.; p, phosphorylated.

confocal laser-scanning microscope (LSM780; Zeiss AG). The primary and secondary antibodies are listed in Table I.

*RNA analysis*. RNA-Sequencing (RNA-Seq) libraries were generated using RNA extracted from the HCM-MEC010 cell line, as previously described (27), with the TruSeq Stranded mRNA Library Prep kit for Illumina, Inc., following the manufacturer's instructions, then sequenced on a NovaSeq 6000 System (Illumina, Inc.). The analysis was performed by Takara Bio, Inc.

*Hematoxylin and eosin-staining*. A section of the hard palate was fixed in 10% formalin solution at room temperature for 24 h and embedded in paraffin. Sections (5-$\mu$m thick) were cut from the paraffin blocks and stained with hematoxylin (0.09%) for 5 min and eosin (0.13%) for 9 min at room temperature according to standard methods (28). The images were captured using a light microscope (BX51; Olympus Corporation).

*Patient*. A 45-year-old Japanese female noticed spontaneous dull pain and swelling in her hard palate for 1 month and was referred to Hyogo College of Medicine, Nishinomiya, Hyogo, Japan on January, 2019. On examination, diffuse swelling was observed in the right hard palate. There was no trismus. The surface of the mass was smooth and was soft on palpation (Fig. 1A). Bilateral cervical lymph nodes were palpable, but painless and mobile. Magnetic resonance imaging showed an irregular mass measuring 30x20x18 mm in the right hard palate, and resorption in the nasal septum and posterior wall of the maxillary sinus (Fig. 1B). The clinical diagnosis was a malignant tumor of the hard palate. A biopsy was performed intraorally and the lesion was pathologically diagnosed as low-grade MEC using Armed Forces Institute of Pathology (29).

## Results

The patient was admitted to Hyogo College of Medicine, Nishinomiya, Hyogo, Japan and treated by partial resection of the hard palate, supraomohyoid neck dissection and reconstruction using an anterolateral thigh flap under general anesthesia. Hematoxylin and eosin-stained tumor tissue microscopically showed an overlying stratified squamous epithelium, mucous cells and squamous cells that were polygonal-to-ovoid in shape with eosinophilic cytoplasms (Fig. 1C). The mucous cells were cuboidal or goblet-like and tended to line the cysts. The

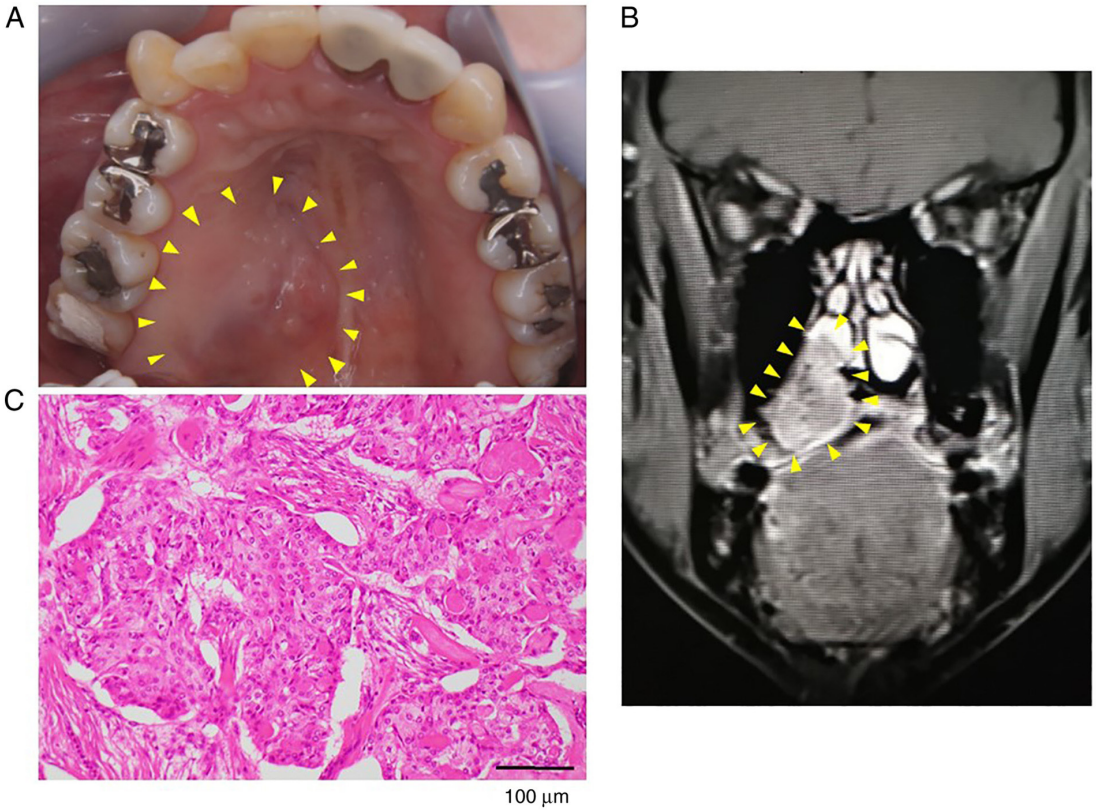


Figure 1. Clinical findings. (A) Normal-colored volumetric tissue with a smooth surface ~30x20 mm in diameter located on the right side of the hard palate (yellow arrowheads). (B) Magnetic resonance imaging showing an irregular mass measuring 30x20x18 mm in the right hard palate and resorption in the nasal septum and posterior wall of the maxillary sinus (yellow arrowheads). (C) Microscopic view showing epidermal cells with very few mucous cells and minimal cystic changes suggestive of mucoepidermoid carcinoma with hematoxylin and eosin staining.

squamous cells formed solid sheets. The tumor was diagnosed as mucoepidermoid carcinoma, low-grade type, pT4aN0M0 MEC of the hard palate. All dissected cervical lymph nodes showed no metastatic cells. At the 30-month follow up, the patient's prognosis was excellent and she had maintained a disease-free status.

*Establishment of a MEC cell line from a patient tumor.* A new MEC cell line, termed HCM-MEC010 was established, which maintained a cobblestone epithelial-like morphology for at least 30 passages (Fig. 2A and B). To confirm that the HCM-MEC010 cell line was derived from the tumor sample of the patient, STR profiling was performed using the DNA extracted from the high-passage HCM-MEC010 cell line and the blood from the patient. Genotypic analysis confirmed that the cell line was derived from the tumor and no contamination with other cell types was detected (EV, 1.0). (Table SI; Figs. S1 and S2).

*RT-PCR analysis reveals that HCM-MEC010 cells express the CRTC1-MAML2 fusion gene.* As the *CRTC1-MAML2* gene fusion is common in MEC (9), the fusion event was analyzed in the HCM-MEC010 cell line using RT-PCR. Fig. 3A shows the translocation event between chromosomes 11 and 19, while Fig. 3B shows the RT-PCR amplified fragments (lane 1, 101 bp and lane 2, 196 bp) using primer sets 1 or 2, respectively. The fusion transcript of *CRTC1* and *MAML2* genes was confirmed using Sanger sequencing (Fig. 3C). This revealed the fusion products of *CRTC1* exon 1 and *MAML2* exon 2 with the predicted splicing event, indicating that a translocation event had occurred between the first introns of *CRTC1* and *MAML2*.

*Protein expression in the HCM-MEC010 cell line.* Next, the protein expression of the epithelial and mesenchymal markers in the HCM-MEC010 cell line was confirmed using immunofluorescent staining. EGFR and E-cadherin were expressed on the cell membrane in the HCM-MEC010 cells, while N-cadherin expression was only faintly detected. Vimentin expression was also detected in HCM-MEC010 cells (Fig. 4).

*HCM-MEC010 cells express AREG and show EGFR activation.* As the AREG-EGFR signaling cascade has been identified as a *CRTC1-MAML2* fusion gene target (18), AREG expression and the status of the EGFR cascade was analyzed in the HCM-MEC010 cell line. The human tongue SAS cell line was used as a comparison as the SAS cell line contains a mutation in the *HER4* gene, which encodes one of the other types of human EGFR, and the authentic *EGFR* pathway is not involved in cell proliferation (30). EGFR was expressed in both cell types, but the AREG expression level was much higher in the HCM-MEC010 cell line compared with that in the SAS cell line (Fig. 5). Furthermore, EGFR was phosphorylated (p) in the HCM-MEC010 cell line compared with that in the SAS cell line, indicating the activation of the EGFR pathway. In addition, the expression level of AKT and p-AKT was lower in the HCM-MEC010 cell line

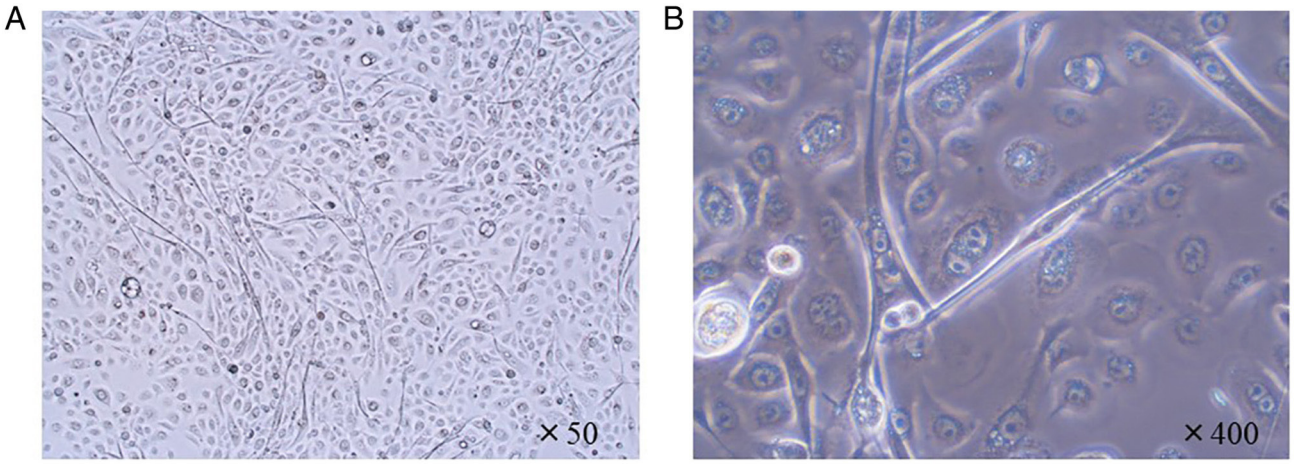


Figure 2. Morphology of the established HCM-MEC010 cells. Microscopic findings of the MEC cell line under (A) low power and (B) high power. MEC, mucoepidermoid carcinoma.

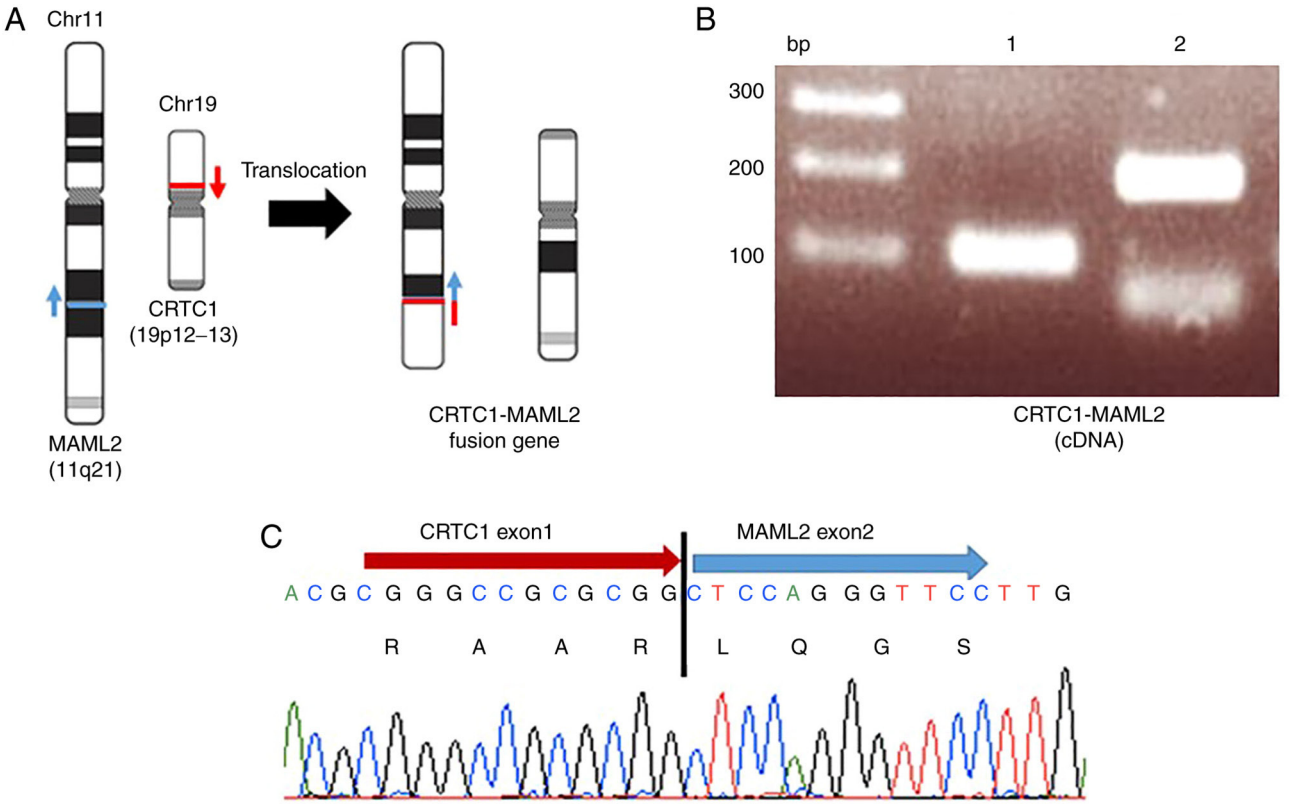


Figure 3. Gene structure of the identified *CRTC1-MAML2* fusion gene. (A) Schematic of *CRTC1-MAML2* gene fusion. (B) Reverse transcription-PCR analysis showing the presence of a *CRTC1-MAML2* fusion transcript. A 101 bp fragment (lane 1) and 194 bp fragment (lane 2) can be seen. (C) Direct sequencing of the *CRTC1-MAML2* fusion gene in the mucoepidermoid carcinoma cell line. *CRTC1*, CREB-regulated transcription coactivator 1; *MAML2*, Notch coactivator mastermind-like gene 2.

compared with that in the SAS cell line. In the SAS cell line, AKT can be phosphorylated by both the AREG-EGFR and HER4 pathways (31,32), and high levels of AKT phosphorylation in the SAS cell line must represent an additive effect of HER4 pathway activation (33). E-cadherin was expressed at higher levels in the HCM-MEC010 cell line compared with that in the SAS cell line. Vimentin expression was detected in small amounts in both the HCM-MEC010 and SAS cell lines (Fig. 5).

*RNA-seq analysis of the HCM-MEC010 cell line revealed epidermoid characteristics*. To further characterize the HCM-MEC010 cell line, RNA-Seq analysis was performed. MEC is known to be composed of a mixture of mucous, epidermoid, and intermediate cells (34). RNA-Seq analysis revealed the high expression level of genes in the keratin family, including *KRT5*, *KRT14*, *KRT6A*, *KRT17*, and *KRT7*. Table II lists the top 200 expressed genes. However, expression of the mucous cell marker *MUC* was not detected. These

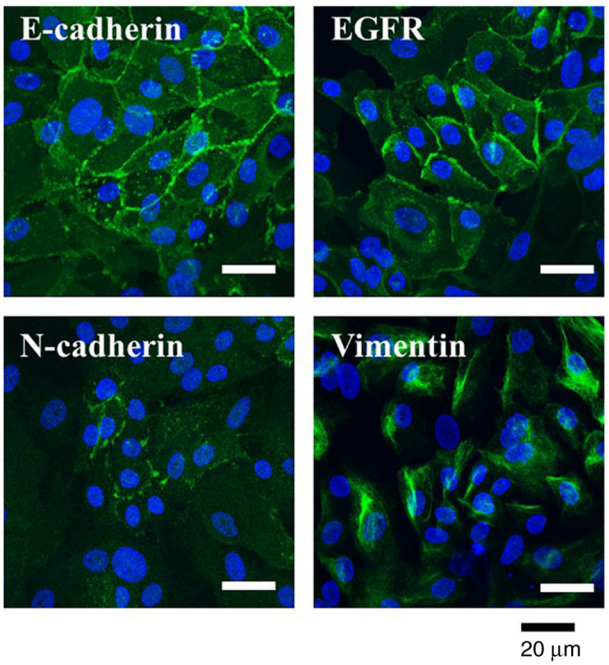


Figure 4. Immunofluorescence staining analysis in the HCM-MEC010 cell line. E-cadherin and EGFR were expressed at high levels. Low expression of N-cadherin and vimentin were observed. EGFR, epidermal growth factor receptor.

results, together with the cell morphology results, suggest that the HCM-MEC010 cell line is considered to be of epidermoid, but not mucinous, origin.

## Discussion

The isolation of primary tumor cells from patient samples is the first step for several genetic, biochemical and pharmacological experiments relevant to personalized cancer treatment (35). However, such studies are limited due to cell availability. The establishment of a cancer cell line is a traditional, but still powerful and informative method of studying human cancer. The present study reports the establishment of a MEC cell line with a *CRTC1-MAML2* fusion gene.

Several studies have shown that the presence of the *CRTC1/3-MAML2* fusion gene confers an improved prognosis, with improved disease-free survival and fewer distant metastasis in MEC (36,37). There are rare exceptions to this rule, including fusion-positive high-grade MEC with multiple additional genetic variations, such as mutations in *CDKN2A*, that have been associated with a poor prognosis (38).

The function of the *CRTC1-MAML2* fusion gene has been intensively studied. Its transformation ability was identified using the RK3E cell line (39) and its importance for tumor state maintenance has also been demonstrated. Initially, it was hypothesized to cause tumor growth by the constitutive activation of Notch signaling via the *MAML2* gene portion. Furthermore, the N terminus CRTC1 domain-mediated aberrant activation of cAMP/CREB signaling has also been identified as a cause of tumor formation (14,40). The interaction between AP-1 and MYC oncoprotein with CRTC1–MAML2 fusion proteins has been reported (41), suggesting that the *CRTC1-MAML2* fusion gene regulates several different

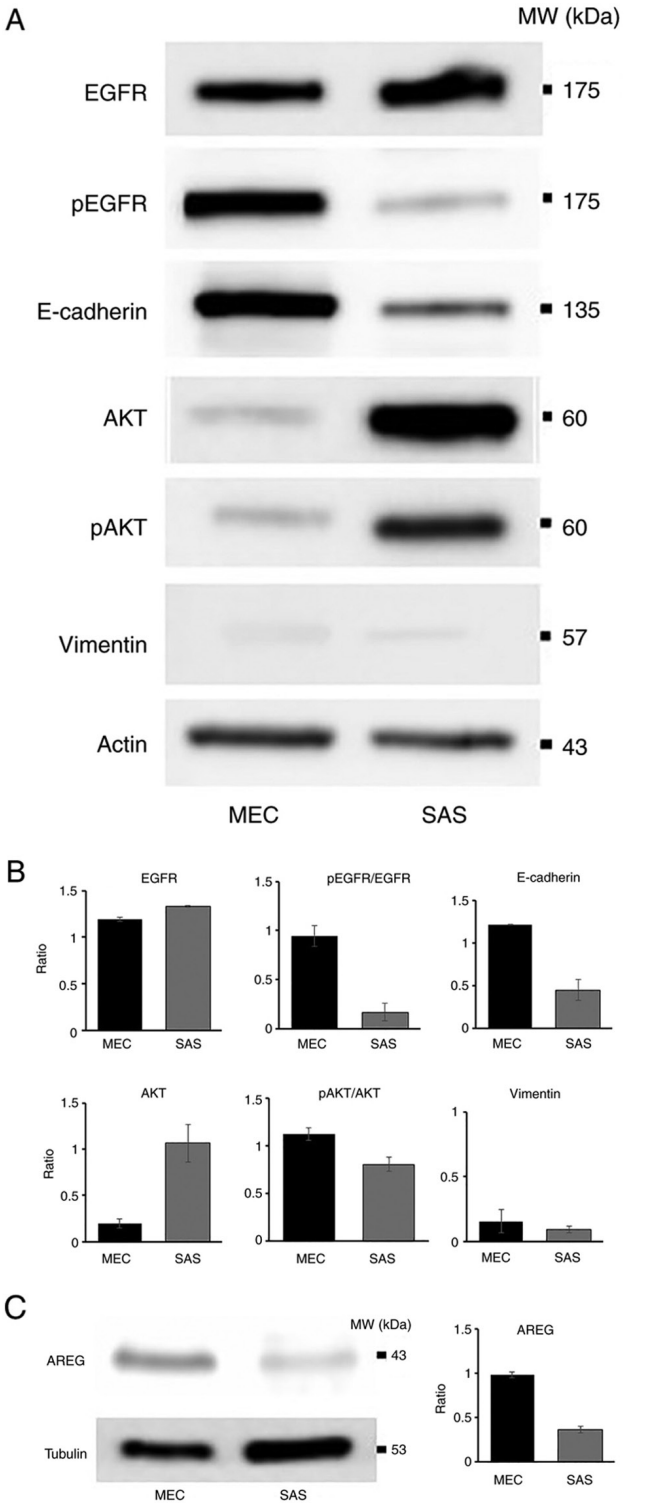


Figure 5. Western blot analysis of EGFR-AKT signaling in the HCM-MEC010 and SAS cell lines. (A) Western blot analysis of EGFR, p-EGFR, E-cadherin, AKT, p-AKT, vimentin and actin and the results were (B) analyzed using densitometry. (C) Western blot analysis of AREG and tubulin. MEC, muco-epidermoid carcinoma; p, phosphorylated.

signaling pathways. *AREG* is a known cAMP/CREB-regulated gene, whose expression positively correlates with that of *CRTC1-MAML2* in MEC (42). As AREG-EGFR signaling was identified as one of the *CRTC1-MAML2* fusion gene targets, EGFR signaling could represent the mechanism of action by which the fusion gene promotes carcinogenesis.

Table II. RNA-Sequencing data for the MEC cell line.

| Entrez gene ID | Gene symbol | Description | TPM |
|---|---|---|---|
| 6280 | S100A9 | S100 calcium binding protein A9 | 25012.1582 |
| - | RNR2 | - | 19355.6582 |
| 6277 | S100A6 | S100 calcium binding protein A6 | 15991.2832 |
| 1915 | EEF1A1 | Eukaryotic translation elongation factor 1 α 1 | 13189.91406 |
| 9168 | TMSB10 | Thymosin β 10 | 11790.02637 |
| 3852 | KRT5 | Keratin 5 | 10268.93652 |
| 6590 | SLPI | Secretory leukocyte peptidase inhibitor | 9873.083008 |
| 6222 | RPS18 | Ribosomal protein S18 | 9865.65332 |
| 3861 | KRT14 | Keratin 14 | 9010.899414 |
| 301 | ANXA1 | Annexin A1 | 8724.607422 |
| 302 | ANXA2 | Annexin A2 | 8183.348633 |
| 6130 | RPL7A | Ribosomal protein l7a | 7233.334961 |
| 3853 | KRT6A | Keratin 6A | 7038.019531 |
| 6205 | RPS11 | Ribosomal protein S11 | 6782.036133 |
| 3872 | KRT17 | Keratin 17 | 6779 |
| 6282 | S100A11 | S100 calcium binding protein A11 | 6758.137695 |
| 57402 | S100A14 | S100 calcium binding protein A14 | 6577.536133 |
| 6136 | RPL12 | Ribosomal protein L12 | 6571.3125 |
| 6202 | RPS8 | Ribosomal protein S8 | 5322.165039 |
| 23521 | RPL13A | Ribosomal protein l13a | 5300.688477 |
| 6175 | RPLP0 | Ribosomal protein lateral stalk subunit P0 | 5200.693848 |
| 6144 | RPL21 (2) | Ribosomal protein L21 | 5167.824219 |
| 7114 | TMSB4X | Thymosin β 4 X-linked | 4858.939453 |
| 6201 | RPS7 | Ribosomal protein S7 | 4807.380371 |
| 6281 | S100A10 | S100 calcium binding protein A10 | 4705.583984 |
| 6206 | RPS12 | Ribosomal protein S12 | 4461.196777 |
| 6230 | RPS25 | Ribosomal protein S25 | 4362.669922 |
| 6122 | RPL3 | Ribosomal protein L3 | 4190.952148 |
| 2597 | GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | 4084.755371 |
| 4502 | MT2A | Metallothionein 2A | 4024.866699 |
| 3855 | KRT7 | Keratin 7 | 3954.171631 |
| 6194 | RPS6 | Ribosomal protein S6 | 3893.395996 |
| 6152 | RPL24 | Ribosomal protein L24 | 3876.068848 |
| 6142 | RPL18A | Ribosomal protein l18a | 3788.035645 |
| 60 | ACTB | Actin β | 3734.336914 |
| 6156 | RPL30 | Ribosomal protein L30 | 3719.687988 |
| 6279 | S100A8 | S100 calcium binding protein A8 | 3664.906982 |
| 10399 | RACK1 | Receptor for activated C kinase 1 | 3650.605225 |
| 100133941 | CD24 | CD24 molecule | 3593.078369 |
| 6191 | RPS4X | Ribosomal protein S4 X-linked | 3478.193848 |
| - | RNR1 | - | 3421.098877 |
| 2950 | GSTP1 | Glutathione S-transferase pi 1 | 3338.100586 |
| 6187 | RPS2 | Ribosomal protein S2 | 3280.663086 |
| 6207 | RPS13 | Ribosomal protein S13 | 3170.464111 |
| 11224 | RPL35 | Ribosomal protein L35 | 3153.293701 |
| 1937 | EEF1G | Eukaryotic translation elongation factor 1 γ | 3140.122559 |
| 6125 | RPL5 | Ribosomal protein L5 | 3137.963623 |
| 6170 | RPL39 | Ribosomal protein L39 | 3100.071045 |
| 4637 | MYL6 | Myosin light chain 6 | 3067.146484 |
| 3868 | KRT16 | Keratin 16 | 3044.359619 |
| 4736 | RPL10A | Ribosomal protein l10a | 2961.195801 |
| 6141 | RPL18 | Ribosomal protein L18 | 2929.649658 |

Table II. Continued.

| Entrez gene ID | Gene symbol | Description | TPM |
|---|---|---|---|
| 1476 | CSTB | Cystatin B | 2910.897217 |
| 6124 | RPL4 | Ribosomal protein L4 | 2865.387207 |
| 4070 | TACSTD2 | Tumor associated calcium signal transducer 2 | 2787.421387 |
| 6147 | RPL23A | Ribosomal protein l23a | 2730.734131 |
| 71 | ACTG1 | Actin γ 1 | 2705.085693 |
| 220 | ALDH1A3 | Aldehyde dehydrogenase 1 family member A3 | 2588.035156 |
| 6135 | RPL11 | Ribosomal protein L11 | 2561.839844 |
| 3880 | KRT19 | Keratin 19 | 2536.187012 |
| 6132 | RPL8 | Ribosomal protein L8 | 2522.498047 |
| 6181 | RPLP2 | Ribosomal protein lateral stalk subunit P2 | 2490.632813 |
| 3866 | KRT15 | Keratin 15 | 2464.382324 |
| 6699 | SPRR1B | Small proline rich protein 1B | 2444.126465 |
| 6159 | RPL29 | Ribosomal protein L29 | 2439.016113 |
| 2512 | FTL | Ferritin light chain | 2432.441895 |
| 6193 | RPS5 | Ribosomal protein S5 | 2432.29126 |
| 6233 | RPS27A | Ribosomal protein s27a | 2403.434326 |
| 6129 | RPL7 | Ribosomal protein L7 | 2332.271973 |
| 6273 | S100A2 | S100 calcium binding protein A2 | 2289.59375 |
| 6133 | RPL9 | Ribosomal protein L9 | 2237.880371 |
| 1475 | CSTA | Cystatin A | 2159.565186 |
| 6128 | RPL6 | Ribosomal protein L6 | 2119.131592 |
| 2495 | FTH1 | Ferritin heavy chain 1 | 2094.474121 |
| 3921 | RPSA | Ribosomal protein SA | 2085.400391 |
| 5266 | PI3 | Peptidase inhibitor 3 | 2079.049805 |
| 2171 | FABP5 | Fatty acid binding protein 5 | 2073.613281 |
| 5052 | PRDX1 | Peroxiredoxin 1 | 2053.132568 |
| 3956 | LGALS1 | Galectin 1 | 2031.178833 |
| 6143 | RPL19 | Ribosomal protein L19 | 2021.314087 |
| 25818 | KLK5 | Kallikrein related peptidase 5 | 1822.719238 |
| 3939 | LDHA | Lactate dehydrogenase A | 1803.661499 |
| 6176 | RPLP1 | Ribosomal protein lateral stalk subunit P1 | 1802.172241 |
| 51458 | RHCG | Rh family C glycoprotein | 1785.147339 |
| 6303 | SAT1 | Spermidine/spermine N1-acetyltransferase 1 | 1763.299316 |
| 9982 | FGFBP1 | Fibroblast growth factor binding protein 1 | 1742.557251 |
| 7178 | TPT1 | Tumor protein, translationally-controlled 1 | 1741.52832 |
| 6227 | RPS21 | Ribosomal protein S21 | 1726.189087 |
| 3934 | LCN2 | Lipocalin 2 | 1720.297241 |
| 3315 | HSPB1 | Heat shock protein family B (small) member 1 | 1668.157104 |
| 1973 | EIF4A1 | Eukaryotic translation initiation factor 4A1 | 1623.40625 |
| 1938 | EEF2 | Eukaryotic translation elongation factor 2 | 1612.361694 |
| 5055 | SERPINB2 | Serpin family B member 2 | 1610.25 |
| 2810 | SFN | Stratifin | 1591.703979 |
| 6703 | SPRR2D | Small proline rich protein 2D | 1568.223389 |
| 26986 | PABPC1 | Poly(A) binding protein cytoplasmic 1 | 1534.452637 |
| 6204 | RPS10 | Ribosomal protein S10 | 1532.445679 |
| 10410 | IFITM3 | Interferon induced transmembrane protein 3 | 1529.12146 |
| 6189 | RPS3A | Ribosomal protein S3A | 1509.361816 |
| 6154 | RPL26 | Ribosomal protein L26 | 1432.493286 |
| 3918 | LAMC2 | Laminin subunit γ 2 | 1429.380249 |
| 83442 | SH3BGRL3 | SH3 domain binding glutamate rich protein like 3 | 1395.721313 |
| 6139 | RPL17 | Ribosomal protein L17 | 1375.231934 |

Table II. Continued.

| Entrez gene ID | Gene symbol | Description | TPM |
|---|---|---|---|
| 1933 | EEF1B2 | Eukaryotic translation elongation factor 1 β 2 | 1351.424194 |
| 10974 | ADIRF | Adipogenesis regulatory factor | 1348.772461 |
| 6134 | RPL10 | Ribosomal protein L10 | 1336.026611 |
| 5268 | SERPINB5 | Serpin family B member 5 | 1335.237183 |
| 6700 | SPRR2A | Small proline rich protein 2A | 1285.784912 |
| 10094 | ARPC3 | Actin related protein 2/3 complex subunit 3 | 1270.268311 |
| 2152 | F3 | Coagulation factor III, tissue factor | 1268.36792 |
| 2197 | FAU | FAU ubiquitin like and ribosomal protein S30 fusion | 1255.56189 |
| 9124 | PDLIM1 | PDZ and LIM domain 1 | 1252.652954 |
| 64065 | PERP | P53 apoptosis effector related to PMP22 | 1252.282227 |
| 4869 | NPM1 | Nucleophosmin 1 | 1247.643188 |
| 7295 | TXN | Thioredoxin | 1169.833984 |
| 3553 | IL1B | Interleukin 1 β | 1166.45752 |
| 5054 | SERPINE1 | Serpin family E member 1 | 1154.025146 |
| 6171 | RPL41 | Ribosomal protein L41 | 1152.395996 |
| 25824 | PRDX5 | Peroxiredoxin 5 | 1133.30603 |
| 6173 | RPL36A | Ribosomal protein l36a | 1111.359619 |
| 5315 | PKM | Pyruvate kinase M1/2 | 1092.81897 |
| 1072 | CFL1 | Cofilin 1 | 1085.361328 |
| 6289 | SAA2 | Serum amyloid A2 | 1073.526978 |
| 4071 | TM4SF1 | Transmembrane 4 L six family member 1 | 1063.068237 |
| 506 | ATP5F1B | ATP synthase F1 subunit β | 1047.457275 |
| 5834 | PYGB | Glycogen phosphorylase B | 1047.218994 |
| 928 | CD9 | CD9 molecule | 1021.081299 |
| 10628 | TXNIP | Thioredoxin interacting protein | 1021.076111 |
| 103910 | MYL12B | Myosin light chain 12B | 1012.033325 |
| 3854 | KRT6B | Keratin 6B | 1011.945374 |
| 3688 | ITGB1 | Integrin subunit β 1 | 1004.073792 |
| 3312 | HSPA8 | Heat shock protein family A (Hsp70) member 8 | 1000.302063 |
| 6288 | SAA1 | Serum amyloid A1 | 999.111145 |
| 1382 | CRABP2 | Cellular retinoic acid binding protein 2 | 986.4415283 |
| 6224 | RPS20 | Ribosomal protein S20 | 975.680481 |
| 10109 | ARPC2 | Actin related protein 2/3 complex subunit 2 | 966.9124146 |
| 1992 | SERPINB1 | Serpin family B member 1 | 952.090332 |
| 306 | ANXA3 | Annexin A3 | 951.5344238 |
| 4501 | MT1X | Metallothionein 1X | 939.5453491 |
| 5660 | PSAP | Prosaposin | 936.7683105 |
| 6286 | S100P | S100 calcium binding protein P | 924.9679565 |
| 567 | B2M | β-2-microglobulin | 919.1690674 |
| 3914 | LAMB3 | Laminin subunit β 3 | 918.9204102 |
| 1308 | COL17A1 | Collagen type XVII α 1 chain | 916.5231323 |
| 824 | CAPN2 | Calpain 2 | 912.717041 |
| 2706 | GJB2 | Gap junction protein β 2 | 904.8463745 |
| 3860 | KRT13 | Keratin 13 | 894.9153442 |
| 3646 | EIF3E | Eukaryotic translation initiation factor 3 subunit E | 893.5683594 |
| 5479 | PPIB | Peptidylprolyl isomerase B | 883.137207 |
| 7316 | UBC | Ubiquitin C | 875.6885986 |
| 3326 | HSP90AB1 | Heat shock protein 90 α family class B member 1 | 871.9744263 |
| 642587 | MIR205HG | MIR205 host gene | 864.2874146 |
| 468 | ATF4 | Activating transcription factor 4 | 850.9224243 |
| 140576 | S100A16 | S100 calcium binding protein A16 | 849.9338989 |
| 6155 | RPL27 | Ribosomal protein L27 | 841.65802 |

Table II. Continued.

| Entrez gene ID | Gene symbol | Description | TPM |
|---|---|---|---|
| 6228 | RPS23 | Ribosomal protein S23 | 837.4863281 |
| 25984 | KRT23 | Keratin 23 | 837.0656738 |
| 54541 | DDIT4 | DNA damage inducible transcript 4 | 831.8173218 |
| 112694756 | LOC112694756 | Uncharaterized LOC112694756 | 831.1845093 |
| 9349 | RPL23 | Ribosomal protein L23 | 826.6482544 |
| 7184 | HSP90B1 | Heat shock protein 90 β family member 1 | 826.4506836 |
| 1337 | COX6A1 | Cytochrome c oxidase subunit 6A1 | 820.6051025 |
| 1974 | EIF4A2 | Eukaryotic translation initiation factor 4A2 | 800.7364502 |
| 6188 | RPS3 | Ribosomal protein S3 | 796.1228638 |
| 6157 | RPL27A | Ribosomal protein l27a | 790.3303833 |
| 5757 | PTMA | Prothymosin α | 790.0863037 |
| 826 | CAPNS1 | Calpain small subunit 1 | 783.6133423 |
| 5328 | PLAU | Plasminogen activator, urokinase | 780.4100342 |
| 2023 | ENO1 | Enolase 1 | 778.8522949 |
| 1509 | CTSD | Cathepsin D | 771.4251709 |
| 10476 | ATP5PD | ATP synthase peripheral stalk subunit d | 768.3088989 |
| 7534 | YWHAZ | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ | 767.7701416 |
| 292 | SLC25A5 | Solute carrier family 25 member 5 | 758.4469604 |
| 5216 | PFN1 | Profilin 1 | 753.312439 |
| 1340 | COX6B1 | Cytochrome c oxidase subunit 6B1 | 751.3442383 |
| 8407 | TAGLN2 | Transgelin 2 | 741.7597046 |
| 689 | BTF3 | Basic transcription factor 3 | 738.1211548 |
| 374 | AREG | Amphiregulin | 735.1116333 |
| 10376 | TUBA1B | Tubulin α 1b | 732.8063965 |
| 6210 | RPS15A | Ribosomal protein s15a | 728.9209595 |
| 3909 | LAMA3 | Laminin subunit α 3 | 723.6885986 |
| 7086 | TKT | Transketolase | 713.4926147 |
| 5650 | KLK7 | Kallikrein related peptidase 7 | 708.7366333 |
| 4323 | MMP14 | Matrix metallopeptidase 14 | 702.4146118 |
| 4312 | MMP1 | Matrix metallopeptidase 1 | 700.8983154 |
| 6229 | RPS24 | Ribosomal protein S24 | 700.0944824 |
| 10653 | SPINT2 | Serine peptidase inhibitor, Kunitz type 2 | 695.8338623 |
| 4831 | NME2 | NME/NM23 nucleoside diphosphate kinase 2 | 694.8643799 |
| 10971 | YWHAQ | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein τ | 692.3873291 |
| 5478 | PPIA | Peptidylprolyl isomerase A | 682.8765869 |
| 7980 | TFPI2 | Tissue factor pathway inhibitor 2 | 679.0671997 |
| 6146 | RPL22 | Ribosomal protein L22 | 678.4135132 |
| 3945 | LDHB | Lactate dehydrogenase B | 671.2799683 |
| 351 | APP | Amyloid β precursor protein | 665.9901733 |
| 1508 | CTSB | Cathepsin B | 665.0159302 |
| 10209 | EIF1 | Eukaryotic translation initiation factor 1 | 664.9918213 |
| 8673 | VAMP8 | Vesicle associated membrane protein 8 | 659.6922607 |
| 7416 | VDAC1 | Voltage dependent anion channel 1 | 659.1289063 |
| 4946 | OAZ1 | Ornithine decarboxylase antizyme 1 | 656.2600098 |
| 6168 | RPL37A | Ribosomal protein l37a | 649.401123 |

TPM, transcript per million.

These observations suggest an overall role for EGFR in the pathogenesis of MEC and the EGFR pathway could be a possible therapeutic target. As several drugs target this pathway, AREG–EGFR signaling was analyzed in the HCM-MEC010 cell line in the present study. The HCM-MEC010 cell line was found to express AREG and phosphorylate EGFR. Immunofluorescence analysis localized EGFR expression to the HCM-MEC010 cell membrane. These data suggest that the EGFR ligand, AREG, activated EGFR in an autocrine manner; therefore, antibodies that block AREG-EGFR binding or drugs that interfere with EGFR activation could be used for *CRTC1-MAML2* fusion-positive MEC treatment. However, further analysis is required to identify suitable therapies.

MECs are composed of mucin-producing, epidermoid, and intermediate cells; however, RNA-Seq analysis of the HCM-MEC010 cell line detected little expression of *MUC* genes in the mucous cell marker family, indicating that mucin-producing cells and intermediate cells may have been removed during culture. MECs develop in excretory duct cells (43) and the mixture of three different cell types in MECs predicts their common origin. Duct and acinar cell differentiation are typically lineage-restricted; however, after irradiation, both duct and acinar cells can differentiate into different cell types (44). It is conceivable that established epidermoid-like cells are competent to differentiate into acinar cells, which is a predicted characteristic of injured duct stem cells. Further analysis will assist in the clarification into the origin of MECs. Cancer stem cells have been hypothesized to be involved in tumor formation (43). The results of the present study potentially indicate these cells may be of the same origin.

In conclusion, a MEC cell line, HCM-MEC010, with a *CRTC1-MAML2* gene fusion was established. This cell line showed typical MEC characteristics, including AREG expression and EGFR activation; therefore, it could be used to assist in the identification of EGFR-targeted drugs for the treatment of *CRTC1-MAML2* fusion gene-harboring MEC.

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**Availability of data and materials**

The datasets generated and/or analyzed during the current study are not publicly available due to a pending patent application, but are available from the corresponding author on reasonable request.

**Authors' contributions**

KN, SK, KaY, KT, HK and YN conceived and designed the present study. KN, SK, KaY, YF, KyY and YN performed the experiments. KN, SK, KoY and YN analyzed the data. KN, SK and YN wrote, reviewed, and revised the manuscript. All authors read and approved the final manuscript. KN and SK confirm the authenticity of all the raw data.

**Ethics approval and consent to participate**

The current study was approved by the Institutional Review Board of Hyogo College of Medicine (Hyogo, Japan) and was conducted in accordance with the Declaration of Helsinki. The patient provided written informed consent to participate.

**Patient consent for publication**

The patient provided written informed consent for the publication of their case study.

**Competing interests**

The authors declare that they have no competing interests.

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