MOLECULAR DETECTION OF MECT1-MAML2 FUSION GENE IN MUCOEPIDERMOID CARCINOMA WITH ORDINARY AND VARIANT HISTOLOGY: A STUDY USING ARCHIVAL PARAFFIN EMBEDDED TISSUE

TAKAHIKO KASAI, MAIKO TAKEDA, YASUNORI ENOMOTO,
MASATO TAKANO, KOUHEI MORITA and AKITAKA NONOMURA
Department of Diagnostic Pathology, Nara Medical University
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Abstract : Mucoepidermoid carcinoma (MEC) has been characterized by t (11; 19)(q21; p13). This chromosomal translocation has been recently shown to generate a MECT1-MAML2 fusion gene. MEC can pose diagnostic challenges when they are of high-grade, of variant histologic appearance and occurring in an unusual site. The aim of this study was to evaluate the frequency of the MECT1-MAML2 fusion gene among primary salivary gland MECs and extrasalivary gland MECs, together with some histological variants and its role as a possible diagnostic adjunct, comparing the salivary gland tumors including Warthin's tumor(WT), pleomorphic adenoma(PA), and adenoid cystic carcinoma(ACC). Using a reverse transcription-polymerase chain (RT-PCR)-based approach, we assayed for the MECT1-MAML2 transcript in 39 cases for which paraffin-embedded tumor tissue with adequate RNA was available. These included 19 MECs, 10 WTs, five PAs, and five ACCs. The MECT1-MAML2 fusion gene transcript was detected in 16 (84.2%) of 19 MECs. These positive cases included two cases of MEC with WT-like areas, a sclerosing MEC and a clear cell MEC. Three negative cases were high-grade MECs. Two of them were not easy to distinguish from squamous cell carcinoma. The MECT1-MAML2 fusion gene was negative in all cases of WT, PA and ACC. The potential usefulness of MECT1-MAML2 fusion gene expression as a molecular marker in the diagnosis of MEC is supported.

Key words : mucoepidermoid carcinoma, MECT1-MAML2 fusion gene, RT-PCR, paraffin-embedded tissue

INTRODUCTION

Mucoepidermoid carcinoma (MEC) of the salivary glands represents 15.5% of all salivary gland tumors and 22 to 41% of the malignant forms[1]. Histologically, MEC is composed of mucous-secreting cells, epidermoid cells, and intermediate cells in variable combinations. It is classified into low-, intermediate-, and high-grade types on the basis of morphologic and cytologic features[1, 2]. MEC occasionally displays morphologic variations with a minimum or complete absence of more typical morphologic features, which can present diagnostic difficulties[1, 3]. Among these features are a predominance of clear cells, a spindle cell-like pattern of growth, sebaceous-like differentiation, a predominantly oncocytic appearance, areas mimicking Warthin's tumor (WT) and a sclerosing pattern[1-7]. The differential diagnosis of low-grade MEC included pleomorphic adenoma (PA) with squamous metaplasia, cystadenoma and mucocele[1, 3]. The existence of high-grade MEC and the relevance of its distinction from

poorly differentiated adenocarcinoma or squamous cell carcinoma, have also been challenged[1, 3]. Therefore, it is desirable to identify a molecular marker that is sensitive and specific for MEC.

To date, the karyotypic profileinMEC has been described in 30 cases[8]: innine cases there were rearrangements of 11q14–21 and 19p12–13, mainly as a chromosomal translocation, t(11 ; 19) (q21 ; p12–13)[9], and this was the sole abnormality in three cases[9-11]. The remaining sixcases showed either a more complex translocation involving other chromosomes or other rearrangements[8]. The same abnormality has also been described in MEC originatingin bronchial glands of the lung[11,12]. Interestingly, t(11;19)(q13–21;p12–13) was also reported in WT[13,14], a benign salivary gland neoplasm, which suggested an unexpected cytogenetic association between two otherwise unrelated salivary gland tumors. However, it is known that WT can arise and/or co-exist with MEC, warranting a reappraisal of this association[5,7]. Thetranslocationt(11 ; 19) (q21 ; p13) found in MEC has recently been cloned[15]. Two genes are involved: the mucoepidermoid carcinoma translocated 1(MECT1) gene and a member of the mastermind-like gene family (MAML2) located at 19p13 and 11q21, respectively[15]. This translocation generates a chimeric gene in which exon 1 of MECT1isfused with exons 2–5 of MAML2. This MECT1–MAML2 fusion product disrupts the Notch signaling pathway, activating Notch-target genes independently of exogenous signals, therefore representing a novel mechanism for altered Notch function in tumorigenesis[15]. In addition, recent study of the MECT 1 gene product as a potent co-activator for gene responsive elements indicates that MECT1-MAML2 may disrupt both the Notch and CREB signaling pathways to induce tumorigenesis[16, 17]. Recently, MECT1–MAML2 fusion gene expression has been studied in MEC, using a conventional cytogenetic analysis, RT–PCR with frozen tumor tissue and *in situ* hybridization (ISH)[18]. There are some reports about the detection of the MECT1– MAML2 fusion gene by RT–PCR assay using paraffin-embedded tumor tissue. The correlation between the MECT1–MAML2 fusion gene and variants of MECs and the utility of this chimeric product as an ancillary diagnostic tool have not been examined.

Therefore, we used an RT–PCR assay for the MECT1–MAML2 transcript to explore the prevalence of this fusion transcript in a series of 19 primary MECs, 10 primary WTs, five pleomorphic adenoma(PA)s and five adenoid cystic carcinoma(ACC)s, using paraffin-embedded tumor tissue.

MATERIALS AND METHODS

Case selection

All the previously diagnosed cases of mucoepidermoid carcinoma (MEC), Warthin's tumor (WT), pleomorphic adenoma (PA) and adenoid cystic carcinoma (ACC) in the files of the Department of Diagnostic Pathology, Nara Medical University, were reclassified according to the World Health Organization's criteria[19]. MEC cases were graded according to Goode and Ellis's criteria[1, 20]. All the tumors examined were primary lesions, associated with one metastatic lesion (case 10B) and recurrence (case 19A'). All the tumors were fixed in 10% neutral-buffered formalin. The oldest paraffin block was processed in 1992 (case 10A). In sixteen cases specimens from salivary glands and lungs were obtained from resected tumor specimens. The remaining three lesions (10B, 18, 19), obtained at curettage or open biopsied

Table 1a. Clinicopathologic features and RT-PCR Data from the Tumor Samples Collected

Case	Sex/Age	Histologic Diagnosis			Location	RT-PCR	
						MECT1-MAML2	PGK
1	32/F	MEC	LG		Nasopharynx	+	+
2	56/M	MEC	LG		Oral	+	+
3	51/F	MEC	LG		Tonsil, rt	+	+
4	64/F	MEC	LG	WTLA	Parotid, lt	+	+
5	53/F	MEC	LG	Sclerosing	Oral	+	+
6	75/F	MEC	LG	Clear cell	Submandibular,lt	+	+
7	48/F	MEC	IG		Sublingual, rt	+	+
8	68/M	MEC	IG		Submaxillary, lt	+	+
9	76/F	MEC	IG		Oral	+	+
10 A	70/F	MEC	IG		LLL	+	+
B					Cerebellum, lt	+	+
11	68/F	MEC	IG	WTLA	Parotid, rt	+	+
12	68/M	MEC	HG		Parotid, lt	+	+
13	69/M	MEC	HG		Maxillary sinus, rt	+	+
14	63/M	MEC	HG		RLL	+	+
15	74/F	MEC	HG		Submaxillary, lt	+	+
16	49/M	MEC	HG		Parotid, rt	+	+
17	51/M	MEC	HG		Parotid, rt	-	+
18	67/M	MEC	HG		Maxillary sinus,rt	-	+
19 A	58/M	MEC	HG		Nasal cavity,lt	-	+
A'					Nasal cavity,lt	-	+

MEC, Mucoepidermoid carcinoma; LG, Low grade; IG, Intermediate grade; HG, High grade; A, Primary tumor; A', Local recurrence tumor at 6 month later; B, Metastatic brain tumor at 3 years later; WTLA, Warthin's tumor-like area; RLL, Right lower lobe of lung; LLL, Left lower lobe.

tumors, were examined. Each of the blocks contained over half of the tumor's area. Both in case 4 and in case 11, blocks contained MEC (approximately 60%) and WT-like areas. Paraffin-embedded specimens of ten WTs, five PAs, and five ACCs were also included in this study. The clinical, histopathological, and RT-PCR data are shown in Table 1.

RNA Extraction

Total RNA was extracted from the paraffin embedded tissues, according to a previously reported protocol with some modifications[21, 22]. In brief, 10-μm-thick tissue sections were cut from each block and deparaffinized with two changes of xylene followed by three washes with 100% ethanol. Tissue fragments of each specimen were treated with a cocktail containing 10μL of proteinaseK(100mg/ml) and 200μL of lysis buffer (20mM Tris-HCl(pH8.0), 20mM EDTA and 2% sodium dodecyl sulfate) and incubated overnight at 55℃ until the sections were completely dissolved. To optimize the extraction of RNA, the digested lysate of

each of which was solubilized in 1.0 ml of Trizol reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer's directions. The precipitated RNA was then washed in 70% ethanol, redissolved in 10μL of DEPC-treated water (Invitrogen Life Technologies, Carlsbad, CA), treated with 1μL of DNAase I for 15 minutes at 37℃ to eliminate contaminated genomic DNA, extracted again with 100μL of the Trizol reagent, and subsequently resolubilized in 10μL of DEPC-treated water .

RT-PCR Analysis

For the synthesis of the first-strand cDNA, 4μl of total RNA, 4μL of 25nM MgC_{12}, 2μL of 10xPCR buffer (Applied Biosystems, Foster City, CA), 1μL of 50μM random hexamers (Invitrogen), 2μL of 10nM of dNTPmix (Invitorogen), 20units of RNAase inhibitor (Invitorogen), and 100 units of reverse transcriptase (Superscript II, Invitrogen) were added to a final reaction volume of 20μL. The RT reaction was performed at 42℃ for 40 minutes and then the mixture was heated at 70 ℃ to inactivate the reverse transcriptase. The oligonucleotide primer sets used for the paraffin-embedded tissue to amplify a segment from the MECT1-MAML2 cDNA starting in exon 1 from the MECT1 gene and finishing in exon 2 from the MAML2 gene were sense MECT1-FWD (agatggcgacttcgaacaat) and antisense MAML2-REV (gctgttggcaggagataggt) with the expected PCR product size being 182-bp. PCR was performed in a final reaction volume of 50μL containing 2μL of RT product, 1.5mM of $MgCl_2$, 1xPCR buffer (Applied Biosystems), 0.1mM of dNTP mix, 0.5μM each of the sense and antisense primer and 0.5 units of Gold Taq DNA polymerase (Applied Biosystems). PCRs were performed for 40 cycles of amplification, using the following thermal cycle profile: 95℃ for 45 seconds, 56℃ for 45 seconds and 72℃ for 1minute. After the last cycle, a final extension at 72℃ for 10 minutes was carried out and then the temperature was held at 4℃. Negative controls for cDNA synthesis and PCRs, in which the template was replaced by sterile water, were included in each experiment. RNA integrity and efficiency of cDNA synthesis were confirmed in each sample by RT-PCR for the house-keeping gene phosphoglycerate kinase (PGK), using previously published oligonucleotide primers[23]. Amplification with these primers yielded a 189-bp product. PCR products were analyzed and purified by electrophoresis in a 2% agarose gel stained with ethidium bromide. All the experiments were repeated, at least twice, with different batches of mRNA extracted at different times, from the same tumor. In the cases that presented the MECT1-MAML2 rearrangement, the PCR product was sequenced (ABI Prism 377 DNA Sequencer, using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit version1.1; Applied Biosystems, Foster City, USA).

RESULTS

Clinicopathological features

Table 1 summarizes the salient clinical and pathologic information. The patients with MEC studied ranged in age from 32 to 76 years at first presentation (mean 61.0 years). Nine tumors were located in major salivary glands (parotid : 5 cases, submaxillary : 2 cases, submandibula ar : 1 case) and two cases in the lungs. Seven cases seemed to involve minor salivary glands in oral and nasopharyngeal regions.

The cases selected were reclassified as low-grade (n=6), six intermediate-grade (6), and

Table 1b. Clinicopathologic features and RT-PCR Data from the Tumors

Case	Sex/Age	Histologic Diagnosis	Location	RT-PCR	
				MECT1-MAML2	PGK
20	54/M	WT	Parotid, lt	-	+
21	64/M	WT	Parotid, rt	-	+
22	63/M	WT	Parotid, lt	-	+
23	76/M	WT	Parotid, lt	-	+
24	67/M	WT	Parotid, rt	-	+
25	48/M	WT	Parotid, lt	-	+
26	75/M	WT	Parotid, lt.	-	+
27	67/M	WT	Parotid, lt	-	+
28	53/M	WT	Parotid, rt	-	+
29	52/M	WT	Parotid, rt	-	+
30	54/M	PA	Parotid, rt.	-	+
31	70/F	PA	Parotid, lt	-	+
32	33/F	PA	Submandibular, rt	-	+
33	53/F	PA	Parotid, rt	-	+
34	46/F	PA	Lower lip	-	+
35	50/F	ACC	External ear, lt	-	+
36	59/M	ACC	Submandibular, rt	-	+
37	51/M	ACC	RUL	-	+
38	67/M	ACC	Lower lip	-	+
39	58/M	ACC	Oral cavity	-	+

WT, Warthin's tumor; PA, Pleomorphic adenoma; ACC, Adenoid cystic carcinoma; RUL, Right upper lobe of lung

high-grade (7) MECs. Since adequate follow-up data were not available, we couldn't evaluate the relation between the MECT1-MAML2 fusion gene expression and prognosis in this study.

Two cases had conventional MEC areas (Case 4 : low grade, Case11 : intermediate grade), associated with WT-like areas composed of a glandular epithelial component with characteristic eosinophilic cytoplasm and a lymphoid stroma (Fig.1. A, B, C). In Case 5, the majority (approximately 70%) of the tumor was composed of a central paucicellular area of dense hyaline sclerosis (Fig.1. D, E). One of the low-grade MEC (case 6) was composed of prominent clear cells (approximately 80%), associated with a small amount of intermediate and mucous cells (Fig.1, F).

Detection of MECT1-MAML2 fusion gene by RT-PCR

The results of RT-PCR are summarized in Table 1. We detected the MECT1-MAML2 fusion transcript in 16 (84.2%) of 19 MECs (Fig. 2). Sequence analysis of the positive RT-PCR products confirmed the presence of the same rearrangement in the sixteen cases. Sixteen cases of MEC with positive RT-PCR products included variant histology, two cases of Warthin-like area, one sclerosing type, and one clear cell type. The MECT1-MAML2

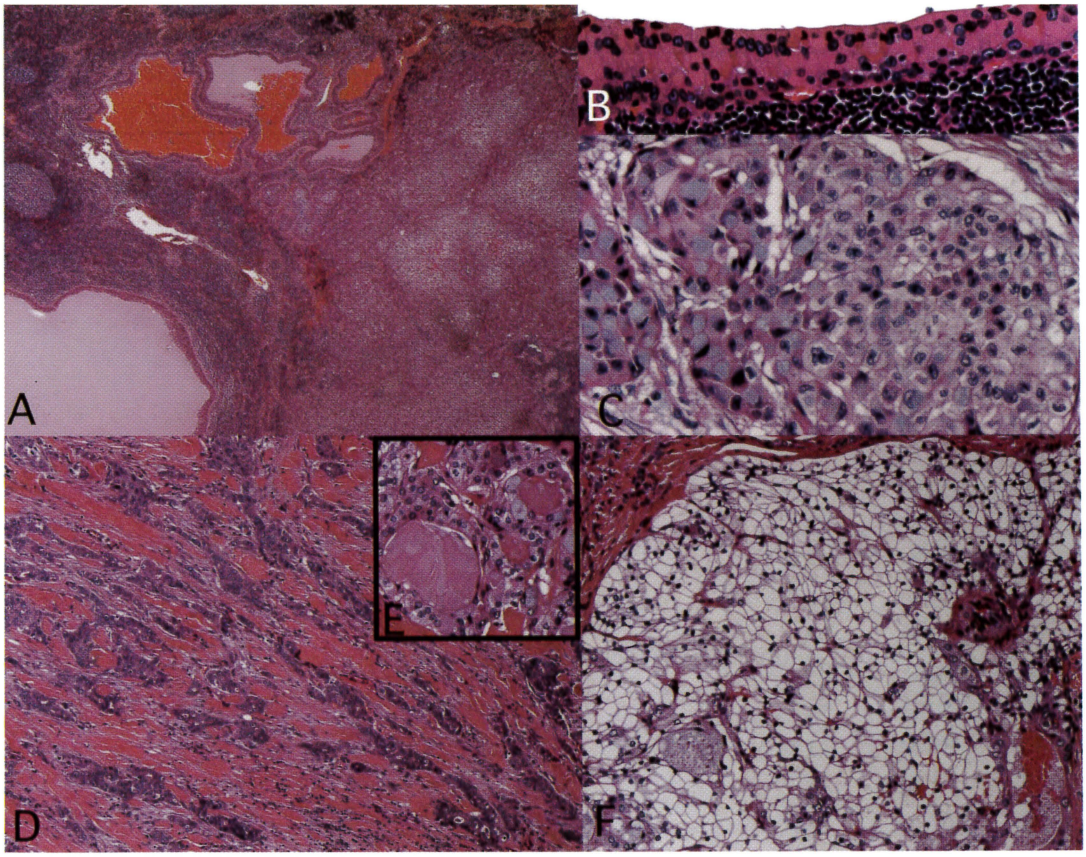

Fig. 1. Variants of mucoepidermoid carcinoma
A, B, C; Mucoepidermoid carcinoma (MEC) with Warthin's tumor (WT)-like area (case 4).
A) MEC (left side) showing solid growth and WT-like (right side) area showing cystic epithelial element with lymphoid stroma.
B) The cystic lesion lined double and multilayered with oncocytic cells surrounded by lymphoid stroma in the WT-like area.
C) MEC characterized by intermediate or squamous cells, and mucous cells.
D, E: Sclerosing MEC (case 5)
D) Sclerosing MEC displaying solid or trabecular tumor nests within marked sclerotic stroma.
E) The tumor nests showing intermediate and mucous cells with tubular formation.
F: Clear cell MEC with most of tumor cells showing a clear cytoplasm (case 6).

fusion gene was detected in primary and metastatic lesions in Case 10. Sequencing analysis of the positive RT–PCR products confirmed the presence of the same arrangement in 16 cases with the product of the fusion between MECT1 exon 1 and MAML2 exon 2. Otherwise, three negative cases of MEC were initially diagnosed as high-grade. Two of them were indistinguishable from squamous cell carcinoma. There was no expression of the fusion gene either in ten WTs, in five PAs, or in five ACCs. The 189-bp RT–PCR product for the PGK housekeeping gene was detected in all 41 paraffin tumor blocks of 39 cases, indicating adequate sampling and confirming mRNA integrity (Fig. 2).

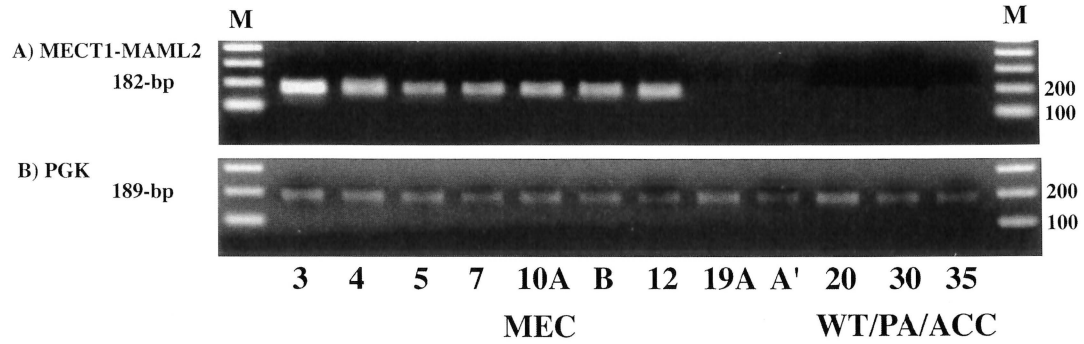

Fig. 2.

RT-PCR detection of MECT1-MAML2 fusion gene transcript, using primers in MECT1 exon 1(sense) and MAML2 exon 2 (antisense) oligonucleotide primers, and control PGK transcripts.

A: a 182-bp fragment corresponding to MECT1-MAML2 was detected in MECs (data shown only for case 3,4,5,7,10A, 10B, 12), but not in high-grade MEC (case 19 A, A') and tenWTs (data shown only for case 20), five PAs (data shown only for case30), and five ACC (data shown only for case35).

B: The detection of a 189-bp PGK RT-PCR product confirmed the mRNA integrity in all of the samples.

M; 100 bp DNA ladder.

DISCUSSION

The diagnosis and classification MECs have until now been based mainly on histologic criteria[1, 3, 19]. Low-grade MEC can resemble benign lesions, ex, mucocele, mucin pools and sialometaplasia in the inflammatory or non-inflammatory process in salivary gland. High-grade MEC may have significant morphologic overlap with squamous cell carcinoma and poorly differentiated adenocarcinoma. MECs can also contain lymphoid stroma, in which case the differential diagnosis includes Warthin's tumor[1, 5, 7]. When they contain prominent clear cells, they may resemble acinic cell carcinoma, clear cell oncocytoma, clear cell adenocarcinoma, epithelial-myoepithelial carcinoma, and myoepithelial carcinoma[2, 6]. MEC may also display an intense sclerosing pattern that obscures its typical morphologic feature[4]. Therefore, tumor-specific genetic alterations may be particularly attractive targets in the diagnostic setting when histopathological features and conventional ancillary techniques are insufficient to render an unambiguous diagnosis of MEC.

Cytogenetic studies of sarcoma have recognized a number of reciprocal translocations that correlate with specific tumor types[24-26]. Molecular cloning of the translocation breakpoints in several types of sarcomas has identified fusions between the breakpoint-spanning gene from each chromosome[24, 26]. The resulting genes encode chimeric fusion gene products that appear to function in transformation by dysregulating gene transcription or affecting cellular signal transduction pathways[24, 25]. The chimeric RNAs produced from the gene fusions can now be detected by RT–PCR, creating specific diagnostic assays for each of these tumors. For some gene fusions, molecular detection can be performed using RNA from routine formalin-fixed, paraffin-embedded material, which has increased the practical use of this methodology[22, 27].

The chromosomal translocation t(11 ; 19)(q21 ; p12–13) is the most frequently detected aberration (30%) in MEC, either as an unique abnormality or together with other chromosomal abnormalities[8]. In addition to standard cytogenetics, the molecular cloning of t (11; 19)(q21; p12–13) in two cell lines generated from lung and parotid gland MEC has now allowed for specific RT–PCR and ISH[15]. Martins et al demonstrated that these analyses were sensitive methods for detecting MECT1–MAML2 fusion gene in MEC[18].

To evaluate the incidence of the MECT1–MAML2 fusion gene, we selected a series of 19 MECs, occurring in nine major and seven minor salivary glands and two lungs in the RT–PCR analysis, using paraffin-embedded tumor tissue. Our RT–PCR analysis detected MECT1–MAML2 in 16 (84.5%) of 19 cases, in comparison with the rate 70% of detection that Martins reported[18]. Our rate of detection may have been better than that of Martins because the MECT1–MAML2 fusion gene amplification area that we designed was short.

Ten WT cases were included in this study because a similar translocation t (11; 19)(q13-21; p12–13), has been reported in some of these tumors[13, 14]. Otherwise, we did not detect MECT1–MAML2 expression in 10 WTs using RT–PCR assay, as in the previous report[18]. In addition, Enlund et al reported that one case classified as WT and exhibiting a t (11; 19) expressed the MECT1–MAML2 fusion gene[28]. WT and MEC are two distinctive salivary gland tumors that do not share clinicopathological features, and their general histogenesis is also distinct[1, 19]. However, there are some reports of the co-existence of these histological types in the same gland lesion[5, 7], which might be an explanation for the finding of common genetic alterations[18]. Martins et al reported two cases of typical MEC with WT-like areas, and that MECT1–MAML2 expression might be a rare event in WT samples that do not contain concomitant evidence for MEC[18]. The MECT1–MAML2 fusion gene was expressed in our two cases with WT-like areas. Both components of MEC and WT-like areas were included in the two paraffin blocks in our cases, and it was not possible to clearly determine which component was expressed. We detected the MECT1–MAML2 fusion gene in both sclerosing and clear cell types of MEC. But there has been no report about this fusion gene expression in such variant MEC. In addition, MECs occur in the anal canal, esophagus, uterus, thymus, thyroid and skin[3]. Previous reports, as well as our analysis, searched only for MECT1–MAML2 fusion gene expression in MEC located in salivary glands and lungs [8–12, 15, 18, 29, 30].

In our three cases of MEC, there was no expression of the MECT1–MAML2 fusion gene. Histopathologically, two high-grade cases were reminiscent of squamous cell carcinoma. Two negative cases reported by Martin et al were high-grade MECs as well as our result[18]. But our one case is composed of mucous and intermediate cells and we reclassified it as high-grade MEC. According to previous reports[8, 31–33], there are other cytogenetic aberrations in MECs, such as rearrangements affecting 6q21–25, loss of chromosome Y, and gains of chromosomes 2, 3, 5, 6, 18, 20, and X [8, 10, 29, 30, 32, 34]. Some of these aberrations occurred together with t (11; 19) which might indicate they are secondary changes. However, 6q rearrangements are not associated with the t (11; 19) translocation. Moreover, 6q rearrangements were found in squamous cell carcionomas of salivary gland[32, 35, 36]. Some of the MECT1–MAML2 fusion gene negative cases in our study without cytogenetic analysis, may have another gene aberration. Further study is needed to explain the pathways of these gene aberrations of

MEC in comparison with the gene aberrations of other tumors in its differential diagnosis.

In conclusion, our data confirms the frequent expression of the MECT1-MAML2 fusion gene in MEC, supporting the usefulness of RT-PCR using paraffin-embedded tissue for the diagnosis of MEC, especially in histologically challenging cases.

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