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Clinicopathological significance of the *CRTC3–MAML2* fusion transcript in mucoepidermoid carcinoma

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Mucoepidermoid carcinoma is the most common primary malignancy of the salivary gland. We and others showed that *CRTC1–MAML2* gene fusion was associated with favorable clinicopathological tumor features. Recently, a novel gene fusion, *CRTC3–MAML2*, was reported as a rare gene alteration in a case of mucoepidermoid carcinoma. However, its frequency and clinicopathological significance remains unclear. In all, 101 cases of mucoepidermoid carcinoma and 89 cases of non-mucoepidermoid carcinoma of the salivary gland were analyzed, and RNA was extracted from formalin-fixed, paraffin-embedded specimens. In the *CRTC* family, there have been three genes, *CRTC1*, *CRTC2*, and *CRTC3*. We developed reverse transcription-polymerase chain reaction (RT-PCR) assays for *CRTC1–MAML2*, *CRTC2–MAML2*, and *CRTC3–MAML2* fusions. Clinicopathological data of the patients were obtained from their clinical records. Of 101 cases of mucoepidermoid carcinoma, 34 (34%) and 6 (6%) were positive for *CRTC1–MAML2* and *CRTC3–MAML2* fusion transcripts. However, in the 89 cases of non-mucoepidermoid carcinoma, neither transcript was noted. In the former cases, *CRTC1–MAML2* and *CRTC3–MAML2* fusions were mutually exclusive. The other fusion, *CRTC2–MAML2*, was not detected. We confirmed that the clinicopathological features of *CRTC1–MAML2*-positive mucoepidermoid carcinomas indicated an indolent course. *CRTC3–MAML2*-positive mucoepidermoid carcinomas also had clinicopathologically favorable features; all cases showed a less advanced clinical stage, negative nodal metastasis, no high-grade tumor histology, and no recurrence or tumor-related death after surgical resection of the tumor. It is interesting to note that patients with *CRTC3–MAML2*-positive tumors (mean 36 years of age) were significantly younger that those with the *CRTC1–MAML2* fusion (55 years) and those with fusion-negative tumors (58 years). In conclusion, *CRTC3–MAML2* fusion, which is mutually exclusive with *CRTC1–MAML2* fusion and specific to mucoepidermoid carcinoma, may be detected more frequently than previously expected. Mucoepidermoid carcinomas possessing *CRTC3–MAML2* fusion may be associated with favorable clinicopathological features and patients may be younger than those with *CRTC1–MAML2* fusion or those with no detectable gene fusion.
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Mucoepidermoid carcinoma, representing 5% of all salivary gland tumors and 20% of the malignant forms, is the most frequent primary malignancy of the salivary gland in both adults and children.[1]

A subset of this carcinoma has been associated with a recurring chromosomal translocation, t(11;19)(q21;p13), which is often the sole cytogenetic alteration.[2] This translocation generates a fusion protein comprised of the N-terminal cAMP response element-binding (CREB) protein-binding domain of CREB-regulated transcription coactivator 1 (*CRTC1*, also called *MECT1*, *TORC1*, or *WAMP1*) at 19q21 and the C-terminal transcriptional activation domain of the Notch coactivator mastermind-like gene 2 (*MAML2*) at 11q21.[3–6] Recent data suggest that *CRTC1–MAML2*-induced activation of CREB is critical for cell transformation.[3,5,6] We recently showed that the *CTCR1–MAML2* fusion was detected in approximately 40% of primary salivary gland mucoepidermoid carcinomas, and was associated with a distinct tumor subset that has favorable clinicopathological features and an indolent clinical course.[7] Similar observations have been reported by other research groups.[8,9]

In the *CRTC* family, there are two other *CRTC1*-related human genes, *CRTC2* at 1q21 and *CRTC3* at 15q26, and the *CRTC1* gene has 32% homology with *CRTC2* and *CRTC3* genes.[10] Recently, Fehr *et al*[11] screened 66 mucoepidermoid carcinomas and found that the same part of the *MAML2* gene that participates in *CRTC1–MAML2* fusion was fused to the *CRTC3* gene in a case involving a middle-aged woman. However, the frequency and clinicopathological significance of the *CRTC3–MAML2* fusion remains to be clarified. Another gene in the *CRTC* family, *CRTC2*, has not been reported to be involved in tumorigenesis in mucoepidermoid carcinoma or other tumors. In this study, we screened a large series of cases of mucoepidermoid carcinoma and non-mucoepidermoid carcinoma for *CRTC1–MAML2*, *CRTC2–MAML2*, and *CRTC3–MAML2*, and studied clinicopathological features of fusion-positive cases.

Materials and methods

Case Selection

Mucoepidermoid carcinomas of the major and minor glands were retrieved from the pathology files of Nagoya City University Graduate School of Medical Sciences; Aichi Cancer Center Central Hospital; Okayama University School of Medicine, Dentistry, and Pharmaceutical Sciences; and Aichi-Gakuin University School of Dentistry. Tumors originating in the lung or other sites were not included in this study. All cases were carefully reviewed according to criteria of the World Health Organization for the classification of head and neck tumors,[1] and 101 cases were included in this study. Some of the cases in this series were also included in our previous study.[7] Formalin-fixed, paraffin-embedded specimens of the resected tumors were obtained from all cases. In addition, we also collected typical cases of adenoid cystic carcinoma ($n=8$), oral primary squamous cell carcinoma ($n=22$), pleomorphic adenoma ($n=21$), and Warthin's tumor ($n=38$). Informed consent was obtained, and the study was approved by the institutional review board of Nagoya City University and conducted in accordance with the Declaration of Helsinki. Clinicopathological data were obtained from the medical records. Mucoepidermoid carcinomas were histologically classified according to a three-grade system,[1] which has been widely used for grading this carcinoma affecting both major and minor salivary glands. The tumor grade was determined from the sum of the point values assigned to each of five histological elements: cystic component, neural invasion, necrosis, mitosis, and anaplasia (Supplementary 1).

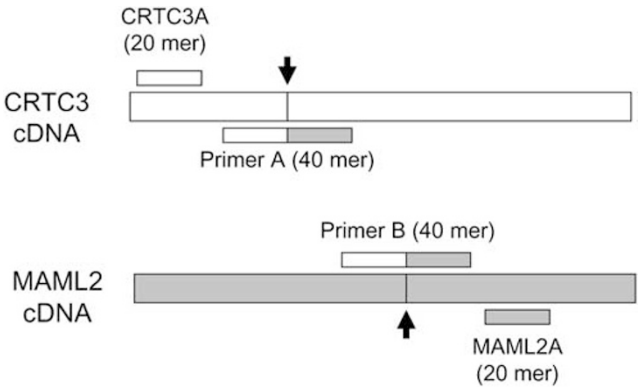

Figure 1 Artificial generation of *CTCR3–MAML2* fusion cDNA from *CRTC3* and *MAML2* cDNA. Primer A (40 mer) for *CRTC3* cDNA amplification includes 20 mer of the *MAML2* sequence on the 3′ side, and primer B (40 mer) for *MAML2* cDNA containes 20 mer of the *CRTC3* sequence on the 5′ side. After amplification of the *CRTC3* gene with primer CRTC3A and primer A and amplification of the *MAML2* gene with primer B and MAML2A, both polymerase chain reaction (PCR) products are mixed together, and the fusion gene is amplified by PCR using primers CRTC3A and MAML2A. Arrows indicate breakpoints.

Positive Controls for the *CRTC1*, *CRTC2*, and *CRTC3* Fusion Transcripts

Clinical samples known to possess the *CRTC1–MAML2* fusion were used as the positive control for the *CRTC1–MAML2* fusion transcript.[7] For positive controls for the other two fusions (*CRTC2–MAML2* and *CRTC3–MAML2*), we synthesized their cDNAs *in vitro*, as we previously described.[12] Figure 1 and Table 1 show the artificial generation of *CRTC3–MAML2* cDNA using a series of polymerase chain reaction (PCR) primers. Briefly, a *CRTC3*-side gene fragment was amplified by the PCR using CRTC3A (20 mer) and a primer A (40 mer) containing both *CRTC3* (20 mer) and *MAML2* (20 mer) sequences. Similarly, a *MAML2*-side fragment of the fusion cDNA was amplified by using a primer B (40 mer) containing both 20-mer *MAML2* and 20-mer *CRTC3* sequences and a MAML2A primer (20 mer). The

Table 1 Sequences of primers

Primer	*Sequence (5′–3′)*
CRTC1A (outer)	tcgcgctgcacaatcagaag
CRTC1B (inner)	gaggtcatgaaggacctgag
CRTC2A (outer)	ttgcgctgcagaagcagcgt
CRTC2B (inner)	ggaggtgatgatggacatcg
CRTC3A (outer)	tcgcgctgcacacgcagaga
CRTC3B (inner)	cagagacaggccgaggagac
MAML2A (outer)	ggtcgcttgctgttggcagg
MAML2B (inner)	ttgctgttggcaggagatag

CRTC3-side and *MAML2*-side PCR products were diluted to 1:1000, then mixed in a 1:1 ratio, and subjected to PCR using CRTC3A and MAML2A primers. The objective fusion product thus produced was subcloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), and its sequence was confirmed. It was then used as the positive control for *CRTC3–MAML2* fusion. Following this strategy, *CRTC2–MAML2* cDNA was synthesized and used as a positive control. The breakpoints of *CRTC2* and *CRTC3* were set so that the exon 1 (*CRTC2* and *CRTC3*) was fused to *MAML2* exon 2.[7,11] No *CRTC2–MAML2* fusion has been reported, but we assumed that *CRTC2* exon 1 would fuse to *MAML2* exon 2, based on the homology between *CRTC1*, *CRTC2*, and *CRTC3* genes and the constant breakpoint of the *MAML2* gene when involved in creating the fusion.

Detection of the *CRTC1–*, *CRTC2–*, and *CRTC3–MAML2* Fusion Transcripts

CRTC1–,*CRTC2–*, and *CRTC3–MAML2* fusion transcripts were detected using a method consisting of one-tube reverse transcription (RT)-PCR and nested PCR.[7] Total RNA was extracted from formalin-fixed, paraffin-embedded specimens as previously described.[7] Deparaffinized tissue sections were incubated at 56°C overnight in protease K digestion buffer, and RNA was extracted using concentrated phenol/guanidine isothiocyanate (Trizol LS; Gibco BRL, Friendswood, TX, USA), followed by DNase I treatment (Takara, Otsu, Japan). Then, 5 μl of the extracted RNA were heated to 70°C and placed on ice. The RT-PCR mixture containing outer primers was added. The thermocycler was programmed for an initial RT incubation of 30 min at 42°C and then for 10 min at 95°C for the inactivation of RT as well as for the activation of DNA polymerase. This was followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The products were then diluted 1:50 with water, and subjected to a nested PCR using inner primers. The amplification conditions consisted of 35 cycles at 95°C for 30 s and at 60°C for 30 s. Primers used in this study are shown in Table 1. Primers used for *CRTC1–MAML2*, *CRTC2–MAML2* and *CRTC3–MAML2* transcripts were newly designed. As an internal control for RNA quality, the ubiquitously expressed β-actin mRNA fragment (190 bp) was amplified. All specimens were shown to possess RNA of satisfactory quality. The normal salivary gland tissue was used as a negative control.

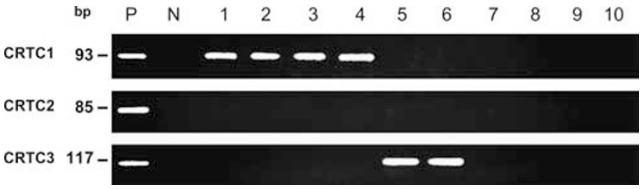

Figure 2 Detection of *CRTC1–MAML2* fusion (indicated as *CRTC1*, 93 bp), *CRTC2–MAML2* (*CRTC2*, 85 bp), and *CRTC3–MAML2* (*CRTC3*, 117 bp). P, positive control; N, negative control; bp, base pair; lanes 1–4, tumors positive for *CRTC1–MAML2* fusion; lanes 5, 6, tumors positive for *CRTC3–MAML2* fusion; lanes 7–10, tumors with no detectable *MAML2*-associated fusion.

Statistical Analysis

Statistical evaluation of data from two groups was carried out using the Fischer's exact test and Student's *t*-test. All analyses were two-tailed. To identify the parameters significantly associated with disease-free and overall survivals, the survival rate was calculated by the Kaplan–Meier method, and the statistical difference was estimated using log-rank test. A value of $P<0.05$ for each test was regarded as statistically significant, and $0.05<P<0.1$ as marginally significant. All the analyses were performed using the statistical package JMP v5 (SAS Institute, Cary, NC, USA).

Results

Of 101 cases of mucoepidermoid carcinoma, *CRTC1–MAML2* and *CRTC3–MAML2* fusion transcripts were detected in 34 (34%) and 6 (6%) cases, respectively (Figure 2). These two fusions were mutually exclusive. All fusion transcripts were fused in-frame, and none of the positive cases showed any atypical transcript, such as an insertion or deletion, as confirmed by direct sequencing. The *CRTC2–MAML2* fusion transcripts were not detected in any case of mucoepidermoid carcinoma studied. Non-mucoepidermoid carcinoma tumors, including adenoid cystic carcinomas, squamous cell carcinomas, pleomorphic adenomas, and Warthin's tumors were negative for the three fusion transcripts investigated.

Table 2 shows a summary of the clinical features, treatment, and the outcome of six patients with *CRTC3–MAML2*-positive mucoepidermoid carcinomas. Two patients were male and four were female patients. The ages ranged from 24 to 53 years (mean 36, median 32). Three tumors were found in the major salivary gland (the parotid), and the

Table 2 Clinicopathological features of patients with *CRTC3–MAML2* fusion-positive mucoepidermoid carcinoma

Case	*Age (years)*	*Sex*	*Tumor site*	*Tumor size (mm)*	*Nodal status*	*TNM*	*Clinical stage*	*Histological grade*	*Cystic component*	*Neural invasion*	*Necrosis*	*Mitoses (10 HPF)*	*Anaplasia*	*Treatment*	*Follow-up (months)*	*Outcome*
1	25	F	Parotid	18 × 15	Negative	T1N0M0	I	Low	5%	No	No	1	No	Resection	147	NED
2	27	F	Parotid	26 × 20	Negative	T2N0M0	II	Intermediate	10%	No	No	3	Yes	Resection	55	NED
3	24	F	Hard plate	27 × 20	Negative	T2N0M0	II	Low	30%	No	No	0	No	Resection	60	NED
4	47	M	Parotid	27 × 25	Negative	T2N0M0	II	Low	60%	No	No	0	No	Resection	113	NED
5	53	M	Oral floor	22 × 18	Negative	T2N0M0	II	Low	10%	No	No	1	No	Resection	28	NED
6	37	F	Retromolar	10 × 10	Negative	T1N0M0	I	Low	5%	No	No	0	No	Resection	39	NED

HPF, high-power filed; NED, no evidence of disease.

Figure 3 Histological findings of a *CRTC3–MAML2* fusion-positive mucoepidermoid carcinoma with low-grade histology (**a** and **b**, case 6). Focal anaplastic change is found in case 2 (**c**).

remaining three were found in the minor salivary gland (the hard palate, the oral floor, and the retromolar area). Results of laboratory tests including serum lactate dehydrogenase level were unremarkable in all six cases. All tumors were surgically

Table 3 Clinicopathological features of mucoepidermoid carcinomas with *CRTC3–MAML2*, *CRTC1–MAML2*, and no detectable gene fusion

Factor	*MAML2 fusion partner*			P		
	CRTC3 (n = *6*)	*CRTC1* (n = *34*)	*None* (n = *61*)	*CRTC3 vs CRTC1*	*CRTC3 vs none*	*CRTC1 vs none*
Age (years)						
Mean	36	55	58	0.01	0.0006	N.S.
Sex						
Male	2	15	38	N.S.	N.S.	N.S.
Female	4	19	23			
Tumor site						
Major	3	15	20	N.S.	N.S.	N.S.
Minor	3	19	41			
Tumor size						
<2 cm	2	15	16	N.S.	N.S.	0.007
>2 cm	4	19	45			
Nodal status						
Positive	0	2	24	N.S.	0.08	0.0003
Negative	6	32	37			
Clinical stage						
I or II	6	31	34	N.S.	0.07	0.0004
III or IV	0	3	27			
Histological grade						
Low+intermediate	6	34	37	N.S.	0.08	<0.0001
High	0	0	24			

N.S., non-significant.

resected without additional treatment, including chemotherapy or radiotherapy. Four tumors were more than 20 mm in diameter. Tumor metastasis to the regional lymph nodes was negative. Histologically, the fusion-positive tumors tended to grow in a solid pattern, and the cystic component was <20% in four cases. No case showed neural invasion, necrosis, or increased mitotic figures. One case showed focal anaplasia (case 2). The histological grade was low in five cases and intermediate in one, with no high-grade tumor (Figure 3). Two patients had clinical stage-I disease and four had stage-II disease. During the follow-up (28–147 months, median 57.5 months), all patients were alive with no evidence of tumor recurrence, and no tumor-related death was recorded.

Table 3 shows a clinicopathological comparison of *CRTC3–MAML2* tumors with *CRTC1–MAML2* or fusion-negative tumors. As compared with fusion-negative tumors, *CRTC3–MAML2* tumors occurred in younger patients (36 years *vs* 58 years, $P = 0.0006$) and tended to show negative nodal status, a less advanced clinical stage, and lower histological grade (marginally significant). Both *CRTC1–MAML2* and *CRTC3–MAML2* tumors had favorable clinicopathological features. However, patients with the *CRTC3–MAML2* fusion were significantly younger than those with the *CRTC1–MAML2* fusion (36 years *vs* 55 years, $P = 0.01$). Disease-free and overall survival is shown in Figure 4. For disease-free survival, patients with *CRTC3–MAML2* and *CRTC1–MAML2* fusions showed better prognosis than those with no detectable gene fusion, ($P = 0.06$ and $P = 0.03$, respectively). There was no significant difference in disease-free survival between patients with *CRTC3–MAML2* and *CRTC1–MAML2* fusions. For overall survival, all patients with *CRTC3–MAML2* and *CRTC1–MAML2* fusions were alive at the last follow-up. Patients with *CRTC3–MAML2* and *CRTC1–MAML2* fusions showed longer overall survival than those with no detectable gene fusion ($P = 0.09$ and $P = 0.0004$, respectively).

Discussion

In this study, we screened 101 cases of mucoepidermoid carcinoma and 89 cases of non-mucoepidermoid carcinoma tumors for *CRTC1–*,*CRTC2–*, and *CRTC3–MAML2* fusions using RT-PCR. Positive controls for these assays were either obtained from clinical tumor samples or synthesized *in vitro* in our laboratory. The *CRTC1–MAML2* and *CRTC3–MAML2* fusion transcripts were detected in 34 and 6% of mucoepidermoid carcinoma cases, respectively. These two fusions were mutually exclusive. The rate of positives for *CRTC3–MAML2* fusion in our series was four times higher than the 1.5% that

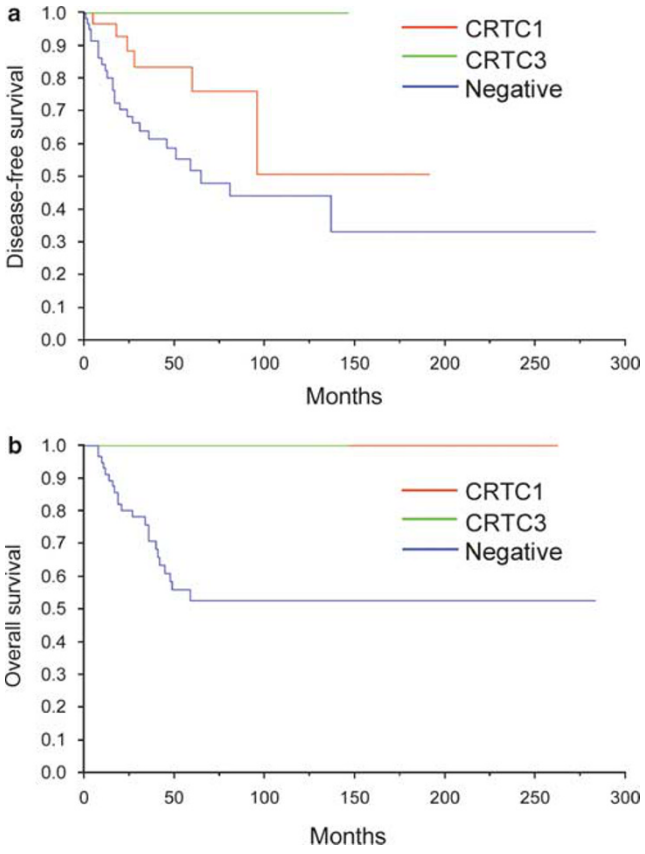

Figure 4 Disease-free (**a**) and overall (**b**) survival of patients with *CRTC1–MAML2*, *CRTC3–MAML2*, and no detectable gene fusion. Five-year disease-free survival for patients with *CRTC3–MAML2, CRTC1–MAML2*, and fusion-negative tumors is 100, 76, and 52%, respectively. Five-year overall survival for patients with *CRTC3–MAML2, CRTC1–MAML2*, and fusion-negative tumors is 100, 100, and 53%, respectively.

was reported by Fehr *et al*[11] Several factors that might explain this discrepancy include race (Asian *vs* Caucasian), geographical factors, case collection bias, and technical differences. Although Fehr *et al* used conventional RT-PCR, we used nested PCR after RT using gene-specific primers, assuming that our approach may be theoretically more sensitive. The *CRTC2* gene is another gene of the *CRTC* family,[10] and we failed to obtain evidence that *CRTC2–MAML2* fusion has a role in mucoepidermoid carcinomas or other salivary gland tumors.

It is interesting to note that *CRTC3–MAML2* fusion was found only in mucoepidermoid carcinoma cases, but not in non-mucoepidermoid carcinoma tumors. This observation suggests that this fusion may be specific to mucoepidermoid carcinoma, an observation similar to our previous findings as well as those of other groups who showed that *CRTC1–MAML2* fusion was found in this carcinoma but not in other salivary tumors.[13,7] Some researchers have reported that the *CRTC1–MAML2* fusion was found in a subset of Warthin's tumors,[8,9] whereas others have reported that the fusion occurred only in Warthin's tumors with atypical features.[14] All Warthin's tumors that we examined were typical in histology, and no atypical cases were included. Whether atypical Warthin's tumors possess *CRTC3–MAML2* fusion may be a subject of future investigation.

Our study showed that mucoepidermoid carcinomas positive for *CRTC3–MAML2* fusion had favorable clinicopathological features with no nodal metastasis and a less advanced clinical stage. Although one carcinoma showed focal anaplasia and was given an intermediate grade, five other carcinomas were scored as low grade. It is worth noting that during the follow-up period (median 57.5 months), all patients were alive with no evidence of tumor recurrence after surgery without additional radiation or chemotherapy. These findings suggest that in mucoepidermoid carcinomas positive for *CRTC3–MAML2* fusion, extensive surgery may not be necessary, and function-preserving tumor resection may well be warranted. However, we do not exclude a possibility that a subset of *CRTC3–MAML2* fusion-positive tumors would progress to high-grade tumors, which may be a heterogeneous group and probably include both *de novo* high-grade ones and those transformed from low-grade tumors.[15] Some high-grade mucoepidermoid carcinomas have been positive for *CRTC1–MAML2* fusion.[9]

In the same subtype of malignant tumor, a difference in the gene partners fused to one pivotal gene can have major clinicopathological implications. In alveolar rhabdomyosarcoma, patients with *PAX7–FKHR* fusion have a better prognosis than those with *PAX3–FKHR* fusion.[16] Synovial sarcoma patients with tumors positive for *SS18–SSX2* fusion show a longer overall survival than those with *SS18–SSX1* fusion. In addition, the former tumors are characterized by an exclusively monophasic tumor histology, whereas the latter tumors exhibit a monophasic or biphasic tumor histology.[17,18] In the current study, we presented preliminary data showing that patients with mucoepidermoid carcinoma harboring *CRTC3–MAML2* fusion may be younger than those with *CRTC1–MAML2* fusion. This finding is important for clarifying mucoepidermoid carcinoma oncogenesis, and further investigation is warranted.

In conclusion, we showed that *CRTC3–MAML2* fusion was detected in 6% of mucoepidermoid carcinomas and that the fusion-positive cases showed favorable clinicopathological features and no tumor recurrence after surgical resection alone. It is interesting to note that patients with this gene fusion were comparatively younger than those with the *CRTC1–MAML2* fusion or those with no detectable gene fusion. As *CRTC3–MAML2* fusion was found only in mucoepidermoid carcinomas, and as all patients with this fusion presented the favorable clinicopathological features and an indolent clinical course, the *CRTC3–MAML2* fusion may define a distinct clinicopathological subgroup.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)