Modern Pathology (2013) 26, 213–222
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Genomic profiles and *CRTC1–MAML2* fusion distinguish different subtypes of mucoepidermoid carcinoma

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Mucoepidermoid carcinoma is the most common salivary gland malignancy, and includes a spectrum of lesions ranging from non-aggressive low-grade tumors to aggressive high-grade tumors. To further characterize this heterogeneous group of tumors we have performed a comprehensive analysis of copy number alterations and *CRTC1–MAML2* fusion status in a series of 28 mucoepidermoid carcinomas. The *CRTC1–MAML2* fusion was detected by RT-PCR or fluorescence *in situ* hybridization in 18 of 28 mucoepidermoid carcinomas (64%). All 15 low-grade tumors were fusion-positive whereas only 3 of 13 high-grade tumors were fusion-positive. High-resolution array-based comparative genomic hybridization revealed that fusion-positive tumors had significantly fewer copy number alterations/tumor compared with fusion-negative tumors (1.5 *vs* 9.5; $P = 0.002$). Twelve of 18 fusion-positive tumors had normal genomic profiles whereas only 1 out of 10 fusion-negative tumors lacked copy number alterations. The profiles of fusion-positive and fusion-negative tumors were very similar to those of low- and high-grade tumors. Thus, low-grade mucoepidermoid carcinomas had significantly fewer copy number alterations/tumor compared with high-grade mucoepidermoid carcinomas (0.7 *vs* 8.6; $P < 0.0001$). The most frequent copy number alterations detected were losses of 18q12.2-qter (including the tumor suppressor genes *DCC*, *SMAD4*, and *GALR1*), 9p21.3 (including the tumor suppressor genes *CDKN2A/B*), 6q22.1-q23.1, and 8pter-p12.1, and gains of 8q24.3 (including the oncogene *MAFA*), 11q12.3-q13.2, 3q26.1-q28, 19p13.2-p13.11, and 8q11.1-q12.2 (including the oncogenes *LYN*, *MOS*, and *PLAG1*). On the basis of these results we propose that mucoepidermoid carcinoma may be subdivided in (i) low-grade, fusion-positive mucoepidermoid carcinomas with no or few genomic imbalances and favorable prognosis, (ii) high-grade, fusion-positive mucoepidermoid carcinomas with multiple genomic imbalances and unfavorable prognosis, and (iii) a heterogeneous group of high-grade, fusion-negative adenocarcinomas with multiple genomic imbalances and unfavorable outcome. Taken together, our studies indicate that molecular genetic analysis can be a useful adjunct to histologic scoring of mucoepidermoid carcinoma and may lead to development of new clinical guidelines for management of these patients.
Modern Pathology (2013) **26,** 213–222; doi:10.1038/modpathol.2012.154; published online 28 September 2012

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Received 2 April 2012; revised 20 July 2012; accepted 20 July 2012; published online 28 September 2012

Keywords: arrayCGH; *CDKN2A/B*; *CRTC1–MAML2* fusion oncogene; genomic imbalances; histologic subtyping; mucoepidermoid carcinoma

Mucoepidermoid carcinoma is the most common type of salivary gland carcinoma and may be found in both the major and minor salivary glands.[1–3] The histological classification of AFIP adopted by the WHO,[3–5] grade mucoepidermoid carcinoma based on histopathological features including a cystic component, nerve invasion, necrosis, mitotic activity, and cytological pleomorphism. It is recognized that high-grade mucoepidermoid carcinomas are associated with a high risk of recurrences, metastases, and tumor-related deaths, whereas low-grade mucoepidermoid carcinomas usually have an excellent prognosis and only rarely metastasize.[4–7] Nevertheless, all tumors with histologic appearances defined as mucoepidermoid carcinoma are considered malignant.[8] Although efforts have been made to identify clinically useful biomarkers for grading and prognostication[9–12] there is yet no unifying concept on how to classify mucoepidermoid carcinomas.

We previously identified a recurrent t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma.[13,14] Subsequent studies revealed that it results in a *CRTC1–MAML2* fusion in which the N-terminal Notch-binding domain of the coactivator MAML2 (Mastermind-like 2) is replaced by the CREB-binding domain of CRTC1.[15,16] An important molecular consequence of the fusion is the activation of cAMP/CREB target genes (Enlund *et al.*, unpublished data).[17,18] Several studies have now confirmed the initial report by Behboudi *et al*[19] demonstrating that the *CRTC1–MAML2* fusion primarily occurs in low-grade mucoepidermoid carcinomas with a favorable clinical outcome.[12,13,20,21] A second gene fusion involving the sarcoma-associated *EWSR1* gene and the stem cell regulator *POU5F1* was recently identified in mucoepidermoid carcinomas with a more immature morphology compared with *MAML2* positive tumors.[22]

Except for the *CRTC1–MAML2* fusion, little is known about other genomic rearrangements of importance for the genesis and progression of mucoepidermoid carcinoma.[23,24] To identify such alterations, we performed a genome-wide, array-based comparative genomic hybridization (arrayCGH) study of a series of 28 mucoepidermoid carcinomas. Our results demonstrate that low- and high-grade mucoepidermoid carcinomas have different genomic profiles and *CRTC1–MAML2* fusion status. Low-grade tumors have few or no genomic imbalances and are fusion-positive whereas high-grade tumors have numerous genomic imbalances and are often fusion-negative. The results provide additional evidence supporting that mucoepidermoid carcinoma is a heterogeneous tumor entity and that genetic biomarkers may be useful in identifying subgroups with different clinical outcomes.

Materials and methods

Patients and Tissue Specimens

A total of 28 mucoepidermoid carcinomas were analyzed in this study, including 13 archival formalin-fixed paraffin-embedded tumors and 15 fresh-frozen tumors. The samples were retrieved from the files of the Department of Pathology, Haartman Institute, University of Helsinki, Finland, the Department of Pathology, Sahlgrenska University Hospital, Gothenburg, Sweden, and from the Department of Oral Pathology, Guy's Hospital, London, UK. The diagnoses were reviewed by three pathologists and histopathological grading of the tumors was performed according to the WHO classification.[3] Other differential diagnosis of high-grade mucoepidermoid carcinomas, such as salivary duct carcinomas and adenosquamous carcinomas were excluded to the best of the authors' ability using morphology and immunohistochemical criteria described in the WHO classification.[3] Clinical follow-up data were obtained from the patients medical records. Ethical approval was obtained from the National Supervisory Authority for Welfare and Health in Finland (VALVIRA) (Dnro 1451/32/300/04 and 425/05.01.00.06/2009), and ethical approvals from the Ethics Committee of Helsinki University Hospital, Finland (Dnro 410/E9/05), Guy's Research Ethics Committee in London, UK (reference 02/10/14),and from the regional ethics committee in Gothenburg, Sweden (D-no: 178-08).

arrayCGH Analysis

Tumor samples were trimmed to remove adjacent non-neoplastic tissues. Genomic DNA was extracted from formalin-fixed paraffin-embedded and fresh-frozen tumor tissues as previously described.[25,26] A pool of normal female or male genomic DNAs obtained from peripheral blood cells (each from five normal individuals) was used as reference DNA. arrayCGH analysis was performed using the Human Genome CGH Microarray 44K and 244K oligonucleotide arrays (Agilent Technologies, Palo Alto, CA) as previously described and as recommended by the manufacturer.[26] The slides were subsequently scanned using the Agilent microarray confocal scanner G2565AA (Agilent Technologies). Images were analyzed using the Feature Extraction software (v7.5; Agilent Technologies) with intensity-dependent linear normalization to reduce inter-experimental variation.

Data analysis was carried out using Nexus Copy Number software v.4.1 (BioDiscovery, El Segundo, CA). Nexus Copy Number uses the Rank segmenta-

tion algorithm to define non-random regions of copy number alterations across the genome. Sex chromosomes were excluded from the analysis. The significance threshold for segmentation was set to $P = 1.0E-7$ and the log2 ratio thresholds for gain and loss were 0.4 and -0.3, respectively. The log2 ratio thresholds for high copy number gain/amplification and homozygous deletion were 1.0 and -1.0, respectively. A copy number alteration was considered recurrent if three or more tumor samples carried the same copy number alteration with a *p*-value of less than 0.05. Each aberration was checked manually to confirm the accuracy of the call. Regions partially or completely covered by a previously reported copy number variation (Database of Genomic Variants; http://dgvbeta.tcag.ca/dgv/app/news?ref=NCBI36/hg18) were excluded from the analysis.[27]

RT-PCR Analysis

The *CRTC1–MAML2* fusion transcript was detected by nested RT-PCR using primers located in exon 1 of *CRTC1* and exon 2 of *MAML2* as previously described.[16,19]

Fluorescence *In Situ* Hybridization (FISH) Analysis

FISH analyses for detection of the *CRTC1–MAML2* and *EWSR1–POU5F1* gene fusions were performed on 3 μm formalin-fixed paraffin-embedded sections using dual-color break-apart rearrangement probes for the *MAML2* (ZytoVision GmbH, Bremerhaven, Germany) and *EWSR1* genes (Vysis, Downer's Grove, IL) as previously described.[22,28]

To validate recurrent copy number alterations detected by arrayCGH we performed FISH analyses using locus-specific probes for *MALT1* located at 18q21.32 (*MALT1* Break-apart probe; Cytocell, Cambridge, UK) and *MECOM* located at 3q26.2 (*MECOM*/*RUNX1 t*(3;21) Fusion-probe; Kreatech Diagnostics, Amsterdam, The Netherlands). Locus-specific probes for *CCND1* (*IGH*/ *CCND1* Translocation Probe; Cytocell) and *HER2* (Vysis) were used to confirm amplifications involving 11q and 17q sequences. The protocols for pretreatment, hybridization, and posthybridization washes were essentially as recommended by the manufacturers. Fluorescence signals were digitized, processed, and analyzed using the CytoVision image analysis system (Applied Imaging International, Newcastle-Upon-Tyne, UK). Thirty to 300 nuclei were scored from each case.

Results

Clinical and Histopathological Characteristics

The clinical and histopathological data for all mucoepidermoid carcinoma patients are detailed in Table 1. Fifteen of the tumors were classified as low-grade and 13 as high-grade mucoepidermoid carcinomas (Figure 1). The mean age of patients with low-grade tumors was 47 years (range 7–73 years) whereas the corresponding figure for those with high-grade tumors was 64 years (range 29–85 years). Nine of the 13 patients with high-grade mucoepidermoid carcinomas developed distant metastases whereas none of the patients with low-grade tumors developed metastasis during the follow-up period (range 6–23 years). Recurrences were found in 5 of the 28 patients. They included both fusion-positive and negative tumors as well as low- and high-grade tumors.

CRTC1–MAML2 and *EWSR1–POU5F1* Fusion Gene Status

The *CRTC1–MAML2* fusion oncogene was detected by RT-PCR or FISH in 18 of the 28 mucoepidermoid carcinoma cases (64%). All 15 low-grade tumors were fusion-positive (Figure 2a) and 10 of 13 high-grade tumors (77%) were fusion-negative (Table 1 and Figure 2b). The *CRTC1–MAML2*-negative mucoepidermoid carcinomas were also analyzed by FISH for the *EWSR1–POU5F1* fusion using an *EWSR1* dual-color break-apart probe. None of the 10 *MAML2*-negative tumors showed a rearrangement consistent with an *EWSR1* gene fusion (data not shown).

Genomic Profiles in Mucoepidermoid Carcinoma

A detailed description of all copy number alterations identified are presented in Table 1. A total of 122 copy number alterations were recorded in 15 tumors (Figure 3a). The remaining 13 tumors had no detectable copy number alterations. The number of genomic imbalances per tumor was significantly lower in fusion-positive tumors compared with fusion-negative tumors (1.5 *vs* 9.5; $P = 0.002$) (Figure 3b). Twelve of the 18 fusion-positive tumors had normal genomic profiles whereas only one out of 10 fusion-negative tumors lacked copy number alterations. Two high-grade, fusion-positive mucoepidermoid carcinomas had copy number alterations with breakpoints in 11q21 and 19p13 consistent with *CRTC1–MAML2* fusions generated by t(11;19) translocations. The genomic profiles of fusion-positive and fusion-negative tumors were very similar to those of low- and high-grade tumors. Thus, low-grade mucoepidermoid carcinomas had significantly fewer copy number alterations per tumor compared with high-grade mucoepidermoid carcinomas (0.7 *vs* 8.6; $P < 0.0001$) (Figure 3c). The mean number of imbalances for high-grade fusion-positive tumors ($n = 3$) was 5.7 compared with 9.5 in high-grade fusion-negative tumors ($n = 10$). Twelve of 15 low-grade mucoepidermoid carcinomas had apparently normal genomic pro-

Table 1 Clinicopathological data, *CRTC1–MAML2* fusion status, and copy number alterations in 28 mucoepidermoid carcinomas

Case no.	*Sex/age (years)*	*Tumor site*	*Grade*	*Recurrence/ metastasis*	*CRTC1–MAML2 fusion status*	*Array format*	*Copy number alterations*	
							Gains	*Losses*
1	F/38	SMG	Low	−/−	+	44K	N	N
2	F/57	PG	Low	−/−	+	44K	N	N
3	M/25	OSG	Low	+/−	+	44K	N	N
4	F/60	PG	Low	−/−	+	44K	N	N
5	F/27	PG	Low	−/−	+	44K	N	N
6	F/46	OSG	Low	−/−	+	44K	N	N
7	M/63	OSG	Low	+/−	+	44K	N	N
8	M/69	PG	Low	−/−	+	44K	N	N
9	M/38	PG	Low	−/−	+	244K	N	N
10	M/70	PG	Low	−/−	+	244K	N	N
11	F/7	PG	Low	−/−	+	44K	3q22.3-qter	18q11.2-qter, 9p21.3
12	M/73	PG	Low	−/−	+	44K	N	N
13	F/18	OSG	Low	−/−	+	244K	N	N
14	M/68	OSG	Low	NDA	+	244K	6p22.1-21.32, 8q24.3, 9q33.3-q34.3, 11q12.2-q13.2, 12q24.23-q24.31, 19	N
15	F/43	PG	Low	−/−	+	244K	N	5q31.2
16	M/44	OSG	High	−/−	+	244K	8q24.3, 9q33.3-q34.3, 11q12.2-q13.2, 12p13.31, 12q13.1-q14.1, 12q24.31, 19	N
17	F/41	SLG	High	−/−	+	244K	11pter-q21, 19pter-p13.11	N
18	F/29	OSG	High	+/−	+	244K	8, 11pter-q21, 14, 19p13.2-p13.11	7q31.1-q31.2, 7q36.2-qter, 9p21.3-p21.2[b], 18
19	M/48	PG	High	−/+	−	44K	3q25.33-qter, 8p12-qter, 11q13.2-q13.5[a]	1pter-p35.5, 2q21.3-qter, 3p, 4p, 7q11.21-q21.11, 7q22.1-qter, 8p, 11q14.3-qter, 14q12-q32.12, 16q, 17p, 21q22.2.3, 22q
20	F/85	PG	High	−/+	−	44K		1p, 6q, 9p22.3-p21.1, 13q12.11-q13.22, 13q22.3-qter, 18q
21	F/78	PG	High	−/+	−	44K	10p14-p13, 10q21.2-q23.1	4, 5q13.2-q31.1, 9q21.13-q21.33, 18q
22	F/85	SMG	High	−/+	−	44K	N	6q
23	M/43	PG	High	−/+	−	44K	7q11.23-q21.2[a]	8pter-p12, 10q23.31-q23.33, 18q
24	M/85	PG	High	+/+	−	244K	3q22.2-qter, 5p, 6q15-q16.1, 8q11.1-q12.2, 8q21.11-qter, 18q11.2	6q16.1-q27, 9p21.3, 13q32.1-q34, 17q21.31, 18q11.2-qter
25	M/68	PG	High	+/+	−	244K	1q24.2-q32.1, 1q42.2-q43, 2pter-p24.1, 3q26.1-q28, 5q35.2-q35.3, 6p25.2-p23, 7pter-q31.2, 8q24.3, 13q12.3-q13.1, 14q13.2-q21.2, 16q11.2-q21, 17q21.32-qter, 18p11.32-p11.31, 20pter-p12.3	4pter-q22.1, 5pter-q15, 6q22.1-q23.1, 7q31.2-qter, 8pter-p21.1, 8q12.1-q22.1, 9pter-p21.1, 10q11.23-q23.1, 10q23.31-qter, 11p15.4-p15.1, 11q14.1-qter, 14q11.2-q13.2, 14q21.2-q24.1, 18q12.1, 18q12.2-qter
26	M/74	PG	High	−/+	−	244K	N	N
27	M/72	PG	High	−/+	−	44K	2q31.1-q32.2, 5pter-p15.31, 17q12[a], 17q12-q21.2[a]	9p21.3
28	F/81	SMG	High	NDA	−	244K	3q, 5p, 8q, 9p13.3-qter, 11q12.2-q13.2, 19	2, 3p, 5q11.1-q31.1, 8p, 9p22.1-p21.2[b], 12, 15q11.2-q15.1, 15q21.1-q22.2, 15q24.2-qter, 18, 21q11.2-q22.11

Abbreviations: N, no copy number alterations; NDA, no data available; OSG, (intra)oral salivary gland; PG, parotid gland; SMG, submandibular gland; SLG, sublingual gland.
[a]High-level amplification.
[b]Homozygous loss.

files. The three remaining tumors showed a total of 10 copy number alterations. In contrast, 12 of the 13 high-grade mucoepidermoid carcinomas contained 1–29 copy number alterations per tumor (Table 1).

At least 13 recurrent minimal common regions of copy number losses and gains were identified in the 28 mucoepidermoid carcinomas (Table 2). A list of the genes located in these minimal common regions is shown in Supporting Information Table S1. The most frequently lost regions were 18q12.2-qter (eight cases; including the tumor suppressor genes *DCC*, *SMAD4*, and *GALR1*), 9p21.3 (seven cases; including the tumor suppressor genes *CDKN2A/B*), 6q22.1-q23.1 (four cases), 8pter-p12.1 (four cases), 5q13.2-q15 (three cases), and 4p (three cases) (Figures 4a and b). The deletions involving 9p21.3 were found in both high- ($n=6$) and low-grade ($n=1$) mucoepidermoid carcinomas and included an approxi-

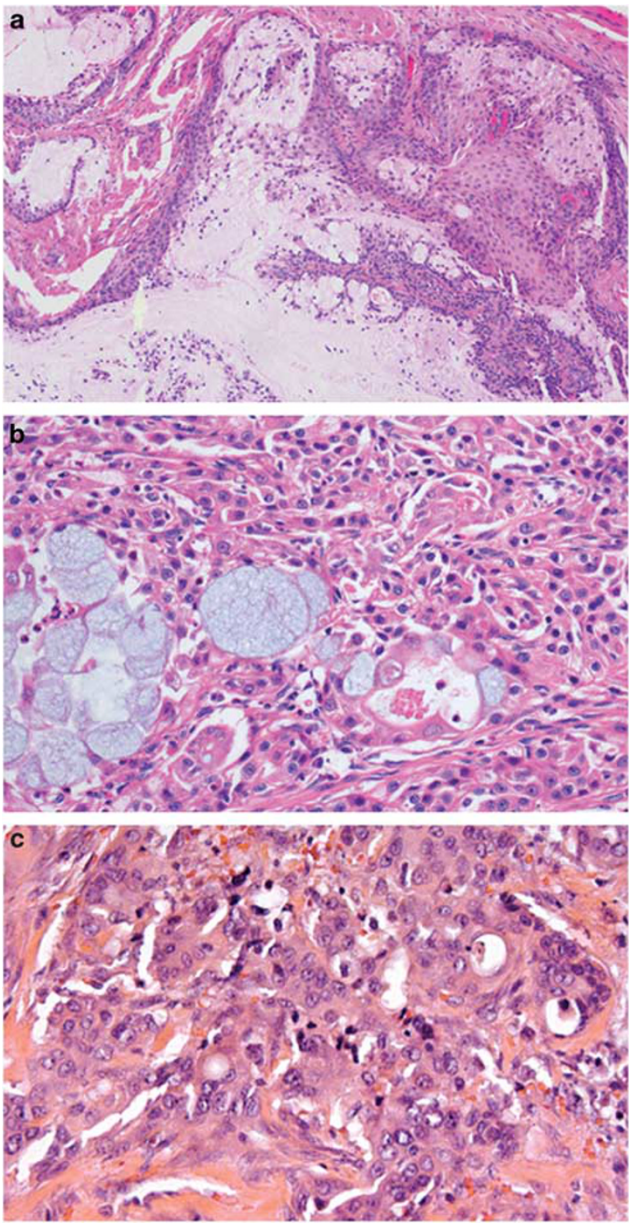

Figure 1 Photomicrographs of mucoepidermoid carcinomas. (**a**) Low-power view of case 6 showing a low-grade *CRTC1–MAML2* fusion-positive mucoepidermoid carcinoma. (**b**) High-power view of case 18 showing a high-grade fusion-positive mucoepidermoid carcinoma. (**c**) High-power view of case 23 showing a high-grade fusion-negative mucoepidermoid carcinoma.

mately 60 kb minimal common region containing *CDKN2A/B*. Two tumors had homozygous deletions of 2.3 and 6.2 Mb, respectively, involving the *CDKN2A/B* genes. The most frequently gained regions were 8q24.3 (seven cases; including the *MAFA* candidate oncogene), 11q12.3-q13.2 (six cases), 3q26.1-q28 (five cases), 19p13.2-p13.11 (five cases), 8q11.1-q12.2 (four cases; including the *LYN*, *MOS*, and *PLAG1* oncogenes), 5pter-p15.31 (three cases), and 9q33.3-q34.3 (three cases) (Figure 4c). Recurrent gain of one chromosome 19 was seen in three tumors, two of which were fusion-positive. Three high-grade, fusion-negative mucoepidermoid carcinomas had amplifications with single continuous amplicons at 7q11.23-q21.2, 11q13.2-q13.5, and 17q12-q21.2, respectively (Table 1). A list of all genes located within these three amplicons is shown in Supporting Information Table S2. The 11q13.2-13.5 amplicon included the *CCND1* gene and the 17q12-q21.2 the *ERBB2* gene.

We also analyzed separately the arrayCGH profiles for the 18 *CRTC1–MAML2* fusion-positive mucoepidermoid carcinoma cases. Six tumors contained 1–8 copy number alterations per tumor whereas 12 cases had normal genomic profiles. The following copy number alterations were detected in ≥ 3 mucoepidermoid carcinoma samples: gains of 11q12.2-q13.2 (four cases), 19p13.2-p13.11 (four cases), 8q24.3 (three cases), and 9q33.3-q34.3 (three cases).

Validation of Copy Number Alterations Using FISH

To confirm the copy number alterations detected by arrayCGH, we performed FISH analysis of tumors with losses involving 18q, gains of 3q, and amplifications of 11q13.2-13.5 and 17q12-q21.2. Using a probe for *MALT1*, located at 18q21.32, we could confirm loss of this locus in all six tumors analyzed (cases 11, 18, 20, 21, 23, and 25). Sixty to seventy-five percent of the tumor cells showed a single *MALT1* signal consistent with loss of one 18q allele (Figure 2c). Similarly, the FISH analysis confirmed gain of the *MECOM* gene located at 3q26.2 in all three cases analyzed (cases 11, 19, and 25). Fifty to ninety-five percent of the tumor cell nuclei displayed three to seven signals consistent with gains of this gene complex (Figure 2d). Amplifications of *CCND1* and *ERBB2* were also confirmed by FISH in cases 19 and 27, respectively. More than 90% of the tumor cell nuclei in both cases showed multiple signals and/or clusters of signals consistent with amplification of *CCND1* (Figure 2e) and *ERBB2* (Figure 2f).

Discussion

Using high-resolution arrayCGH, FISH, and RT-PCR we have performed a comprehensive analysis of genomic imbalances and gene fusion status in a series of histologically and clinically well-characterized mucoepidermoid carcinomas. To our knowledge, this is the largest and most comprehensive array-based CGH study performed on mucoepidermoid carcinoma. Previous analyses of genomic imbalances in mucoepidermoid carcinoma using CGH are limited to one chromosomal-based CGH-study of 16 tumors[23] and one array-based study of 15 tumors[24] (Supporting Information Table S3). Both studies showed similar percentages of *CRTC1–MAML2* fusion-positive tumors (58% *vs* 61%). The most prominent finding in these studies was loss of the *CDKN2A* gene in a subset of fusion-positive

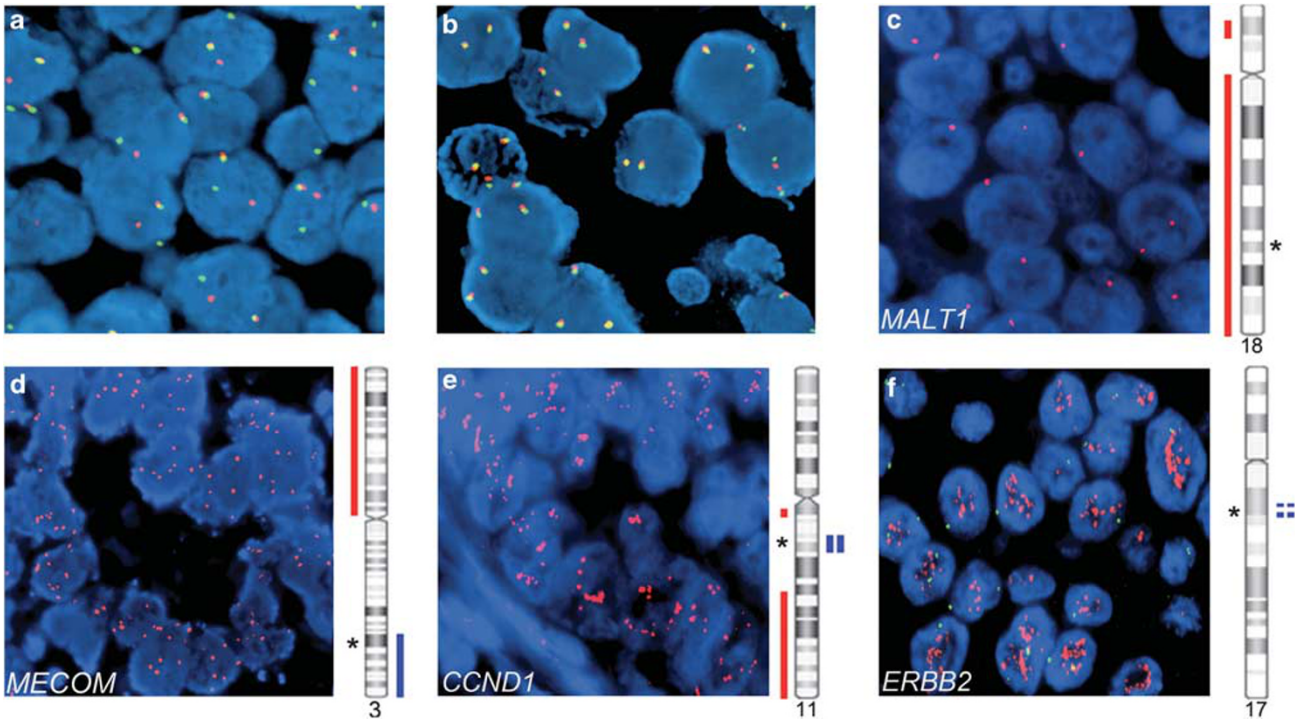

Figure 2 FISH analyses of *CRTC1–MAML2* (**a**, **b**) and copy number alterations detected by arrayCGH (**c–f**) in mucoepidermoid carcinoma. FISH analysis of the *CRTC1–MAML2* fusion gene using a *MAML2* break-apart probe in a fusion-positive (**a**) (case 3; separated green and red signals) and fusion-negative (**b**) (case 21; fused green and red signals) mucoepidermoid carcinoma. arrayCGH and FISH analyses showing loss of the *MALT1* locus at 18q21 (one red signal/nucleus) in case 18 (**c**), gain of the *MECOM* locus at 3q26 (multiple red signals/nucleus) in case 19 (**d**), amplification of *CCND1* at 11q13 (multiple single and clustered red signals) in case 19 (**e**), and amplification of *ERBB2* at 17q12 (multiple single and clustered red signals) in case 27 (**f**). The locations of the genes on the respective chromosomes are indicated by an asterics. Losses are indicated by red lines to the left of each chromosome and gains with blue lines to the right of each chromosome.

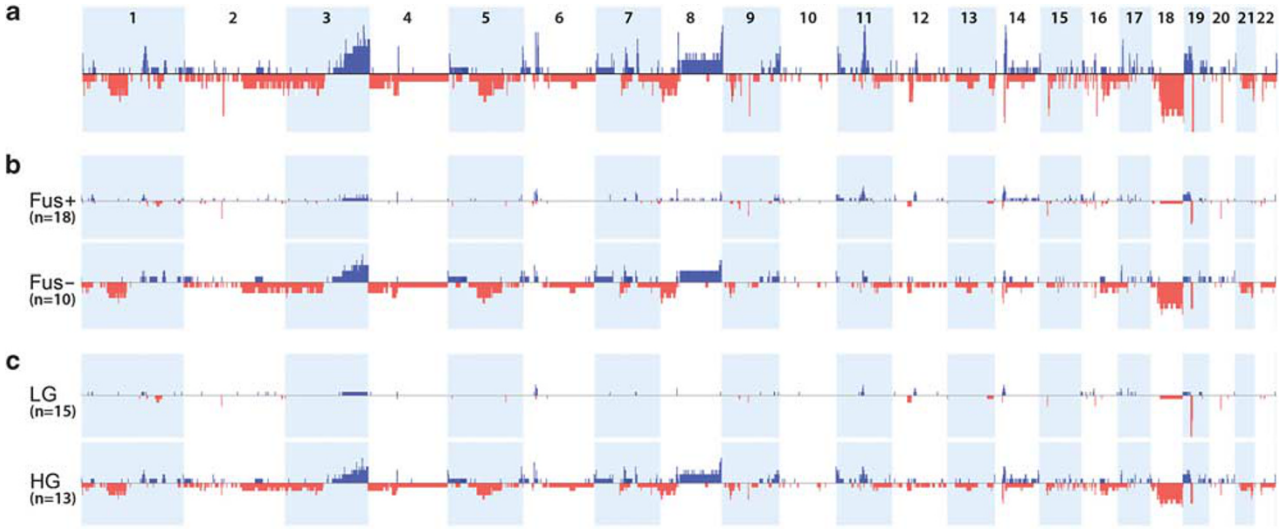

Figure 3 Genome-wide frequency plot of copy number alterations in 28 mucoepidermoid carcinomas. A total of 122 copy number alterations were detected across the genome (sex chromosomes excluded). Losses (red) were more common than gains (blue) (**a**). Distribution of the different copy number alterations in *CRTC1–MAML2*-positive (Fus +) and -negative (Fus −) tumors (**b**) and in low-grade (LG) and high-grade (HG) mucoepidermoid carcinomas. The number of genomic imbalances per tumor was significantly lower in fusion-positive tumors compared with fusion-negative tumors (1.5 *vs* 9.5; $P=0.002$). Similarly, LG mucoepidermoid carcinomas had significantly fewer copy number alterations per tumor compared with HG mucoepidermoid carcinomas (0.7 *vs* 8.6; $P<0.0001$). Note that the profiles of fusion-positive and LG tumors are very similar and that the profiles of fusion-negative and HG tumors are almost identical.

Table 2 Recurrent minimal common regions of gains and losses in 18 fusion-positive and 10 fusion-negative mucoepidermoid carcinomas

Chromosome band	*Region coordinates*	*Region length (bp)*	*CNA*	*No. of tumors*	*No. of genes*	*Candidate genes*
3q26.1-q28	Chr 3: 169 910 015–192 895 824	22 985 809	Gain	5	205	*MECOM, SKIL, ECT2, BCL6*
4p	Chr 4: 0–48 904 229	48 904 229	Loss	3	394	
5pter-p15.31	Chr 5: 0–7 968 073	7 968 073	Gain	3	77	*TERT*
5q13.2-q15	Chr 5: 70 708 950–95 413 966	24 705 016	Loss	3	177	*ENC1, THBS4*
6q22.1-q23.12	Chr 6: 114 705 548–130 872 124	16 166 577	Loss	4	93	*PTPRK*
8pter-p12	Chr 8: 0–35 922 584	35 922 584	Loss	4	362	*DLC1, MTUS1*
8q11.1-q12.2	Chr 8: 47 067 661–62 167 963	15 100 302	Gain	4	73	*LYN, MOS, PLAG1*
8q24.3	Chr 8: 143 579 131–146 097 093	2 517 962	Gain	7	119	*MAFA*
9p21.3	Chr 9: 21 940 840–21 996 612	55 722	Loss	7	4	*CDKN2A, CDKN2B*
9q33.3-q34.3	Chr 9: 127 261 274–137 929 022	10 667 748	Gain	3	252	*SET, ABL1*
11q12.2-q13.2	Chr 11: 60 245 706–67 162 746	6 917 040	Gain	6	333	*VEGFB, FOSL1, RIN1*
18q12.2-qter	Chr 18: 33 201 038–76 117 153	42 916 115	Loss	8	232	*DCC, SMAD4, GALR1*
19p13.2-p13.11	Chr 19: 11 529 876–18 630 355	7 100 479	Gain	5	317	*JUNB, JUND*

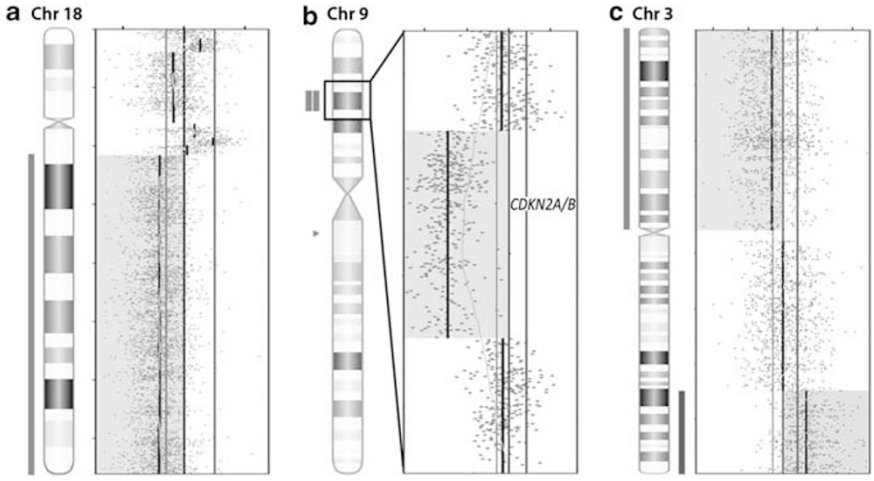

Figure 4 arrayCGH profiles of mucoepidermoid carcinomas demonstrating recurrent copy number losses involving 18q in case 24 (**a**), and 9p21.3 (including the tumor suppressor genes *CDKN2A/B*) in case 28 (**b**), and copy number gain of 3q in case 19 (**c**). The latter case showed also loss of 3p.

tumors and that loss of this gene was associated with an unfavorable prognosis.[24]

In the present study comprising 28 tumors, 12 of 15 low-grade mucoepidermoid carcinomas had apparently normal genomic profiles, which is consistent with the well-known non-aggressive clinical behavior of most low-grade mucoepidermoid carcinomas.[6,10] None of these tumors metastasized and only two recurred during the follow-up period. Moreover, all low-grade tumors were positive for the *CRTC1–MAML2* gene fusion, whereas only three of the 13 high-grade mucoepidermoid carcinomas were fusion-positive.[7,12,19–21] The remaining 10 fusion-negative, high-grade tumors were also negative for the *EWSR1–POU5F1* fusion previously identified in high-grade mucoepidermoid carcinomas.[22] These findings support the notion that low-grade mucoepidermoid carcinomas are fusion-positive and genetically stable tumors with few genomic imbalances. The fact that *CRTC1–MAML2* is a potent oncogene with effects on critical signaling pathways (Enlund *et al*, unpublished data)[17,29] might at least partly explain why these tumors contain relatively few copy number alterations.

To identify genetic events that may cooperate with *CRTC1–MAML2* in mucoepidermoid carcinoma tumorigenesis or disease progression we performed an unbiased search for recurrent copy number alterations in fusion-positive mucoepidermoid carcinomas. In the six fusion-positive tumors with genomic imbalances we detected four copy number alterations that were found in ≥ 3 cases, that is gains of 11q12.2-q13.2, 19p13.2-p13.11, 8q24.3, and 9q33.3-q34.3. Two of these gains, 11q12.2-q13.2 and 19p13.2-p13.11, were only recurrent in fusion-positive tumors, suggesting that they may harbor genes which can cooperate with *CRTC1–MAML2* in mucoepidermoid carcinoma tumorigenesis. Of interest, it was recently shown that gain of 11q13.1 in fusion-positive mucoepidermoid carcinomas is associated with unfavorable prognosis.[23] The 11q12.2-q13.2 and 19p13.2-p13.11 segments contain several

cancer-associated genes such as *RIN1*, *FOSL1*, and *VEGFB* (11q12.2-q13.2) and *JUNB* and *JUND* (19p13.2-p13.11) that are duplicated/rearranged and/or overexpressed in various forms of epithelial cancers.[30–32] Whether the 11q and 19p gains in mucoepidermoid carcinoma target any of these genes remains, however, to be shown.

The most frequent copy number alterations detected in the 28 mucoepidermoid carcinomas were losses of 18q12.2-qter, 9p21.3, 6q22.1-q23.1, and 8pter-p12.1, and gains of 8q24.3, 11q12.3-q13.2, 3q26.1-q28, 19p13.2-p13.11, and 8q11.1-q12.2. The frequencies of these copy number alterations varied from 11 to 29%, suggesting that there are no high-frequency copy number alterations in mucoepidermoid carcinoma and that a given copy number alteration therefore is likely to be of pathogenetic importance only for a subset of patients. The most common copy number alteration was loss of 18q12.2-qter found in 29% of the mucoepidermoid carcinomas. Heterozygous loss of 18q has been reported in several types of carcinomas, for example colon cancer,[33] pancreatic carcinoma,[34] and head and neck squamous cell carcinoma.[35] These losses, including the tumor suppressor genes *SMAD4*, *DCC*, and *GALR1*, are associated with tumor progression and unfavorable prognosis.[33,34,36] Of interest, all but one of our mucoepidermoid carcinomas with loss of 18q were high-grade tumors that developed metastases (five cases) and/or recurrences (three cases).

The second most common copy number loss included an approximately 60 kb minimal common region within 9p21.3, harboring the tumor suppressor genes *CDKN2A*/*B*. In two of the tumors the deletions were homozygous. The 9p deletions were detected in one low- and six high-grade mucoepidermoid carcinomas. All five high-grade tumors with known follow-up data developed metastases and/or recurrences. These findings are partly in agreement with recent data showing that fusion-positive mucoepidermoid carcinomas with inactivating *CDKN2A* deletions have an unfavorable prognosis.[24] Taken together, the present and previous studies show that *CDKN2A* deletions do occur in both fusion-positive and fusion-negative mucoepidermoid carcinomas and that they are associated with an unfavorable outcome. Deletion or hypermethylation of *CDKN2A* is a frequent oncogenic event in various types of carcinomas, including for example lung cancer, head and neck squamous cell carcinoma, and salivary duct carcinoma.[37–39] Further studies will be needed to confirm the significance of *CDKN2A* deletions in mucoepidermoid carcinomas with and without *CRTC1–MAML2* gene fusion.

The most frequent copy number gain was a 1.4 Mb minimal common region in 8q24.3 that was gained in seven tumors. This region is also frequently gained in several other types of carcinomas.[40,41] An interesting candidate target gene of these gains is *MAFA*, an oncogene with transforming properties that is overexpressed in multiple myeloma and a subtype of T-cell lymphoma.[42] We also detected recurrent gains of 8q11.1-q12.2 (harboring the *LYN*, *MOS*, and *PLAG1* oncogenes) in four high-grade tumors. This is of special interest because we recently showed that gain of a 1.4 Mb segment in 8q12.1, containing the *PLAG1* gene, is of importance for malignant transformation of benign salivary pleomorphic adenomas.[43,44]

In summary, we have shown that low-grade mucoepidermoid carcinomas have normal or near-normal genomic profiles, express the *CRTC1–MAML2* fusion, and have a favorable clinical outcome. In contrast, the majority of high-grade tumors had multiple genomic imbalances, were negative for the *CRTC1–MAML2* fusion, and developed frequent metastasis and/or recurrences. On the basis of these results we propose a subdivision of mucoepidermoid carcinomas, in (i) low-grade, fusion-positive mucoepidermoid carcinomas with no or few genomic imbalances and a favorable prognosis, (ii) high-grade, fusion-positive mucoepidermoid carcinomas with multiple genomic imbalances and an unfavorable prognosis, and (iii) high-grade, fusion-negative tumors with multiple genomic imbalances and an unfavorable clinical outcome. The latter tumors likely constitute a heterogeneous group of diverse high-grade adenocarcinomas with some mucoepidermoid carcinoma-like morphologic features. Taken together, the present and previous studies indicate that molecular genetic analysis can be a useful adjunct to histologic scoring of mucoepidermoid carcinoma and may lead to development of new clinical guidelines for management of these patients and ultimately also to new therapeutic strategies.

Acknowledgements

This study was supported by grants from the Finnish Cancer Society, Helsinki University Hospital Fund, Maritza and Reino Salonen Foundation, Finska Läkaresällskapet (Helsinki), Swedish Cancer Society, BioCARE—a National Strategic Research Program at University of Gothenburg, and the Royal Academy of Arts and Sciences in Gothenburg.

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)