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From Cell Biology to Tissue Engineering

Immunohistochemical expression profiles of mucin antigens in salivary gland mucoepidermoid carcinoma: MUC4- and MUC6-negative expression predicts a shortened survival in the early postoperative phase

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Summary. In mucoepidermoid carcinoma (MEC), the most common salivary gland carcinoma, there is a lack of novel prognostic markers, but post-operative early recurrence strongly affects the clinical course and a poor outcome. It is critical to predict which MEC patients are prone to develop recurrence/metastases. Mucins play pivotal roles in influencing cancer biology, thus affecting cell differentiation, adhesion, carcinoma invasion, aggressiveness and/or metastatic potential. Our aim is to elucidate the significance of expression profiles for mucins, particularly MUC4 and MUC6, and their correlations with various clinicopathological features and recurrence in salivary gland MECs. We performed immunohistochemical analyses on patients with surgically resected primary MEC using antibodies against mucin core proteins MUC4/8G7 and MUC6/CLH5 in 73 paraffin-embedded samples. Recurrence was noted in 15 of 73 (20.5%) patients. MUC4 or MUC6 expression was considered to be negative when <30% or 0% of the MEC cells showed positive staining, respectively. MUC4- and/or MUC6-negative expression respectively and variably showed a significant relationship to pathological tumor high-grade, the presence of lymphovascular invasion, lymph node metastasis and/or tumor-related death. In addition, MUC4 showed significantly negative co-expression with MUC6. Kaplan-Meier analyses revealed that not only single MUC4/6-negative expression but also the combination of both predicted significantly shorter disease-free and disease-specific survivals in MECs, especially within the first two years postoperatively. Therefore, each mucin plays a pivotal role in the pathogenesis of MEC progression. The detection of MUC4 and/or MUC6 might be a powerful parameter in the clinical management of MECs in the early postsurgical phase.

Key words: Mucoepidermoid carcinoma (MEC), Immunohistochemistry, MUC4, MUC6, Disease-free survival (DFS), Disease-specific survival (DSS).

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Introduction

Mucoepidermoid carcinoma (MEC) has been regarded as the most common primary salivary gland malignancy worldwide, accounting for approximately 30%-40% of all salivary carcinomas (Coca-Pelaz et al., 2015). The World Health Organization defines MEC as 'a malignant glandular epithelial neoplasm characterized

by mucous, intermediate and epidermoid cells, with columnar, clear cell and oncocytoid features' (Goode and El-Naggar, 2005). MECs are histopathologically subdivided into three categories of low, intermediate and high grade, according to the cystic component rate, mitotic figure count, neural involvement, necrosis and anaplasia, based on the classic Armed Forces Institute of Pathology (AFIP) criteria proposed by Goode et al. (Goode et al., 1998). Although it has been suggested that this histopathological grading system is closely associated with the clinical outcome of MECs, the system is currently criticized for its inconsistent reproducibility and lack of predictive accuracy (Aro et al., 2011; Coca-Pelaz et al., 2015). Indeed, some low-grade MECs can show an unexpectedly aggressive course with recurrence and/or distant metastases, leading to a poor prognosis, whereas patients with high-grade MECs might experience substantially different (better or worse) clinical outcomes (Aro et al., 2011; Herd et al., 2012; Coca-Pelaz et al., 2015). Furthermore, MECs can occasionally pose a diagnostic challenge to clinicians, due to their vague, painless symptomatology, nonspecific imaging findings and/or grossly misleading appearances (Eversole, 1970; Coca-Pelaz et al., 2015). Although most MEC patients are successfully treated by surgical resection and radiation, there are very few alternative treatment modalities available for inoperable cases discovered at an already advanced stage or for postsurgical recurrence, resulting in a poor quality of life and prognosis (Chen et al., 2007; Coca-Pelaz et al., 2015). In addition, the molecular and genetic factors influencing the clinical picture of MECs, such as epigenetic modulation, remain to be clarified. Given this background, it is critical to predict which MEC patients are prone to develop recurrence/metastases with biologically aggressive behavior before and after surgery. However, no practical, accurate, reliable prognostic biomarkers are available at present, although several are being evaluated.

Glycosylation is a major type of post-translational modification of secretory and cellular proteins, altering the physicochemical properties and biological activities of these proteins (Brockhausen, 1999). As such, the initiation/progression of cancers is considered to be closely correlated with not only frequent aberrant glycosylation but subsequent alterations in cell-surface carbohydrate antigens, leading to changes in the functions of glycoproteins and transforming cellular phenotypes (Brockhausen, 1999). Mucin-type O-glycosylation encompasses diverse classes of glycoproteins and mucins, which are high-molecular-weight glycoproteins, comprising up to 80% of the total carbohydrate antigens in mammals (Brockhausen, 1999; Hollingsworth and Swanson, 2004; Yonezawa et al., 2008). More than 20 different human mucins have been identified, with two types established: membrane-bound mucins, including MUC1, MUC3 and MUC4; and gel-forming secreted mucins, including MUC2, MUC5AC and MUC6 (Yonezawa et al., 2011). Intriguingly, mucins tend to show a tissue-specific expression but display variably deregulated, aberrant expression patterns of one or more types in various carcinomas, including salivary carcinomas (Ho et al., 1995; Yonezawa et al., 2008, 2011).

It is noteworthy that mucin expression might be reduced along with a loss of organ specificity during malignant transformation and subsequent progression; in contrast, other new mucins may be aberrantly expressed, closely related to the presentation of shortened irregular glycan structures and easily influencing cell differentiation, adhesion, invasion and/or metastasis while worsening the clinical outcome (Hollingsworth and Swanson, 2004; Yonezawa et al., 2008, 2011). We found that, in contrast to MUC1/2, which may be a useful parameter for evaluating patient outcomes in various carcinomas of the digestive system (Higashi et al., 1999; Yonezawa et al., 2008, 2011; Hiraki et al., 2017), few studies have examined possible correlations between the MUC4/6 expression in malignant neoplasms, including salivary carcinomas, and the associated clinicopathological factors. However, other groups have reported that post-operative MEC patients with immunohistochemically MUC4-positive expression show a significantly better survival duration than those with negative expression, although those studies were conducted in relatively small cohorts (Alos et al., 2005; Handra-Luca et al., 2005). Nevertheless, to our knowledge, findings regarding the clinicopathological significance, especially the potential prognostic value, of mucin expression in MECs are limited and contradictory.

In the present study, we clarified the relationship between the immunohistochemical expression profiles of mucin antigens, focusing on MUC4 and MUC6, in relatively large samples of postsurgical MECs and their clinicopathological characteristics, including the tumor grade and post-operative recurrence and death.

Materials and methods

Patients

Surgically resected MEC tissues were studied in the present study. Pathological reports were reviewed to identify patients who underwent simple tumor extirpation (33 cases) or radical sialoadenectomy with cervical lymph nodes dissection (40 cases) for MEC between 1991 and 2016 at the Department of Pathology, Faculty of Medicine and Kagoshima University. Very few patients who suffered perioperative deaths, defined as death during the patient's initial hospitalization or within 30 days of surgery, were excluded from the study. A total of 73 patients with available follow-up data comprised the cohort of this retrospective study after further excluding those with the following characteristics: (a) other prior or concomitant malignant neoplasms; (b) coexisting medical problems of sufficient severity to shorten life expectancy and (c) treatment with adjuvant chemotherapies or radiotherapies prior to the

surgery. Thirty-two (43.8%) patients received re-operation (2 cases), adjuvant radiotherapy (4 cases), and adjuvant chemotherapy (2 cases) or chemo-radiotherapy (24 cases) after surgery. Regimens for the chemotherapy included cisplatin plus fluorouracil, cisplatin plus fluorouracil plus docetaxel, S-1 (TS-1; Taiho Pharmaceutical, Tokyo, Japan), and so on. Especially, 18 MEC patients with positive margin status received re-operation (1 case), adjuvant radiotherapy (4 cases), and adjuvant chemo-radiotherapy (4 cases) after surgery. All materials in this article were approved by the Ethical Committee of Kagoshima University Hospital (28-179). The surgical margins were considered to be involved when the presence of MECs at the lateral or deep margin was identified or the distance to the non-carcinomatous mucosa margin was substantially less than 1 mm. The duration of disease-free survival (DFS) and disease-specific survival (DSS) was defined as the interval from the date of surgery to recurrence (DFS) and the interval from the date of surgery to death except for patients who died from causes other than MEC (DSS) or the most recent clinic visit, respectively.

Pathological examinations

Three pathologists examined all resected specimens to confirm their histopathological features. The pathologic findings were described using the TNM Classification of Malignant Tumors, 7th edition, published by the International Union Against Cancer (UICC) (Sobin et al., 2009). All MECs were graded based on the three-tiered, low, intermediate and high, histological grading system from the AFIP proposed by Goode et al. (1998); and a grade of low or intermediate was considered to wholly indicate a 'low'-grade MEC. Clinical information was gathered from the patients' records, and no patients had a pre-operative biopsy specimen obtained from the MEC tumor sample. Patients were followed-up and evaluated postoperatively at approximately three- to six-month intervals using a physical examination, head, neck and chest computed tomography (CT) scans and/or measurements of blood cell counts and biochemistry. Additional examinations, including brain CT, magnetic resonance imaging (MRI) and bone scintigraphy, were performed if any symptoms or signs of recurrence were evident. The formalin-fixed, paraffin-embedded tissue blocks came from our Department of Pathology. Normal human salivary gland tissue was taken from non-tumor portions of the surgically-resected specimens and then stained with hematoxylin and eosin (H&E), modified elastic tissue-*Masson trichrome* (E-M) or were subjected to immunohistochemical analyses of sequential sections. The E-M and immunohistochemical Podoplanin (D2-40; DAKO, Glostrup Denmark; diluted 1:1) and S-100 protein (DAKO; diluted 1:20) staining clearly revealed the presence of lymphovascular invasion (LVI) and perineural invasion (ne), respectively (Hiraki et al., 2017). The results of E-M, D2-40 and S-100 protein staining are not shown.

Preparation of antibodies against mucins and secondary antibodies, and immunohistochemistry of tissue samples

Immunohistochemistry for various mucins was performed using the following established antibodies. For the immunohistochemical staining of each mucin, we used human malignant tumor cells of well- to moderately differentiated adenocarcinoma of the pancreas, or human non-carcinomatous epithelium of the pancreas and stomach, appropriately, as positive controls (Yonezawa et al., 2008; Hiraki et al., 2017). These antigens, including mucins, are summarized in Table 1. Next, biotinylated affinity-purified horse anti-mouse IgG and avidin-biotinylated horseradish peroxidase complex (ABC) were purchased as part of the Vectastain Elite ABC kit from Vector Laboratories (Burlingame, CA, USA), as described elsewhere (Higashi et al., 1999; Hiraki et al., 2017).

Evaluation of the immunohistochemical results by scoring

The immunoreactivity for mucins in each case was assessed semi-quantitatively by evaluating the proportion of positive cells compared to the total neoplastic MEC cells. To assess the membranous and intracytoplasmic MUC4 and MUC6 expressions, positive areas that were <30% and 0% of the total were considered to be negatively stained (i.e. positive areas comprising ≥30% and ≥1% of the total were positively stained). We selected and validated these immunohistochemical cut-off scores using a receiver operating characteristic (ROC) curve analysis (Hanley, 1989;

Table 1. List of antibodies, including mucins, used in the present study.

Antigens	Antibodies	Sources of antibodies	Dilution
MUC1 (core peptide)	DF3	Toray-Fuji Bionics	1:50
MUC2 (core peptide)	Ccp58	Novocastra	1:200
MUC4 (core peptide)	8G7	generated by one of authors (S. K. B)	1:3000
MUC5AC (core peptide)	CLH2	Novocastra	1:100
MUC6 (core peptide)	CLH5	Novocastra	1:100
Podoplanin	D2-40	DAKO	1:1
S-100	S-100	DAKO	1:20

Harada et al., 2016; Hiraki et al., 2017), as shown in Fig. 1. All patients were divided into two groups based on the mucin expression as follows: positive when the MUC1, MUC2, MUC4, MUC5AC or MUC6 staining was ≥25%, 5%, 30%, 1% or 1%, respectively, and negative when the staining was less than that. The distribution of the staining for mucins in the MEC and the adjacent non-neoplastic epithelium in each case was also assessed semi-quantitatively and compared.

All histological and immunohistochemical slides were evaluated by two independent observers (certified surgical pathologists in our department; T.H. and S.Y.) using a blind protocol design (the observers were blinded to the clinicopathological data). The agreement between the observers was excellent (more than 90%) for all antibodies investigated, as measured by the interclass correlation coefficient. For the few (less than 1%) instances of disagreement, a consensus score was determined by a third board-certified pathologists (A.T.) in our department (Kawatsu et al., 2014; Harada et al., 2016; Hiraki et al., 2017).

Statistical analyses

The significance of correlations was determined using Fisher's exact test or χ^2 test, where appropriate, in order to assess the relationships between the immunohistochemical expression and the clinicopathological features (Hiraki et al., 2017). Survival curves were plotted with the Kaplan-Meier method and compared with the log-rank test. Hazard ratios and 95% confidence intervals (95% CI) were estimated using univariate or multivariate Cox proportional hazard models (Kitada et al., 2013; Kawatsu et al., 2014; Harada et al., 2016; Hiraki et al., 2017). All statistical tests were two-tailed, with values of $P<0.05$ considered to be significant.

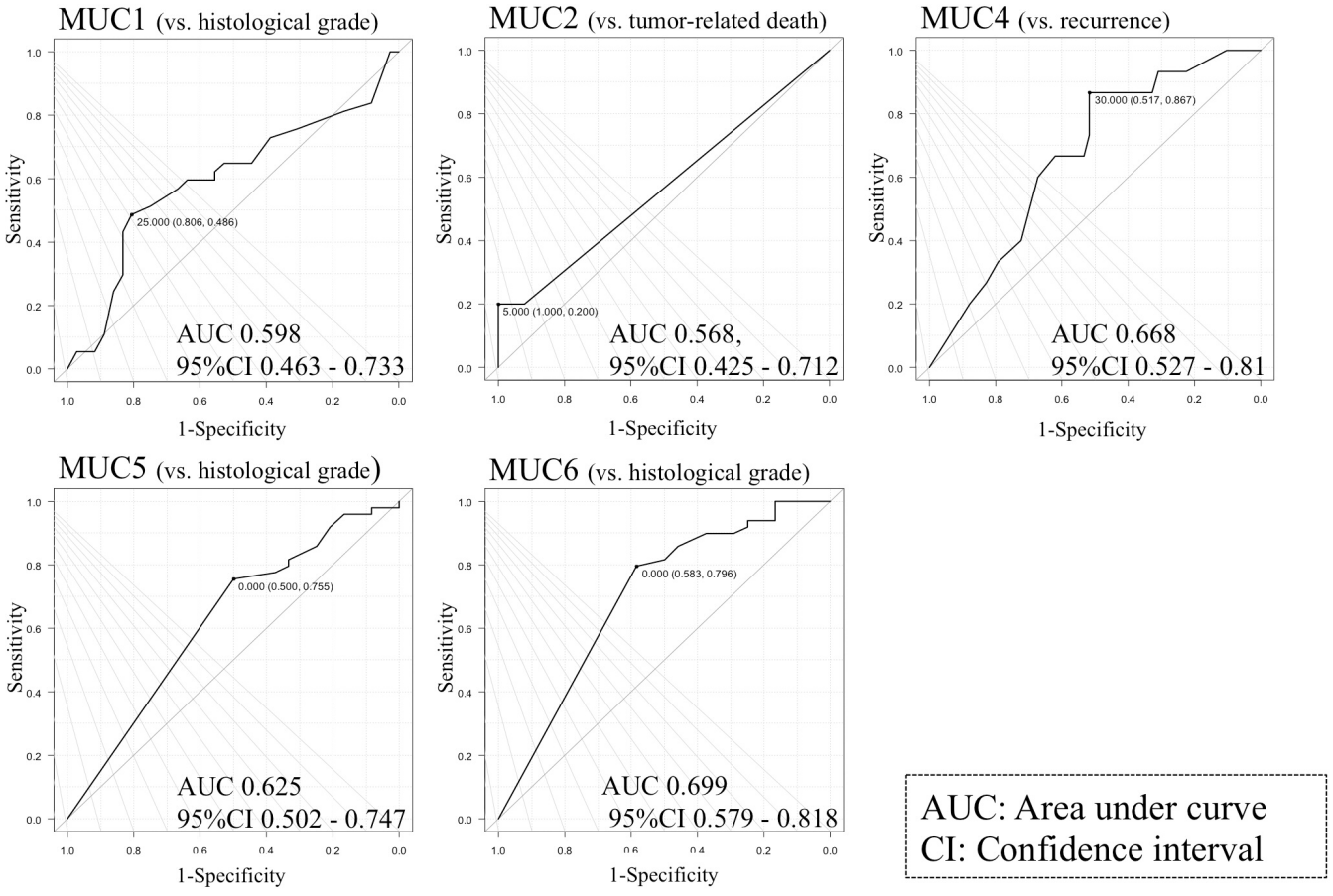

Fig. 1. The results of the receiver operating characteristic (ROC) curve analyses for selecting and validating the immunohistochemical cut-off points for each mucin negativity. We have chosen the cut-off values of each mucin expression using ROC and area under the curve (AUC), because an effective measure of accuracy has been considered as a meaningful interpretation. Finally, we selected 25, 5 30, 1 and 1, respectively, as a cut-off point for MUC1, MUC2, MUC4, MUC5AC and MUC6, since each AUC was the highest among all clinicopathological variables, including tumor size (T stage), lymph node metastasis (N stage), histologic grade, the presence of LVI, ne (perinural involvement), positive margin status (the surgical margins considered to be involved by the presence of MECs at the lateral or deep margin), recurrence or tumor-related death.

All of the above statistical analyses were performed with the EZR (Saitama Medical Center, Jichi Medical University, Japan) graphical user interface for the R software program (The R Foundation for Statistical Computing, version 2.13.0) (Kanda, 2013; Kitada et al., 2013; Hiraki et al., 2017). More precisely, it is a modified version of R commander (version 1.6-3) that incorporates the statistical functions frequently used in biostatistics.

Results

Clinicopathological features

The clinicopathological features of 73 patients with MEC who were able to be evaluated are summarized in Table 2. The range of age at surgery was 12-86 years (average and median were 59 and 62 years, respectively). The collected MECs were located at similar rates in the major (n=37, 50.7%; including 30 cases in parotid gland) and minor (n=36, 49.3%) salivary glands. T1-3 stage, except for T4 (14 patients) with extraparenchymal extension, was defined based on the tumor size at the greatest dimension: T1 (24 patients) for ≤2 cm; T2 (22 patients) for >2-4 cm; and T3 (13 patients) for >4 cm (Sobin et al., 2009). At diagnosis, 26 patients (35.6%) had lymph node metastases, but no patients (0%) had distant metastases. The tumors included 24 low-grade (32.9%), 12 intermediate-grade (16.4%) and 37 high-grade (50.7%) MECs, based on the well-known AFIP criteria (Goode et al., 1998), accounting for the cystic component rate, mitotic figure count, perineural involvement (ne), necrosis and anaplasia. The majority of the patients (n=41; 56.2%) had stage III-IV disease, while the other patients had stage I-II disease (n=32; 43.8%), according to the UICC criteria (Sobin et al., 2009). The resection margins of the majority of those MEC specimens (n=55; 75.3%) were free. Postsurgical follow-up data were available for all 73 patients (average: 42.1 months; range: 1-131 months). Post-operative recurrence was noted in 15 of 73 (20.5%) patients. The median DFS and DSS were 37.8 and 42.7 months, and their DFS and DSS rates were 80.8% and 87.5% at 2 years and 79.5% and 86.1% at 5 years, respectively. Table 3 displays each patient's information in detail.

Mucin expressions in normal salivary gland tissues and MEC specimens

MUC4 and MUC6 expressions were not apparently detectable in the adjacent non-neoplastic epithelium (Figs. 2, 3, left). On immunohistochemistry, MUC4 and MUC6 displayed intracytoplasmic and membranous, and intracytoplasmic expression patterns, respectively, in the typically positively-stained MEC cases, especially in the cystic components (Figs. 2, 3, inset, middle). Accordingly, representative 'low'-grade (low- to intermediate-grade) MEC cases demonstrated strongly positive expression of MUC4/6 (Figs. 2, 3, middle), whereas the histologically invasive fronts in the representative 'high'-grade (high-grade) MEC cases showed completely negative expression of MUC4/6 (Figs. 2, 3, right). MUC4-positive expression was noted in 34 cases (46.6%), and MUC6-positive expression was noted in 24 cases (32.9%). The profiles of all mucin expression patterns and clinicopathological features are presented in Table 3.

Table 2. MEC patients' clinicopathological characteristics.

Characteristic		Patients (n=73)
Age (years)	Average	59.2
	Median	62
	Range	12-86
	>60 years	40
	≤60 years	33
Sex	Male	39
	Female	34
Months after surgery	Average	42.1
	Median	33.0
	Range	1-131
T stage	T1	24
	T2	22
	T3	13
	T4	14
N stage	N0	47
	N1	6
	N2	20
TNM stage	Stage I,II	32
	Stage III,IV	41
Grade	Low	24
	Intermediate	12
	High	37
Cystic components (less than 20%)	(-)	18
	(+)	55
LVI	(-)	28
	(+)	45
Perineural invasion	ne (-)	44
	ne (+)	29
Necrosis	(-)	31
	(+)	42
Anaplasia	(-)	66
	(+)	7
Mitotic figures (more than 4)	(-)	35
	(+)	38
Margin status	(-)	55
	(+)	18
Recurrence	(-)	58
	(+)	15
Location	Major salivary gland	37
	Parotid gland	30
	Submandibular gland	4
	Sublingual gland	3
	Minor salivary gland	36
	Palatinal gland	6
	Other minor gland	30

Table 3. The detailed relationships between mucin expression and each patient's variables.

No.	Sex	Age (years)	Months after surgery	T stage	N stage	TNM stage	Grade	Cystic components (less than 20%)	LVI	Perineural invasion	Necrosis	Anaplasia	Mitotic figures (more than 4)	Surgical margin	Recurrence	MUC1	MUC2	MUC4	MUC5AC	MUC6
1	M	78	12	2	0	II	high	+	+	-	+	-	-	-	-	-	-	-	-	-
2	M	80	23	3	2b	IVA	high	+	+	+	+	+	+	+	+	-	-	-	-	-
3	M	83	45	2	0	II	intermediate	+	+	-	+	-	-	-	-	+	-	+	-	-
4	M	62	33	3	0	III	high	+	+	-	+	-	+	-	+	+	-	-	-	-
5	F	56	127	3	1	III	high	+	+	-	+	-	+	-	+	+	-	-	-	+
6	M	52	127	3	2b	IVA	intermediate	+	+	-	+	-	+	-	-	+	-	+	-	+
7	F	60	127	4a	0	IVA	high	+	+	-	+	+	+	+	+	+	-	-	-	-
8	M	74	127	1	0	I	low	+	-	-	-	-	-	-	-	+	-	-	-	-
9	F	62	127	1	0	I	low	-	-	-	-	-	-	-	-	+	-	+	-	+
10	M	66	127	4a	1	IVA	intermediate	+	+	+	+	-	+	-	+	+	-	-	-	-
11	F	40	127	1	0	I	low	-	-	-	-	-	-	+	-	+	-	+	-	+
12	M	62	127	2	0	II	high	+	+	-	+	-	+	-	-	-	-	-	-	-
13	M	62	127	4a	0	IVA	low	-	-	-	-	-	-	-	-	-	-	+	-	+
14	F	54	127	1	0	I	low	-	-	-	-	-	-	+	-	+	-	-	-	+
15	F	71	127	2	0	II	low	-	-	-	-	-	-	-	-	-	-	+	+	-
16	F	38	127	4a	0	IVA	low	+	-	+	-	-	-	-	-	+	-	+	+	+
17	M	79	127	3	0	III	low	-	-	-	-	-	-	-	-	-	-	-	-	-
18	M	71	127	2	0	II	low	-	-	-	-	-	-	-	-	+	-	+	-	-
19	F	34	127	2	0	II	low	-	-	-	-	-	-	-	-	+	+	+	+	+
20	M	54	127	1	0	I	low	+	-	-	-	-	-	-	-	-	-	-	-	-
21	M	61	127	2	0	II	high	+	+	-	+	+	+	-	-	+	-	-	-	-
22	M	76	127	3	2a	IVA	intermediate	+	+	+	-	-	+	-	-	+	-	+	+	+
23	F	55	127	3	0	III	low	+	-	+	-	-	-	+	-	+	-	+	+	-
24	F	73	127	2	0	II	low	-	-	-	-	-	-	-	-	-	-	+	-	-
25	F	55	127	1	0	I	low	+	-	-	-	-	-	-	-	+	-	-	-	-
26	F	12	127	3	0	III	low	-	-	+	-	-	-	-	-	+	+	+	+	+
27	M	62	127	3	1	III	high	+	+	+	+	-	+	-	+	+	-	-	-	-
28	M	75	127	1	2a	IVA	high	+	+	-	+	-	+	+	-	-	-	-	-	-
29	F	56	127	1	0	I	intermediate	+	+	-	-	-	-	+	-	+	-	-	+	-
30	M	68	127	2	0	II	high	+	+	+	+	+	+	+	-	+	-	-	-	-
31	M	74	127	2	2b	IVA	high	+	+	+	+	-	+	-	-	-	-	-	-	-
32	F	59	127	1	0	I	low	+	-	-	-	-	-	-	-	+	-	+	+	+
33	M	65	127	2	2b	IVA	high	+	+	+	+	-	+	-	+	-	-	-	-	-
34	M	79	127	2	0	II	high	+	+	+	+	-	+	-	-	+	-	+	+	-
35	F	68	127	1	0	I	low	-	-	-	-	-	-	-	-	+	-	+	-	-
36	M	65	127	3	0	III	high	+	+	+	+	-	+	-	-	+	-	+	+	-
37	M	69	127	1	0	I	high	+	-	-	+	-	+	-	-	+	-	-	-	-
38	M	65	127	3	2b	IVA	high	+	+	+	+	-	+	-	-	+	-	+	-	-
39	F	52	127	1	1	III	high	+	+	-	+	-	+	-	-	-	-	+	-	-
40	M	62	127	1	2b	IVA	high	+	+	-	+	-	+	-	+	-	-	+	-	-
41	M	43	127	4a	1	IVA	high	+	+	+	+	-	-	+	-	+	-	-	-	-
42	F	65	127	4a	1	IVA	high	+	+	+	+	+	+	-	-	+	-	-	-	-
43	M	64	127	1	2a	IVA	high	+	+	-	+	-	+	-	-	-	-	+	-	-
44	M	86	127	4a	2b	IVA	high	+	+	+	+	+	+	+	+	+	-	-	-	-
45	F	16	127	1	0	I	high	+	+	+	-	-	-	+	-	+	-	+	+	+
46	F	66	127	2	2c	IVA	high	+	+	+	+	-	+	+	+	+	-	+	-	-
47	F	56	127	4a	2c	IVA	high	+	+	+	+	-	+	-	+	+	-	-	-	-
48	F	49	127	1	0	I	low	+	-	-	-	-	-	-	-	+	-	+	+	+
49	F	85	127	4a	0	IVA	high	+	+	+	+	-	+	-	-	+	-	-	-	-
50	M	73	127	4a	2a	IVA	high	+	+	+	+	-	+	-	-	+	-	-	-	-
51	M	59	127	4a	0	IVA	intermediate	-	+	+	-	-	-	+	-	+	-	+	+	+
52	M	70	127	1	2b	IVA	high	+	+	-	+	-	+	-	-	+	+	-	-	-
53	M	82	127	4a	0	IVA	high	+	+	-	+	-	+	-	-	-	-	+	+	-
54	F	44	127	1	0	I	intermediate	+	-	+	-	-	-	-	-	+	-	+	-	+
55	F	27	127	2	0	II	high	+	+	-	+	-	+	-	-	-	-	+	-	-
56	F	34	127	1	0	I	low	-	-	-	-	-	-	+	-	+	-	+	+	+
57	M	76	127	1	2b	IVA	high	+	+	-	+	-	+	-	-	-	-	-	-	-
58	F	58	127	1	0	I	intermediate	+	-	-	+	-	+	+	-	+	+	-	+	+
59	M	29	127	2	0	II	intermediate	+	+	-	-	-	-	-	-	+	+	+	+	+
60	M	69	127	1	0	I	high	-	+	+	+	-	+	-	+	+	+	+	-	-
61	M	56	127	4a	2c	IVA	high	+	+	+	+	-	+	-	+	-	+	-	+	-
62	F	46	127	1	0	I	low	+	-	-	-	-	-	-	-	+	-	-	+	-
63	F	30	127	2	0	II	low	-	-	-	-	-	-	-	-	+	-	-	-	+
64	M	63	127	2	2b	IVA	intermediate	+	+	-	+	-	-	-	-	+	-	-	-	+
65	F	31	127	2	0	II	intermediate	+	+	-	-	-	-	-	-	-	-	-	-	-
66	F	43	127	2	2c	IVA	high	+	+	+	+	+	+	+	-	+	-	-	-	-
67	M	55	127	4a	2c	IVA	high	+	+	+	+	-	+	+	-	+	-	-	-	-
68	F	65	127	3	2b	IVA	high	+	+	+	+	-	+	+	+	+	-	-	+	-
69	M	64	127	3	0	III	high	+	-	+	+	-	+	-	-	+	-	-	-	-
70	F	31	127	2	0	II	low	-	-	-	-	-	-	-	+	+	-	+	+	+
71	M	77	127	1	0	I	low	-	+	-	-	-	-	-	-	+	-	+	+	+
72	F	59	127	2	0	II	low	-	-	-	-	-	-	-	-	+	-	+	+	+
73	F	59	127	2	0	II	intermediate	+	-	-	+	-	-	-	-	+	-	-	+	+

Association of mucin expression, especially MUC4 and MUC6, with the clinicopathological features

The relationship between MUC4 or MUC6 expression and clinicopathological characteristics is summarized in Table 4. MUC4-negative expression had a significantly strong correlation with pathologically high tumor grade and regional lymph node metastasis (P=0.006 and 0.02, respectively) but not with T classification, LVI, perineural invasion (ne), margin status or recurrence (P>0.05) in the overall cohort (Table 4). In addition, there were no significant differences between the age, gender and tumor location between the MUC4-negative and MUC4-positive groups. Furthermore, immunohistochemically MUC4-positive expression in both membranous and intracytoplasmic patterns was evident especially in the cystic components of MECs, whereas the invasive fronts including the elements of perineural involvement (+) were completely negative for MUC4 (Fig. 2), as clearly demonstrated by S-100 protein staining (data not shown). The postsurgical DFS of MEC patients with MUC4-negative expression (median: 32.4 months) was significantly shorter than in patients with MUC4-positive expression (median: 44.1 months), especially within the first 2 years (P=0.03, Fig. 4A) postoperatively (P=0.03 at 5 years, data not shown). Accordingly, there was significant difference in postoperative DSS within the first 2 (P=0.04, Fig. 4B) and 5 (P=0.04, data not shown) years between the MEC patients with negative (median: 39.0 months) and positive (median: 45.8 months) MUC4 expression status.

In contrast, MUC6 revealed only intracytoplasmic immunohistochemical expression patterns (Fig. 3). MUC6-negative expression in the present 73 MECs was significantly related to high-grade histopathological tumor, the presence of LVI, nodal metastasis, and postoperative tumor-related death (P=0.003, <0.001, 0.02 and 0.03, respectively) (Table 4). However, MUC6-negative expression had an insignificantly close relationship with the location, T stage, perineural invasion, margin status and postoperative recurrence (P>0.05) (Table 4). Similar to MUC4, MUC6-postive expression was conspicuous, particularly in the cystic components of MEC, whereas the invasive fronts

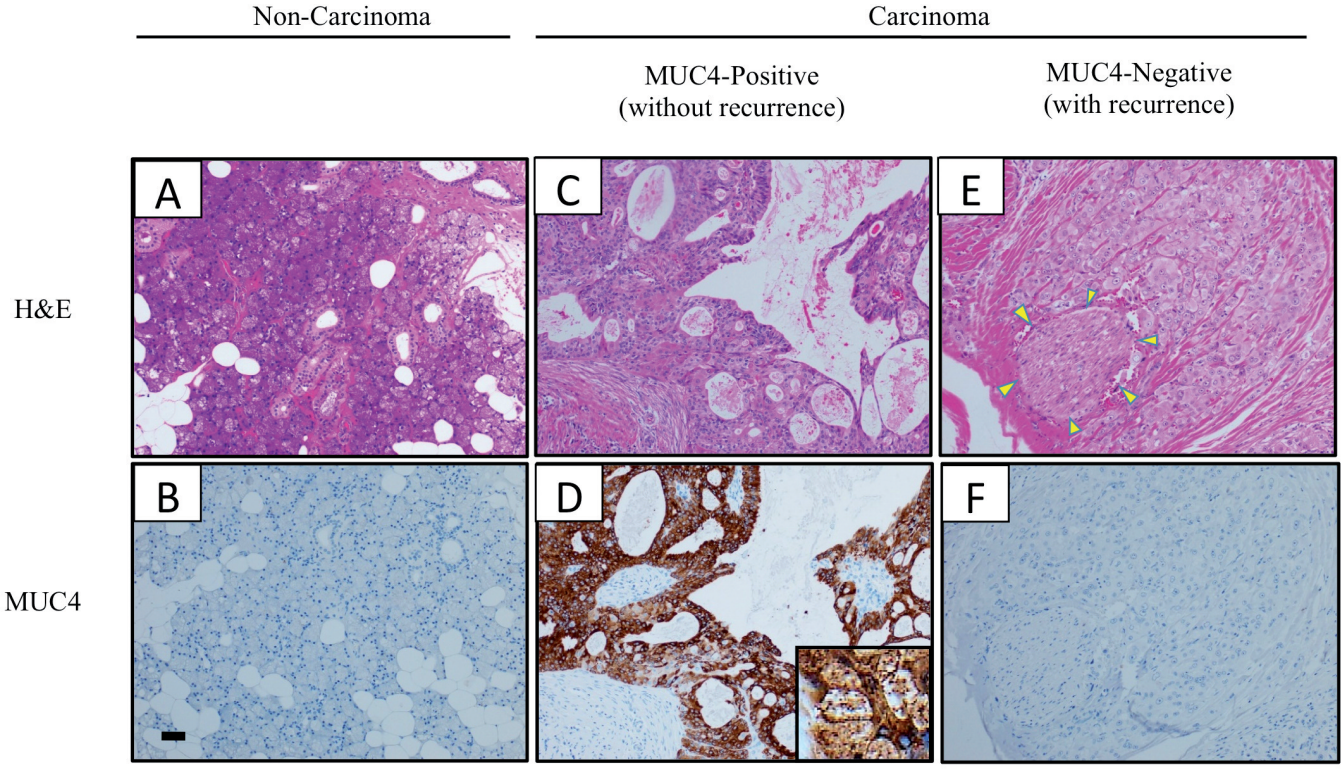

Fig. 2. The membranous and intracytoplasmic MUC4 expression patterns showed specifically positive staining on immunohistochemistry, potentially associated with no early recurrence in the low-grade MEC patients. Representative images of the immunohistochemical staining of MUC4 in the non-neoplastic epithelium **(A, B)** and low- **(C, D)** to high- **(E, F)** grade MECs. Positive expression for MUC4 in the human low-grade MEC samples is shown **(C, D)**, demonstrating a membranous and intracytoplasmic staining pattern **(D)**, especially in the cystic components (not only mucin-producing cells but also epidermoid and/or intermediate cells) of strongly MUC4-positive cases without postoperative recurrence **(C, D)** (Case No. 9), compared to the completely negative cytoplasmic staining pattern **(F)** in the high-grade MEC tumor cases with perineural invasion (ne; arrowheads) **(E)** and postsurgical early recurrence **(E, F)** (Case No. 2). In contrast, negative MUC4 expression **(B)** was evident in the adjacent non-carcinomatous salivary gland epithelium **(A, B)** (normal; Case No. 9). H&E: hematoxylin and eosin. Scale bar: 100 μm.

including the elements of LVI were completely negative for MUC6 (Fig. 3), as clearly shown on E-M staining (data not shown). The postsurgical DFS of MEC patients with MUC6-negative expression (median: 30.0 months) was significantly shorter than in those with MUC6-positive expression (median: 54.0 months), especially within the first 2 years (P=0.02, Fig. 4C) postoperatively (P=0.02 at 5 years, data not shown). Correspondingly, there was a significant difference in the postoperative DSS within the first 2 (P=0.02, Fig. 4D) and 5 (P=0.02, data not shown) years between the MEC patients with negative (median: 34.6 months) and positive (median: 57.5 months) MUC6 expression status.

However, regarding the profiles of the other mucins (MUC1, MUC2 and MUC5AC) and their associations with the clinicopathological characteristics, including DFS and DSS, we observed no significant differences between the patients with negative and positive

Table 4. Detailed correlations between each negative MUC4 and MUC6 expression and the clinicopathological variables.

Variables		Total	(%)	MUC4 Expression Negative (n=39)	MUC4 Expression Positive (n=34)	P value
Age	>60	40	54.8	23	17	0.49
	≤60	33	45.2	16	17	
Sex	Male	39	53.4	24	15	0.16
	Female	34	46.6	15	19	
Location						
	Major salivary gland	37	50.7	19	18	0.82
	Minor salivary gland	36	49.3	20	16	
T stage						
	T1,2	46	63.0	22	24	0.23
	T3,4	27	37.0	17	10	
N stage						
	N0	47	64.4	20	27	0.02
	N1, N2	26	35.6	19	7	
Grade	Low, intermediate	36	49.3	7	17	0.006
	High	37	50.7	32	17	
Cystic components (less than 20%)						
	(-)	18	24.7	3	15	<0.001
	(+)	55	75.3	36	19	
LVI	(-)	28	38.4	11	17	0.09
	(+)	45	61.6	28	17	
Perineural invasion						
	ne(-)	44	60.3	22	22	0.49
	ne(+)	29	39.2	17	12	
Necrosis						
	(-)	31	42.5	9	22	<0.001
	(+)	42	57.5	30	12	
Anaplasia						
	(-)	66	90.4	32	34	0.01
	(+)	7	9.6	7	0	
Mitotic figures (more than 4)						
	(-)	35	47.9	13	22	0.01
	(+)	38	52.1	26	12	
Margin status						
	(-)	55	75.3	27	28	0.28
	(+)	18	24.7	12	6	
Recurrence						
	(-)	58	79.5	28	30	0.15
	(+)	15	20.5	11	4	
Tumor-related death						
	(-)	63	86.3	31	32	0.09
	(+)	10	13.7	8	2	
MUC6 expression						
	negative	49	67.1	33	16	0.001
	positive	24	32.9	6	18	

Variables		Total	(%)	MUC6 Expression Negative (n=49)	MUC6 Expression Positive (n=24)	P value
Age	>60	40	54.8	35	5	<0.001
	≤60	33	45.2	14	19	
Sex	Male	39	53.4	32	7	0.006
	Female	34	46.6	17	17	
	Location					
	Major salivary gland	37	50.7	22	15	0.21
	Minor salivary gland	36	49.3	27	9	
T stage						
	T1,2	46	63.0	29	17	0.44
	T3,4	27	37.0	20	7	
	N stage					
	N0	47	64.4	27	20	0.02
	N1, N2	26	35.6	22	4	
Grade	Low, intermediate	36	49.3	10	14	0.003
	High	37	50.7	39	10	
Cystic components (less than 20%)						
	(-)	18	24.7	6	12	<0.001
	(+)	55	75.3	43	12	
LVI	(-)	45	55.5	12	16	<0.001
	(+)	36	44.5	37	8	
Perineural invasion						
	ne(-)	44	60.3	26	18	0.08
	ne(+)	29	39.2	23	6	
	Necrosis					
	(-)	31	59.3	12	19	<0.001
	(+)	42	40.7	37	5	
	Anaplasia					
	(-)	66	90.4	42	24	0.09
	(+)	7	9.6	7	0	
Mitotic figures (more than 4)						
	(-)	48	59.3	15	20	<0.001
	(+)	33	40.7	34	4	
Margin status						
	(-)	55	75.3	37	18	1.00
	(+)	18	24.7	12	6	
Recurrence						
	(-)	58	79.5	36	22	0.12
	(+)	15	20.5	13	2	
Tumor-related death						
	(-)	63	86.3	39	24	0.03
	(+)	10	13.7	10	0	
MUC4 expression						
	negative	39	53.4	33	6	0.001
	positive	34	46.6	16	18	

expression (data not shown) (P>0.05).

Correlations between MUC4- and MUC6-negative expression

There was a significant relationship between the immunohistochemical mucin expression patterns (P=0.001, r=0.40) (Figs. 2, 3 and Table 4), with negative MUC4 expression showing a significantly positive rate of co-expression with negative MUC6 expression (Table 4). When the patients were divided into groups based on their MUC4 and MUC6 expression patterns (negative or positive for each), their immunoprofiles were, respectively, 45.2% negative and negative (33 cases), 8.2% negative and positive (6 cases), 21.9% positive and negative (16 cases) and 24.7% positive and positive (18 cases). Both MUC4- and MUC6-negative expression patterns demonstrated a significantly close correlation with high-grade histopathological tumor, the presence of LVI, and lymph node metastasis (P<0.001, 0.008 and 0.01, respectively) (data not shown). Accordingly, the current MEC patients with both negative MUC4 and MUC6 profiles showed significantly shorter post-operative median DFS (12.0 months) and DSS (23.0 months) than other groups' median DFS (37.5 months) and DSS (42.5 months), especially within the first 2 years (DFS; P=0.01, Fig. 4E) (DSS; P=0.007, Fig. 4F) postoperatively.

MUC4- and MUC6-negative expression does not represent any significant independent prognostic indicators for MEC

To assess whether mucins expression was an independent predictor of the postoperative DFS/DSS, a Cox proportional hazards model was created in a forward fashion including only covariates that had statistically significant correlations with the DFS, using an inclusion threshold of P<0.05 (Table 5). However, even a univariate analysis demonstrated that there were no significant predictors of poorer survival of MECs, including tumor size (T stage), lymph node metastasis (N stage), histologic high-grade, the presence of LVI, ne (perineural involvement), positive margin status (i.e., the

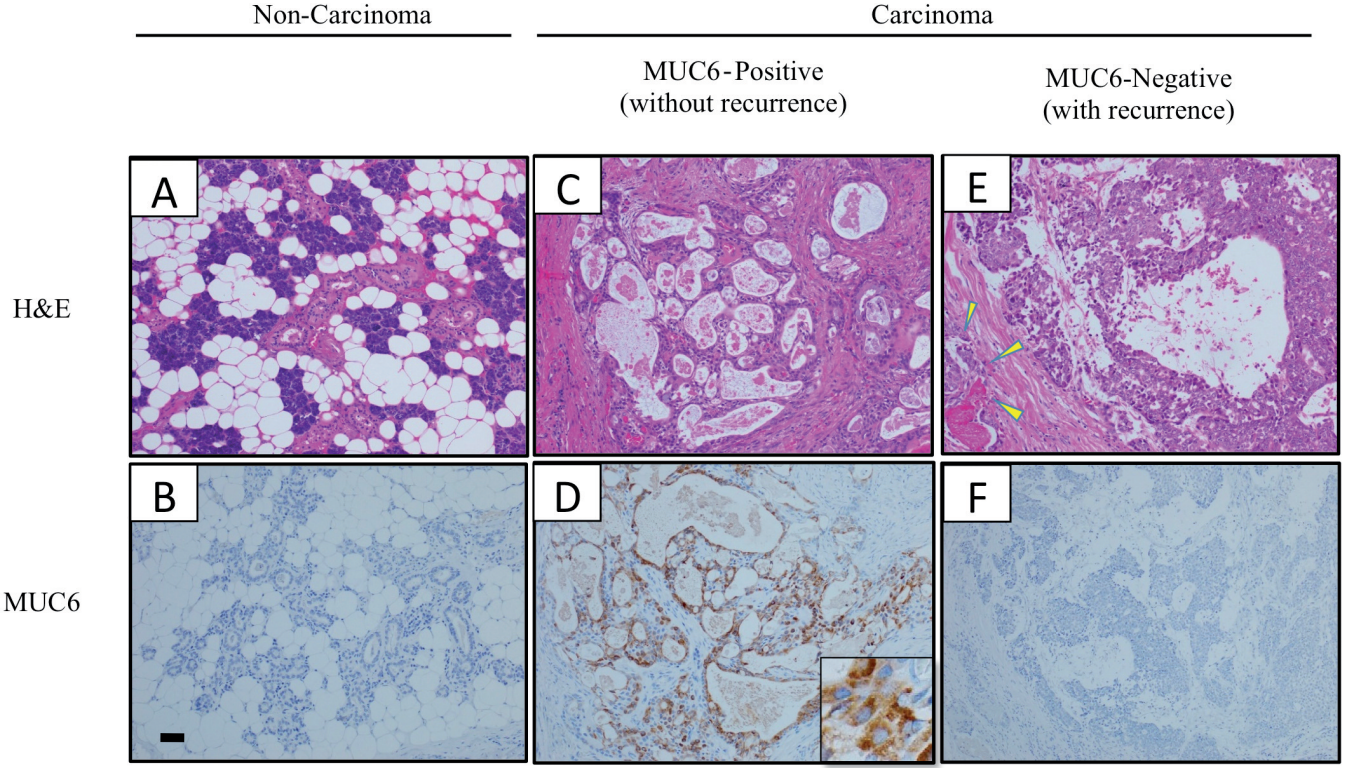

Fig. 3. The intracytoplasmic MUC6 expression patterns show specifically positive staining on immunohistochemistry, potentially associated with no early recurrence in the low-grade MEC patients. Representative images of the immunohistochemical staining of MUC6 in the non-neoplastic epithelium **(A, B)** and low- **(C, D)** to high- **(E, F)** grade MECs. Positive expression for MUC6 in the human low-grade MEC samples is shown **(C, D)**, demonstrating an intracytoplasmic staining pattern **(D)**, especially in the cystic components (not only mucin-producing cells but also epidermoid and/or intermediate cells) of highly MUC6-positive cases without postoperative recurrence **(C, D)** (Case No. 71), compared to the completely negative cytoplasmic staining pattern **(F)** in the high-grade MEC tumor cases with lymphovascular invasion **(E)** (LVI; arrowheads) and postsurgical early recurrence **(E,F)** (Case No. 7). In contrast, negative MUC6 expression **(B)** was evident in the adjacent non-carcinomatous salivary gland epithelium **(A, B)** (normal; Case No. 71). H&E: hematoxylin and eosin. Scale bar: 100 μm.

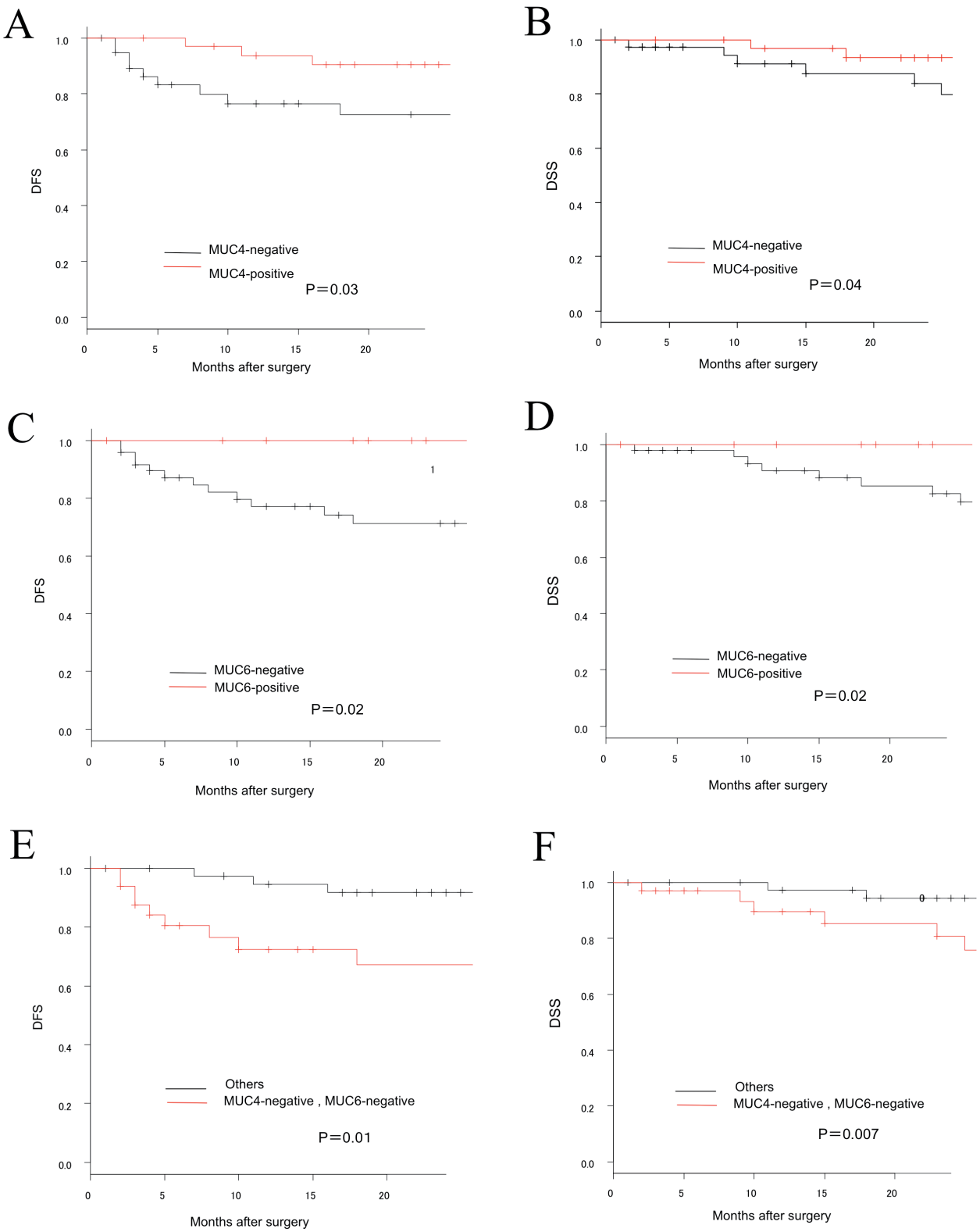

Fig. 4. MUC4- or MUC6-negative expression alone as well as dual MUC4/6-negative expression are associated with a significantly shorter postsurgical DFS and DSS in MEC patients, especially within the first two years postoperatively. **A, B.** Kaplan-Meier curves for the DFS **(A)** and DSS **(B)** in MEC patients within the first two years after surgery, according to the MUC4 expression. **C, D.** Kaplan-Meier curves for the DFS **(C)** and DSS **(D)** in MEC patients within the first two years after surgery, according to the MUC6 expression. **E, F.** Kaplan-Meier curves of the DFS **(E)** and DSS **(F)** in MEC patients within the first two years after surgery, according to both negative MUC4 and negative MUC6 expression. DFS: disease-free survival. DSS: disease-specific survival.

Table 5. The results of the univariate and multivariate analyses of survival in 73 patients with MEC, according to the clinicopathological variables and each negative MUC4 and negative MUC6 expression.

Variables		No. of patients (%)	Univariate			Multivariate		
			HR	95 %CI	P value	HR	95 %CI	P value
Age	>60	40(54.8)	1			1		
	≤60	33(45.2)	2.11	0.71-6.23	0.31	1.43	0.29-6.97	0.03
Sex	Male	39(53.4)	1			1		
	Female	34(46.6)	1.68	0.59-4.75	0.66	0.43	0.11-1.66	0.66
Location	Major salivary gland	37(50.7)	1			1		
	Minor salivary gland	36(49.3)	0.67	0.24-1.89	1.00	0.84	0.18-3.82	0.12
T stage	T1,2	46(63)	1			1		
	T3,4	27(37)	4.19	1.43-12.29	0.61	3.3	0.69-15.99	0.77
N stage	N0	47(64.4)	1			1		
	N1, N2	26(35.6)	7.38	2.32-23.43	0.95	3.55	0.55-23.05	0.55
Grade	Low, intermediate	24(32.9)	1			1		
	High	49(67.1)	11.38	2.48-52.16	0.94	0.58	0.05-7.04	0.52
Cystic components (less than 20%)	(-)	18(24.7)	1			1		
	(+)	55(75.3)	3.06	0.68-13.72	0.83	0.01	2.72e-4-0.27	0.59
LVI	(-)	28(38.4)	1			1		
	(+)	45(61.6)	12.52	1.63-95.94	0.42	4.27	0.04-5.02e+2	0.94
Perineural invasion	ne(-)	44(60.3)	1			1		
	ne(+)	29(39.2)	4.34	1.45-13.05	0.40	0.4	0.06-2.68	0.36
Necrosis	(-)	31(42.5)	1			1		
	(+)	42(57.5)	18.39	1.66-7.56	0.51	2.22	6.51e-4-7.55e+3	0.68
Anaplasia	(-)	66(90.4)	1			1		
	(+)	7(9.6)	3.51	0.96-12.81	0.82	0.62	0.13-2.88	0.94
Mitotic figures (more than 4)	(-)	35(47.9)	1			1		
	(+)	38(52.1)	21.32	2.77-164.20	0.47	45.54	0.01-1.47e+5	0.84
Margin status	(-)	55(75.3)	1			1		
	(+)	18(24.7)	1.54	0.52-4.50	0.26	2.61	0.48-14.08	0.79
MUC4 expression	negative	39(53.4)	1			1		
	positive	34(46.6)	0.31	0.10-1.96	0.40	0.37	0.07-2.03	0.4
MUC6 expression	negative	49(67.1)	1			1		
	positive	24(32.9)	0.20	0.04-0.92	0.18	0.19	0.02-2.36	0.14

surgical margins considered to be involved by the presence of MECs at the lateral or deep margin), negative MUC4 or negative MUC6 expression ($P>0.05$) (DFS: Table 5; and DSS: data not shown). Correspondingly, a multivariate analysis revealed no independent prognostic indicators for the DFS (Table 5) and DSS (data not shown) in the postsurgical MEC patients.

Discussion

The current results in a relatively large cohort of postoperative MEC indicate for the first time that (1) MUC4- and/or MUC6-negative expression respectively and variably showed a significant correlation with invasive/aggressive behavior, manifesting as high-grade pathological tumor, the presence of LVI, lymph node metastasis and/or tumor-related death; (2) MUC4 expression has significantly negative co-expression with MUC6 and (3) not only single MUC4/6-negative expression but also the combination of both can predict a significantly shorter DFS and DSS, especially within the first 2 years postoperatively. MUC4/6-deleted expression is a powerful, but not independent, negative indicator of the DFS/DSS and a potentially poor outcome in patients with postoperative MEC; as such, it is a somewhat novel prognostic marker for the disease. Taken together, these findings suggest that each mucin may play a pivotal role in the pathogenesis of MEC progression, and MUC4 and MUC6 might be expressed simultaneously but function separately.

However, several limitations associated with the present study warrant mention. First, this was a cohort-based, retrospective study at a single institution, which we controlled by random selection of MEC patients and by adherence to strict exclusion criteria. Second, we only conducted immunohistochemical and not detailed molecular analyses. Further in-depth follow-up in much larger cohorts of MEC patients, together with detailed molecular investigations using MEC cell culture lines will be required to confirm the intriguing correlation between negative MUC4/6 expression and a poor

outcome in postsurgical MEC patients.

Recurrence in MEC patients after curative surgery remains a critical problem and can significantly affect the clinical postoperative course and survival (Eversole, 1970; Goode et al., 1998; Chen et al., 2007; Aro et al., 2011; Herd et al., 2012; Coca-Pelaz et al., 2015). The biological invasiveness and aggressiveness of MEC is partly reflected in the ability of carcinoma cells to recur and metastasize, even in low-grade and/or small lesions that are considered to have a relatively good prognosis (Eversole, 1970; Chen et al., 2007; Aro et al., 2011; Herd et al., 2012). Indeed, 7 (29.2%) or 2 (8.3%) of our T1 cases, and 4 (11.1%) or 2 (5.6%) of our 'low'-grade (low to intermediate) MEC patients, may have had slightly occult metastases in the regional lymph nodes, or have shown postsurgical recurrence. Thus far, no reliable predictors of the progressive potential of MECs have been established. The detection of the MUC4/6 expression patterns in not only postsurgical MEC specimens but also preoperative cytology/biopsy samples might therefore allow for improved selection of candidates for adjuvant/neoadjuvant systemic therapies and the need for regional lymph node dissection within the neck, especially in the early phase of the clinical course. It is well known that neck dissection is the most reliable treatment for addressing pathological N stage lesions, even though this method can also induce complications, such as lymphatic leakage or injury of the facial nerve (Harada et al., 2016). The immunohistochemical evaluation of the expression of MUC4/6 on routine cytology/biopsy specimens would therefore tremendously improve the ability to make appropriate preoperative decisions regarding the need for neck lymph node dissection and the subsequent impact on the quality of life in MEC patients. The clinical relevance of the mucin antigens, particularly MUC4/6, should be verified in the future in order to avoid unnecessary surgery and prolong the effects of beneficial surgical treatment for MECs.

In line with our data, other laboratories have reported that the positive expression of MUC4 plays a key role in the favorable clinical course of postoperative MEC patients through the induction of tumor differentiation and a reduction in the recurrent potential of MEC (Alos et al., 2005; Handra-Luca et al., 2005). By contrast, we demonstrated, for the first time, that MUC6-negative expression could also be a powerful adjunctive aid for identifying high-grade malignancy and patients with a poor prognosis, such as those with tumors with fewer cystic components, the presence of LVI, increased mitotic figures, lymph node metastasis, a shorter DFS/DSS and postsurgical tumor-related death, in conjunction with negative MUC4 co-expression. Further supporting these *in vivo* results, MUC6 was able to strictly prohibit the invasion of carcinoma cells through the basement membrane in an *in vitro* study using several carcinoma cell lines (Leir and Harris, 2011). However, our observations disagree with those of other groups on MUC1 immunohistochemistry in MECs (Alos et al., 2005; Handra-Luca et al., 2005; Liu et al., 2014; Siyi et al., 2014). These authors found that patients with increased MUC1 expression show a significantly shorter postoperative survival, which was closely associated with greater progressive activity. Their reports regarding immunohistochemical MUC1 expression actually agree with our serial studies of several other carcinomas in the stomach, esophagus, pancreas and breast and various bile duct tumors (Higashi et al., 1999; Yonezawa et al., 2008, 2011; Hiraki et al., 2017). This discrepancy/divergence may be related, at least in part, to not only (i) the heterogeneity of MECs, but also the methodology of assessment in each study: (ii) the size of the cohort; (iii) the difference of antibodies used against each mucin; (iv) arbitrary or strict selection and validation of the immunohistochemical cut-off scores for MUC1/4/6, not based on any ROC curve analyses; and (v) various glycoforms of mucins core protein antigens, such as underglycosylated, sialylated, and fully glycosylated forms (Yonezawa et al., 2008, 2011). Further experiments are necessary to address methodology standardization for mucins, especially MUC4/6, in clinical specimens after collecting and investigating a much larger number of surgical MEC cases.

In conclusion, the current cohort study demonstrates for the first time that the negative expression of not only MUC4/6 alone but also their dual negative expression is a novel and reliable, but not independent, marker for a shorter DFS/DSS in MEC patients with surgical treatment, particularly within the first 2 years, even after curative treatment. Ultimately, patients with MEC displaying completely negative MUC4/6 expression should be followed up very carefully. Physicians should measure the expression of these crucial MEC-specific biomarkers (MUC4 and/or MUC6) as a useful parameter for guiding the clinical management of postoperative MEC patients, especially in the early phase.

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Compliance with ethical standards. Institutional Review Board approval was obtained from the Ethics Committee of Kagoshima University Hospital (28-179).

Conflict of interest. The authors declare that they have no conflict of interest. No competing financial interests exist.

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