ORIGINAL ARTICLE

Differential mucin phenotypes and their significance in a variation of colorectal carcinoma

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Abstract

AIM: To investigate mucin expression profiles in colorectal carcinoma (CRC) histological subtypes with regard to clinicopathologic variables and prognosis.

METHODS: Mucin (MUC)2 and MUC5AC expressions were assessed by immunohistochemistry for a total of 250 CRC cases that underwent surgical resection. CRCs included 63 well-to-moderately differentiated adenocarcinomas (WMDAs), 91 poorly differentiated adenocarcinomas (PDAs), 81 mucinous adenocarcinoma (MUAs), and 15 signet-ring cell carcinomas (SRCCs). MUC2 and MUC5AC were scored as positive when $\geqslant$ 25% and $\geqslant$ 1% of cancer cells were stained positive, respectively. The human mutL homolog 1 and human mutS homolog 2 expressions were assessed by immunohistochemistry in PDAs to investigate mismatch-repair (MMR) status. Tumors that did not express either of these two were considered MMR-deficient. Results were analyzed for associations with clinicopathologic variables and the prognosis in individual histological CRC subtypes.

RESULTS: MUC2-positive and MUC5AC-positive WMDA percentages were 49.2% and 30.2%, respectively. In contrast, MUC2-positive and MUC5AC-positive PDA percentages were 9.5% and 51.6%, respectively. MUC2 levels tended to decrease and MUC5AC levels tended to increase from WMDA to PDA. In 21 tumors comprising both adenoma and adenocarcinoma components in a single tumor (4 WMDAs, 7 PDAs, and 10 MUAs), MUC2 was significantly downregulated in PDA and MUC5AC was downregulated in PDA and MUA in the adenoma-carcinoma sequence. These results suggested that MUC2 levels might be associated with malignant potential and that MUC5AC expression was an early event in tumorigenesis. Despite worse prognoses than WMDA, high MUC2 expression levels were maintained in MUA (95.1%) and SRCC (71.5%), which suggested a pathogenesis for these subtypes distinct from that of WMDA. No significant associations were found between MUC2 expression and any clinicopathologic variables in any histological subtype. MUC5AC expression in PDA was closely associated with right-sided location ($P = 0.017$), absence of nodal metastasis ($P = 0.010$), low tumor node metastasis stage ($P = 0.010$), and MMR deficiency ($P = 0.003$). MUC2 expression in WMDA was a marginal prognostic factor for recurrence/metastasis-free survival (RFS) by univariate Cox analysis ($P = 0.077$) but not by multivariate Cox analysis ($P = 0.161$). MUC5AC expression in PDA was a significant prognostic factor for RFS by univariate Cox analysis ($P = 0.007$) but not by multivariate Cox analysis ($P = 0.104$). Kaplan-Meier curves and log-rank tests revealed that MUC2 expression was marginally associated with a better WMDA prognosis [$P = 0.064$ for RFS and $P = 0.172$ for overall survival (OS)] but not for PDA. In contrast, MUC5AC expression was significantly and marginally associated with a better PDA prognosis in terms of RFS and OS, respectively

(P = 0.004 for RFS and P = 0.100 for OS), but not for WMDA and MUA.

CONCLUSION: Mucin core protein expression profiles and clinical significance differ according to histological CRC subtypes. This may reflect different pathogeneses for these tumors.

Core tip: Altered mucin expression may be correlated with biological behavior and possibly with the prognosis of colorectal carcinoma (CRC). However, many contradictory results make it difficult to interpret its clinical significance, possibly because of CRC variations. Therefore, we examined mucin (MUC)2 and MUC5AC expressions in different pathological CRC subtypes by immunohistochemistry to determine their true clinical significance. Our results suggest that the expression profiles and the clinical significance of these mucin core proteins are different according to histological subtypes. This may reflect different pathogeneses for these tumors.

INTRODUCTION

Mucins are a diverse family of high-molecular-weight glycoproteins that are widely expressed in epithelial tissues and are characterized by the presence of tandem repeat sequences that are rich in highly *O*-glycosylated serine and threonine residues[1]. Mucins can be classified as either membrane-associated or secretory glycoproteins. To date, a total of 20 human mucins have been identified. Secreted mucins can be gel-forming or non-gel-forming and include mucin (MUC)2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9, and MUC19. Transmembrane mucins include MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, and MUC21; these are anchored to the plasma membranes of various cells through a transmembrane domain. These mucin proteins are encoded by various *MUC* genes[2]. The genes for gel-forming mucins MUC2 and MUC5AC are found in a cluster on chromosome 11p15.5[3]. The *MUC2* gene codes for a typical secretory mucin, which is predominantly found in colorectal goblet cells, and the *MUC5AC* gene is mainly expressed in gas-

tric and tracheal-bronchial mucosa.

Altered expressions of MUC2 and MUC5AC may be significantly correlated with the biological behavior of and, possibly, the prognosis for colorectal carcinoma (CRC). However, many contradictory results make it difficult to interpret their clinical significance. For example, MUC2 expression is significantly decreased according to CRC disease progression[4-6]. MUC2-positive CRC shows a relatively good prognosis or a low incidence of liver and nodal metastasis[7,8]. Suppressing the *MUC2* gene expression in colon carcinoma cell lines in vitro was associated with methylation of its promoter region[7]. In contrast, other studies reported that MUC2 expression was not a significant marker of tumor invasion depth, liver metastasis, or overall survival[9,10]. However, the absence of MUC5AC expression can be a prognostic indicator of a more aggressive colorectal tumor. Highly villous adenoma with severe dysplasia expressed a less MUC5AC than larger adenomas of moderate villous histology and dysplasia[11]. Carcinomas with low grade atypia exhibited a higher incidence of MUC5AC expression as compared with carcinomas showing high grade atypia[6]. Consistently, MUC5AC expression analysis combined with survival analysis has demonstrated that those patients with MUC5AC-negative CRC had lower rates for disease-free status and of overall survival[12].

Most studies analyzed CRC without detailed classifications. However, in the World Health Organization (WHO) classification, CRC consists of various histological subtypes, such as conventional adenocarcinoma, mucinous adenocarcinoma (MUA), signet-ring cell carcinoma (SRCC), squamous cell carcinoma, adenosquamous carcinoma, medullary carcinoma, undifferentiated carcinoma, and other very rare variants[13]. Conventional adenocarcinoma is further sub-classified into well-to-moderately differentiated adenocarcinoma (WMDA) and poorly differentiated adenocarcinoma (PDA) based on the percentage of the area showing a gland-like structure[13]. Most CRCs encountered in the clinic are WMDA and poorly differentiated/undifferentiated carcinomas are rare, accounting for up to 16% of all CRCs in the United States[14,15]. The purpose of this study was to assess MUC2 and MUC5AC expressions in different pathological CRC subtypes by immunohistochemistry and to determine their true clinical significance.

MATERIALS AND METHODS

Patients and tumor samples

For this study, WMDA included all consecutive cases that were surgically resected in Tokyo Kosei Nenkin Hospital from April 1998 to March 2000, but excluded 10 other histological CRC subtypes. These cases included 63 tumors from 63 patients. In addition, a total of 187 histological CRC subtypes other than WMDA were collected from all CRC cases resected in Dokkyo Medical University Koshigaya Hospital between 1990 and 2011 and Tokyo Kosei Nenkin Hospital between 1991 and 2010.
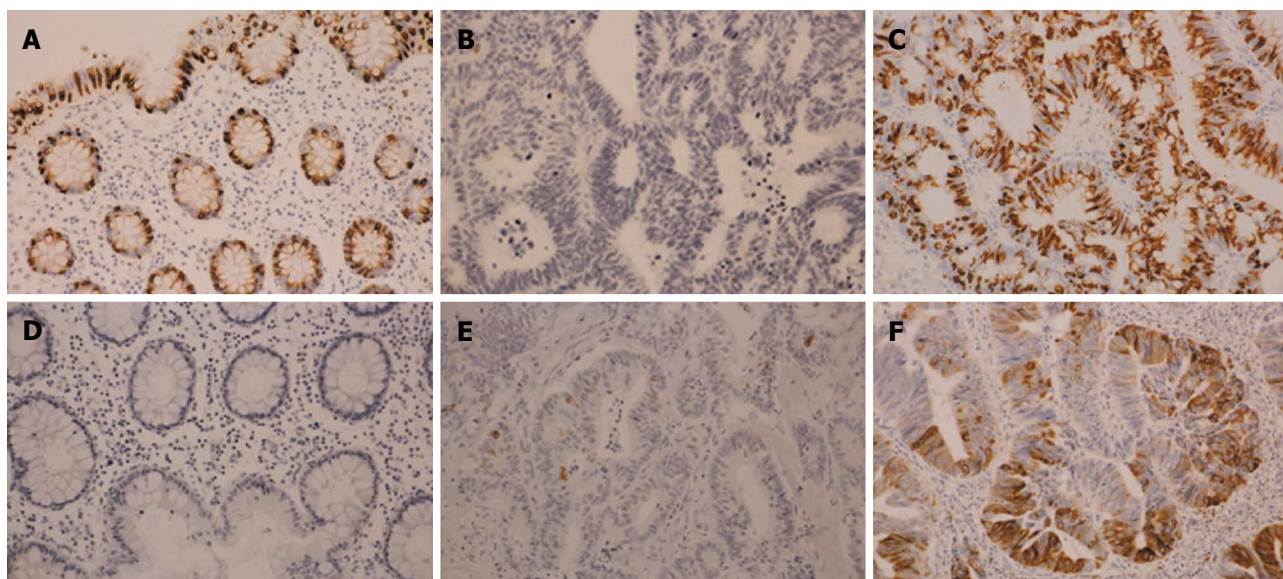
Figure 1 Expression of mucin 2 and mucin 5AC in colorectal carcinomas. A: Mucin (MUC)2 expression in normal colonic mucosa; B: MUC2 expression in cancer: level 0; C: MUC2 expression in cancer: level 4; D: MUC5AC expression in normal colonic mucosa; E: MUC5AC expression in cancer: level 1; F: MUC5AC expression in cancer: level 4 (immunohistochemical staining, × 10).

Formalin-fixed, paraffin-embedded tissue blocks were obtained from the archival material stored in the pathology departments of the both hospitals. A sufficient number of samples to provide for complete investigations were available for all these cases. Patients whose medical records were sufficiently complete were included in survival analysis. Patients with invasive cancers originating from other sites were excluded from the analysis. Clinicopathologic classifications and stage groupings were based on the WHO classification of colorectal tumors and the tumor node metastasis (TNM) staging by the American Joint Committee on Cancer[13,16]. Our study protocol was approved by the ethical review boards of the participating hospitals.

Immunohistochemistry

Tumor specimens were fixed in 10% neutral-buffered formalin for 48 h, embedded in paraffin, and cut into 4-μm-thick sections, and then mounted on silane-coated glass slides. Antigen-retrieval was done by autoclaving (121 ℃) for 5 min in pH 9 Antigen Retrieval Liquid (Nichirei, Tokyo, Japan) for MUC2, MUC5AC, and hMSH2, and by microwave irradiation for 10 min in pH 9 Antigen Retrieval Liquid for human mutL homolog 1 (hMLH1). Primary antibodies used were the mouse monoclonal antibody for MUC2 (1:100 dilution; clone Ccp58, Novocastra, Newcastle Upon Tyne, United Kingdom), the mouse monoclonal antibody for MUC5AC (1:100 dilution; CLH2, Novocastra), the rabbit anti-MLH1 monoclonal antibody (1:400 dilution; EPR3894, GeneTex, San Antonio, TX, United States), and the rabbit anti-MSH2 polyclonal antibody (1:200 dilution; 15520-1-AP, Proteintec, Chicago, IL, United States). Samples were treated overnight with each primary antibody at 4 ℃. Immunos-

taining was performed blindly by an investigator (Fukuda K) who was unaware of the clinical information using an N-Histofine Simple Stain MAX-PO kit (Nichirei).

The immunostaining results for mucin core proteins were assessed semi-quantitatively: 0, no staining; 1, < 5% of cells; 2, 5% to < 25% of cells; 3, 25% to < 50% of cells; 4, ≥ 50% of cells (Figure 1). The immunostaining results for mismatch repair (MMR) proteins were either completely negative (negative) or nearly 100% positive (positive). Immunoreactivity was independently evaluated by two investigators (Fukuda K and Imai Y), and discrepancies were resolved by discussion.

In light of their expression levels in normal colonic mucosa, levels 3-4 for MUC2 and levels 1-4 for MUC5AC were evaluated as positive.

Statistical analysis

Comparisons of two cohorts with or without a specific clinicopathologic variable were made by a χ^2 test with/without a Yates' correction or Fisher's exact probability test based on the expected values in a contingency table. Age was compared with Mann-Whitney U test. Comparisons of the mucin expression levels between adenoma and adenocarcinoma components in a single tumor were made by Wilcoxon signed-rank test for sample numbers of ≥ 6. Univariate analysis by Cox regression analysis was used to identify possible prognostic predictors. Variables for which P values were < 0.10 were entered into multivariate regression analysis (forced entry method). Survival curves were generated using the Kaplan-Meier method, and curves were compared by log-rank test. P value < 0.05 was considered significant. Statistical analysis was performed using IBM SPSS Statistics 20 (IBM, Armonk, NY, United States).

Table 1 Clinicopathologic characteristics in colorectal carcinoma *n* (%)

Variables		WMDA (*n* = 63)	PDA (*n* = 91)	MUA (*n* = 81)	SRCC (*n* = 15)
Gender	Male	39 (61.9)	45 (49.5)	46 (56.8)	6 (40.0)
	Female	24 (38.1)	46 (50.5)	35 (43.2)	9 (60.0)
Age (yr)	Range	32-87	35-92	26-90	30-82
	Median	65	64	71	70
Family history	Yes	5 (7.9)	5 (6.1)	5 (6.3)	2 (13.3)
of CRC	No	58 (92.1)	77 (93.9)	75 (93.7)	13 (86.7)
	Unknown		9	1	
Location	Left-sided	43 (68.3)	38 (41.8)	40 (49.4)	6 (40.0)
	Right-sided	20 (31.7)	53 (58.2)	41 (50.6)	9 (60.0)
Depth	Up tp MP	10 (15.9)	4 (4.4)	5 (6.2)	1 (6.7)
	Beyond MP	53 (84.1)	87 (95.6)	76 (93.8)	14 (93.3)
Venous	Yes	48 (76.2)	77 (85.6)	41 (50.6)	14 (93.3)
invasion	No	15 (23.8)	13 (14.4)	40 (49.4)	1 (6.7)
	Unknown		1		
Lymphatic	Yes	35 (55.6)	82 (91.1)	54 (66.7)	12 (80.0)
invasion	No	28 (44.4)	8 (8.9)	27 (33.3)	3 (20.0)
	Unknown		1		
Nodal	Yes	34 (54.0)	69 (78.4)	37 (46.8)	9 (64.3)
metastasis	No	29 (46.0)	19 (21.6)	42 (53.2)	5 (35.7)
	Unknown		3	2	1
Chemotherapy	Yes	17 (27.0)	42 (53.8)	31 (41.9)	7 (46.7)
	No	46 (73.0)	36 (46.2)	43 (58.1)	8 (53.3)
	Unknown		13	7	
Irradiation	Yes	4 (6.3)	2 (2.6)	3 (4.1)	1 (7.1)
	No	59 (93.7)	76 (97.4)	71 (95.9)	14 (92.9)
	Unknown		13	7	
TNM stage	I / II	29 (46.0)	17 (18.9)	40 (50.0)	4 (26.7)
	III / IV	34 (54.0)	73 (81.1)	40 (50.0)	11 (73.3)
	Unknown		1	1	0

CRC: Colorectal carcinoma; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; SRCC: Signet-ring cell carcinoma; MP: Muscularis propria; TNM: Tumor node metastasis.

Figure 2 Colorectal carcinoma overall survival curves generated by the Kaplan-Meier method. WMDA: Well-to-moderately differentiated adeno-carcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; SRCC: Signet-ring cell carcinoma.

RESULTS

Clinicopathologic characteristics

WMDA cases included 63 tumors from 63 patients. Five of these patients (5 tumors) had a family history of CRC. Additional chemotherapy and irradiation were administered for 17 tumors in 17 patients and 4 tumors in 4 patients, respectively. CRCs other than WMDA included a total of 187 tumors: 91 PDAs (90 patients); 81 MUAs (81 patients); and 15 SRCCs (15 patients). One patient had triple cancers (No. 148: two PDAs and one MUA) and one patient had double cancers (No. 170: one PDA and one MUA). Ten of these patients (12 tumors) had a family history of CRC, one of which was proven to be a hereditary non-polyposis colorectal cancer pedigree (No. 148). Predominant occurrence in females, right-sided location, depth of tumors beyond muscularis propria, lymphatic invasion, nodal involvement (except for MUA), TNM stage III/IV (except for MUA) were more frequent in CRCs other than WMDA as compared with WMDA (Table 1). In addition to surgery, chemotherapy and irradiation were administered for 80 tumors in 80 patients and 6 tumors in 6 patients, respectively.

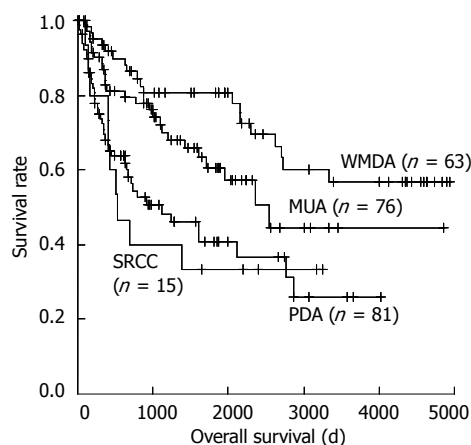The clinicopathologic characteristics of each CRC subtype are summarized in Table 1. CRC patient prognosis was significantly associated with these histological subtypes (Figure 2).

Expression of MUC2 and MUC5AC in CRCs

Expression of the mucin core proteins was assessed individually in different pathological subtypes. MUC2 was expressed in the perinuclear cytoplasm of goblet cells in normal colonic mucosa and diffusely in the cytoplasm of cancer cells. MUC5AC was not expressed in normal colonic mucosa but was occasionally expressed in pericancerous normally appearing colonic mucosa. About half of WMDA cases (49.2%) were positive for MUC2 (levels 3-4), and 30.2% of these cases were positive for MUC5AC (levels 1-4). In contrast, only one tenth of PDA cases (9.5%) were positive for MUC2 and 51.6% of these cases were positive for MUC5AC. PDA was more frequently negative for MUC2 and positive for MUC 5AC than was WMDA. Nearly all MUA cases (95.1%) were positive for MUC2, and over half of these cases (54.3%) aberrantly expressed MUC5AC. Although small in number, 71.5% of SRCC cases were positive for MUC2 and nearly half (46.7%) expressed MUC5AC. These results are summarized in Figure 3.

Expression of the mucin core proteins in the adenoma-carcinoma sequence

Among our study subjects, 21 tumors in 20 patients had an adenoma component indicative of originating from the adenoma-carcinoma sequence. We investigated the sequential expression status of the mucin core proteins. MUC2 expression was found in the adenoma component in all cases, except for one PDA case. MUC2 expression was relatively well maintained in the carcinoma component of WMDA and mildly decreased in some MUA cases. MUC2 expression significantly decreased in the carcinoma component of PDA. MUC5AC was aberrantly expressed in the tubular/tubulovillous adenoma components in all but three cases, and there was a signifi-
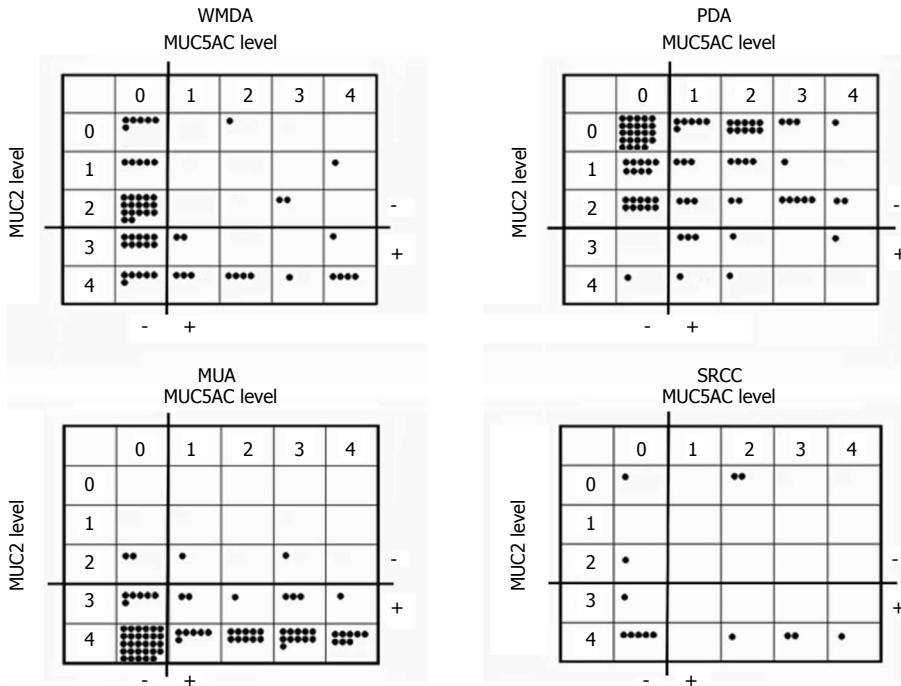
Figure 3 Expression profiles of mucin 2 and mucin 5AC in each colorectal carcinoma histological subtype. Each closed circle indicates one tumor. MUC: Mucin; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; SRCC: Signet-ring cell carcinoma.

Table 2 Expression levels of the mucin core proteins in the adenoma carcinoma sequence

| No. of patient | Histology | | MUC2 | | | MUC5AC | | |
	Adenoma	Carcinoma	Adenoma	Carcinoma	*P* value[1]	Adenoma	Carcinoma	*P* value[1]
208	TV	WMDA	4	3	ND	4	1	ND
225	T	WMDA	4	4		3	2	
237	TV	WMDA	4	4		2	4	
243	TV	WMDA	4	4		4	4	
84	TV	PDA	4	2	0.016	4	0	0.042
86	TV	PDA	4	0		1	0	
87	TV	PDA	4	0		0	0	
102	TV	PDA	4	0		4	0	
116	T	PDA	2	0		2	0	
138	T	PDA	4	0		3	0	
148	T	PDA	3	0		0	0	
15	TV	MUA	4	4	0.063	3	0	0.016
36	TV	MUA	4	4		3	3	
37	TV	MUA	4	4		0	0	
51	TV	MUA	4	4		3	3	
131	T	MUA	4	2		3	1	
148	TV	MUA	4	2		4	0	
151	TV	MUA	4	3		3	1	
155	TV	MUA	4	4		4	2	
175	TV	MUA	4	4		4	2	
191	TV	MUA	4	3		3	0	

[1]Wilcoxon signed-rank test. MUC: Mucin; T: Tubular adenoma; TV: Tubulovillous adenoma; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; ND: Not determined.

cant decrease in MUC5AC expression in the carcinoma components as compared with the adenoma components in PDA and MUA (Table 2).

Expression of the mucin core proteins and clinicopathologic variables

Expression status of the mucin core proteins was ana-

lyzed in association with clinicopathologic variables in each histological CRC subtype that had sufficient numbers for statistical analysis. In a contingency table analysis, no statistically significant associations were found between MUC2 expression and any clinicopathologic variables in any of the histological subtypes. In contrast, MUC5AC expression was significantly associated with right-

Table 3 Expression of the mucin core proteins and clinicopathologic variables *n* (%)

		WMDA			PDA			MUA		
		MUC2			MUC2			MUC2		
		-	+	*P* value	-	+	*P* value	-	+	*P* value
Median age (range), yr		64 (32-82)	64 (34-87)	0.783	64 (35-92)	66.5 (55-86)	0.633	65 (52-88)	71 (26-90)	0.842
Gender	Male	21 (33.3)	18 (28.6)	0.537	41 (45.1)	4 (4.4)	1.000	1 (1.2)	45 (55.6)	0.311
	Female	11 (17.5)	13 (20.6)		42 (46.1)	4 (4.4)		3 (3.7)	32 (39.5)	
Locus	Right-sided	10 (15.9)	10 (15.9)	0.932	47 (51.6)	6 (6.6)	0.461	1 (1.2)	40 (49.4)	0.359
	Left-sided	22 (34.9)	21 (33.3)		36 (39.6)	2 (2.2)		3 (3.7)	37 (45.7)	
Venous invasion	Yes	24 (38.1)	24 (38.1)	1.000	71 (79.0)	6 (6.6)	0.325	3 (3.7)	38 (46.9)	0.616
	No	8 (12.7)	7 (11.1)		11 (12.2)	2 (2.2)		1 (1.2)	39 (48.1)	
Lymphatic invasion	Yes	21 (33.3)	14 (22.2)	0.102	76 (84.4)	6 (6.7)	0.148	1 (1.2)	51 (63.0)	1.000
	No	11 (17.5)	17 (27.0)		6 (6.7)	2 (2.2)		3 (3.7)	26 (32.1)	
Nodal metastasis	Yes	20 (31.7)	14 (22.2)	0.167	64 (72.7)	5 (5.7)	0.362	2 (2.5)	35 (44.3)	1.000
	No	12 (19.0)	17 (27.0)		16 (18.2)	3 (3.4)		2 (2.5)	40 (50.6)	
TNM stage	I / II	12 (19.0)	17 (27.0)	0.167	14 (15.6)	3 (3.3)	0.171	2 (2.5)	38 (48.1)	1.000
	III / IV	20 (31.7)	14 (22.2)		68 (75.5)	5 (5.6)		2 (2.5)	37 (46.8)	
dMMR	Yes	ND			16 (17.6)	3 (3.3)	0.356	ND		
	No				67 (73.6)	5 (5.5)				
		MUC5AC			MUC5AC			MUC5AC		
		-	+	*P* value	-	+	*P* value	-	+	*P* value
Median age (range), yr		64 (32-83)	69 (47-87)	0.099	63 (36-87)	70 (35-92)	0.096	67 (34-87)	72 (26-90)	0.061
Gender	Male	26 (41.3)	13 (20.6)	0.677	22 (24.2)	23 (25.3)	0.919	23 (28.4)	23 (28.4)	0.371
	Female	18 (28.6)	6 (9.5)		22 (24.2)	24 (26.3)		14 (17.3)	21 (25.9)	
Locus	Right-sided	10 (15.9)	10 (15.9)	0.410	20 (22.0)	33 (36.3)	0.017	12 (14.8)	29 (35.8)	0.003
	Left-sided	34 (54.0)	9 (14.3)		24 (26.4)	14 (15.4)		25 (30.9)	15 (18.5)	
Venous invasion	Yes	34 (54.0)	14 (22.2)	0.757	38 (42.2)	39 (43.3)	1.000	16 (19.8)	25 (30.9)	0.224
	No	10 (15.9)	5 (7.9)		6 (6.7)	7 (7.8)		21 (25.9)	19 (23.5)	
Lymphatic invasion	Yes	26 (41.3)	9 (14.3)	0.560	41 (45.6)	41 (45.6)	0.714	25 (30.9)	29 (35.8)	0.875
	No	18 (28.6)	10 (15.9)		3 (3.3)	5 (5.6)		12 (14.8)	15 (18.5)	
Nodal metastasis	Yes	23 (36.5)	11 (17.5)	0.892	40 (45.5)	29 (33.0)	0.010	17 (21.5)	20 (25.3)	0.950
	No	21 (33.3)	8 (12.7)		4 (4.5)	15 (17.0)		19 (24.1)	23 (29.1)	
TNM stage	I / II	21 (33.3)	8 (12.7)	0.892	3 (3.3)	14 (15.6)	0.010	18 (22.8)	22 (27.8)	0.918
	III / IV	23 (36.5)	11 (17.5)		41 (45.6)	32 (35.6)		18 (22.8)	21 (26.6)	
dMMR	Yes	ND			3 (3.3)	16 (17.6)	0.003	ND		
	No				41 (45.1)	31 (34.1)				

MUC: Mucin; WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; MUA: Mucinous adenocarcinoma; dMMR: Mismatch-repair deficiency; ND: Not determined; TNM: Tumor node metastasis.

sided location, absence of nodal metastasis, and lower TNM stage in PDA, and right-sided location in MUA. Furthermore, MUC5AC expression tended to be associated with older age in WMDA, PDA, and MUA, although the difference was not statistically significant. MUC5AC expression was not associated with any clinicopathologic variables in SRCC. These results are summarized in Table 3 (partly not shown).

Expression of the MMR proteins and the mucin core proteins in PDA

In PDA cases, MUC5AC positivity was significantly associated with right-sided location and lower TNM stage, and marginally associated with older age. Survival curve analysis also suggested a better prognosis for MUC5AC-positive PDA cases than for negative ones as described below. These are clinical features associated with high levels of microsatellite instability (MSI; MSI-H)[17-19]. A subset of sporadic CRC cases (approximately 10%-15%) is MSI-H; this is caused by inactivation of the DNA MMR system. Identifying MSI previously required molecular testing, although immunostaining for hMLH1 and hMSH2 has come to be accepted as a practical test to detect MSI[20,21]. Therefore, we investigated MMR status in

PDA cases using immunohistochemistry. Tumors that did not express either of these two were considered MMR deficiency (dMMR).

dMMR was found in 19 PDA cases, 16 of 47 MUC5AC-positive cases and 3 of 44 MUC5AC-negative cases ($P = 0.003$). In contrast, there was no significant association between MUC2 expression and dMMR. Thus, dMMR showed statistically significant association with MUC5AC positivity, although it should be noted that dMMR was found in only one third of MUC5AC-positive tumors and dMMR was also found in one tenth of MUC5AC-negative tumors (Table 3).

Expression of the mucin core proteins and prognosis

The effects of clinicopathologic variables on recurrence/metastasis-free survival (RFS) and overall survival (OS) were investigated using Cox regression analysis for each CRC subtype. For WMDA, TNM stage was the only significant prognostic factor for RFS by univariate and multivariate analysis, but no significant predictor for OS was identified. Next, we included dMMR in the survival analysis for PDA. Univariate Cox regression analysis showed that TNM stage, MUC5AC expression, and dMMR were significant prognostic factors for RFS; however none of

Table 4 Prognostic significance of clinicopathologic variables in well-to-moderately differentiated adenocarcinoma and poorly differentiated adenocarcinoma

		Recurrence/metastasis				Death		
	Parameter	HR (95%CI)	*P* value		Parameter		HR (95%CI)	*P* value
WMDA	Univariate analysis				Univariate analysis			
	Age (over 65 yr)	2.215 (0.974-5.037)	0.058		Age (over 65 yr)		1.984 (0.794-4.955)	0.142
	Gender (male)	0.887 (0.398-1.975)	0.769		Gender (male)		2.194 (0.727-6.617)	0.163
	Location (right-sided)	1.268 (0.559-2.875)	0.570		Location (right-sided)		1.335 (0.524-3.402)	0.545
	TNM stage (Ⅲ/Ⅳ)	3.642 (1.446-9.170)	0.006		TNM stage (Ⅲ/Ⅳ)		2.632 (0.990-6.996)	0.052
	MUC2 positive	0.477 (0.210-1.082)	0.077		MUC2 positive		0.527 (0.207-1.341)	0.179
	MUC5AC positive	1.389 (0.613-3.145)	0.431		MUC5AC positive		1.803 (0.723-4.497)	0.206
	Multivariate analysis				Multivariate analysis			
	Age (over 65 yr)	2.220 (0.965-5.017)	0.061					
	TNM stage (Ⅲ/Ⅳ)	3.473 (1.370-8.805)	0.009					
	MUC2 positive	0.554 (0.243-1.265)	0.161					
PDA	Univariate analysis				Univariate analysis			
	Age (over 65 yr)	0.891 (0.495-1.602)	0.700		Age (over 65 yr)		0.985 (0.955-1.016)	0.331
	Gender (male)	0.624 (0.343-1.138)	0.124		Gender (male)		0.899 (0.484-1.667)	0.734
	Location (right-sided)	0.857 (0.474-1.549)	0.609		Location (right-sided)		0.686 (0.371-1.270)	0.231
	TNM stage (Ⅲ/Ⅳ)	2.647 (1.109-6.320)	0.028		TNM stage (Ⅲ/Ⅳ)		3.208 (1.236-8.330)	0.017
	MUC2 positive	0.936 (0.368-2.379)	0.890		MUC2 positive		1.009 (0.357-2.850)	0.987
	MUC5AC positive	0.433 (0.237-0.793)	0.007		MUC5AC positive		0.599 (0.323-1.112)	0.105
	dMMR	0.373 (0.164-0.847)	0.018		dMMR		0.352 (0.153-0.810)	0.014
	Multivariate analysis				Multivariate analysis			
	TNM stage (Ⅲ/Ⅳ)	1.698 (0.672-4.289)	0.263		TNM stage (Ⅲ/Ⅳ)		2.385 (0.886-6.421)	0.085
	MUC5AC positive	0.586 (0.307-1.117)	0.104		dMMR		0.466 (0.195-1.111)	0.085
	dMMR	0.228 (0.260-1.308)	0.175					

WMDA: Well-to-moderately differentiated adenocarcinoma; PDA: Poorly differentiated adenocarcinoma; dMMR: Mismatch-repair deficiency; TNM: Tumor node metastasis; MUC: Mucin.

these was significant by multivariate analysis. TNM stage and dMMR were also significant predictors for OS by univariate analysis but were not significant by multivariate analysis (Table 4).

For MUA, MUC2 expression was excluded from the analysis because there were very few MUC2-negative MUA cases (*n* = 4 out of 81). The TNM stage was the only significant predictor for RFS, and no variable was a significant predictor for OS (data not shown).

Thus, no significant associations were found between mucin expression and the prognosis of each CRC subtype by multivariate Cox analysis. However, as the data suggested marginal associations between mucin expression and prognosis, and from the need for subsequent discussion, Kaplan-Meier survival curves for associations with mucin expression were generated and assessed by log-rank test for each CRC subtype.

For this analysis of WMDA, marginally better RFS and OS with MUC2-positive tumors were found as compared with negative tumors. The prognosis of MUC5AC-positive WMDA cases tended to be worse as compared to those that were negative, although this difference was not significant (Figure 4A-D). There was also no difference with respect to MUC2 status in PDA. However, MUC5AC expression in PDA was significantly and marginally associated with a better prognosis in terms of RFS and OS, respectively (Figure 4E-H). Because MUC2 positivity was very high in MUA (77 of 81 tumors), its

clinical significance in these settings was not investigated. MUC5AC expression in MUA did not affect its prognosis (Figure 4I and J).

DISCUSSION

MUC2 is normally expressed in the perinuclear cytoplasm of goblet cells in normal colonic mucosa. MUC2 is also expressed in adenomas and mucinous carcinomas[4]. MUC2 downregulation occurs in non-mucinous adeno-car-cinomas that arise within adenomas, whereas cancers that are considered to develop de novo do not express MUC2[4]. Thus, MUC2 levels have been thought to be a predictor of malignant potential. However, despite a poor prognosis, higher levels of MUC2 expression were maintained in MUA and SRCC than in WMDA. This suggests the difficulty with using MUC2 levels as a differentiation marker in these subtypes.

Some investigators reported MUC2 expression in CRC in association with clinical significance, although the association between MUC2 expression and prognosis has been controversial. For example, Matsuda *et al*[9] analyzed 86 CRCs that included 82 WMDA tumors and 4 other variants and reported that the MUC2 expression level was not associated with advanced Dukes' stage and liver metastasis. Baldus *et al*[10] investigated 243 CRCs that included 213 grade Ⅰ-Ⅱ tumors and 30 grade Ⅲ tumors, which also included 22 MUA tumors, and reported that
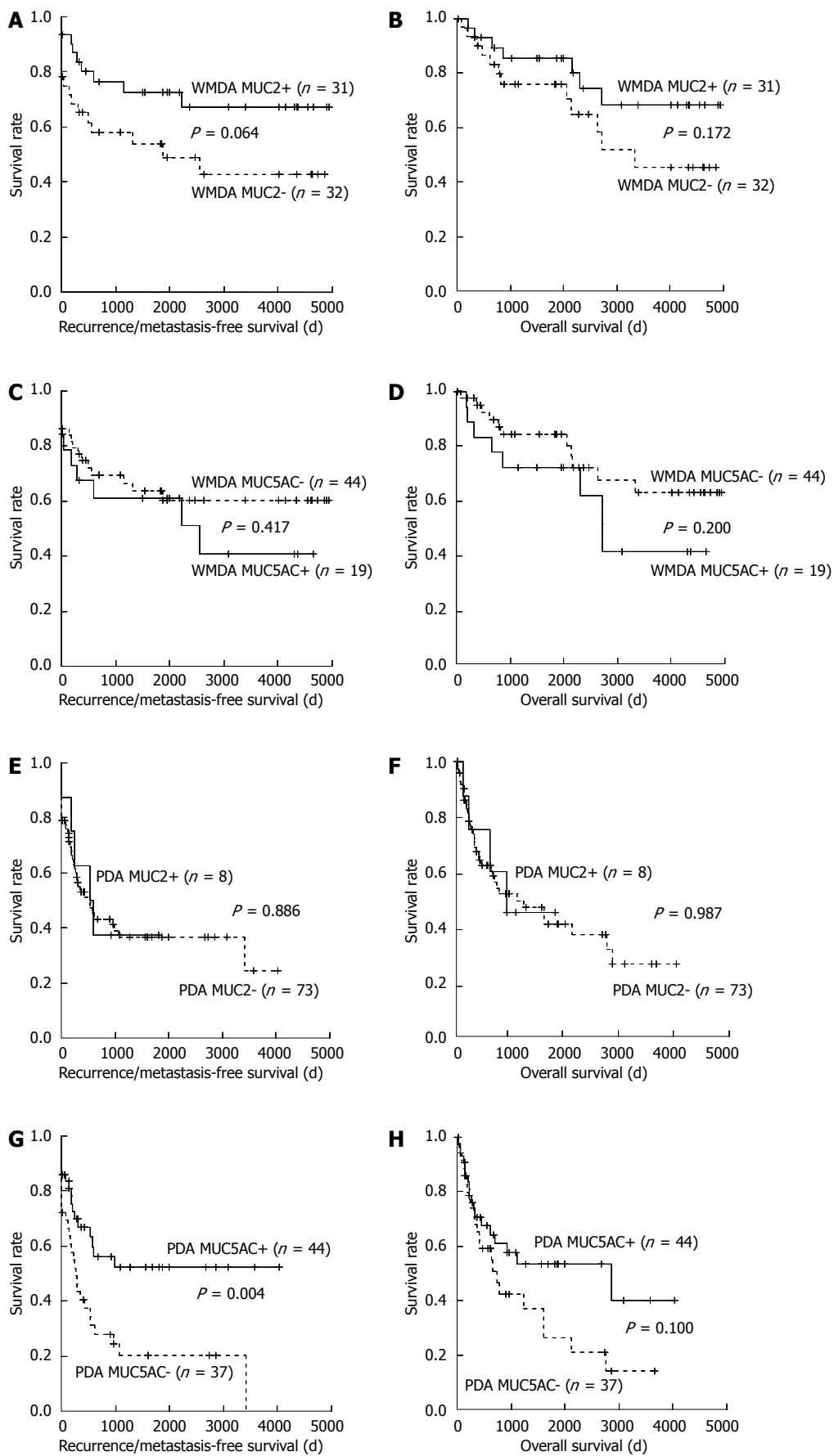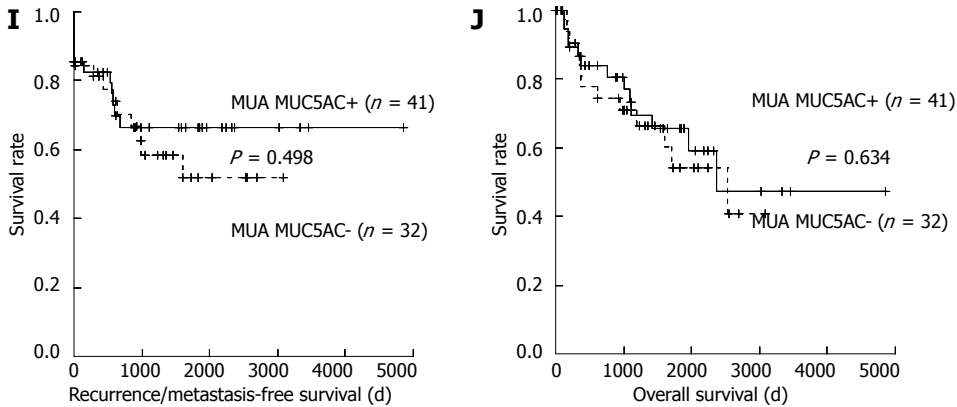
Figure 4 **Survival curves.** A-D: Well-to-moderately differentiated adenocarcinoma (WMDA); E-H: Poorly differentiated adenocarcinoma (PDA); I and J: Mucinous adenocarcinoma (MUA). A: Recurrence/metastasis-free; B: Overall survival curves with or without mucin (MUC) 2 expression of WMDA; C: Recurrence/metastasis-free; D: Overall survival curves with or without MUC5AC expression; E: Recurrence/metastasis-free; F: Overall survival curves with or without MUC2 expression; G: Recurrence/metastasis-free; H: Overall survival curves with or without MUC5AC expression; I: Recurrence/metastasis-free; J: Overall survival curves with or without MUC5AC expression. Curves were generated using the Kaplan-Meier method and compared by log-rank tests. *P* values were derived from comparing mucin-negative and -positive tumors.

MUC2 reactivity was not a marker for worse survival. In contrast, Kang *et al*[8] investigated 301 patients with stage Ⅱ-Ⅲ CRCs, including 200 well-to-moderately differentiated and 101 poorly differentiated cancers (also 266 nonmucinous and 35 mucinous) and reported that a loss of MUC2 expression was associated with a worse overall survival with CRC of stages Ⅱ and Ⅲ. Hanski *et al*[7] showed that a loss of MUC2 expression in CRC owing to promoter methylation was associated with liver and nodal metastasis. On the other hand, a loss of MUC2 expression in CRCs was associated with peritumoral lymphocyte infiltration[22]. Host responses, such as peritumoral lymphocyte infiltration, are known to be associated with a favorable CRC prognosis[23]. MUC2 mucin harbors the sialosyl-Tn antigen that mediates the inhibition of natural killer cell cytotoxicity[24].

However, MUC5AC expression is usually absent in normal colonic mucosa and is only occasionally found in pericancerous normally appearing colonic mucosa. Aberrant MUC5AC expression can be observed in a subset of adenomas and adenocarcinomas[11,25-27]. Microscopically, MUC5AC was detected primarily as focal staining in the cytoplasm and mucous droplets in goblet cells in the normally appearing colonic mucosa but was diffuse in the cytoplasm of cancer cells. MUC5AC expression levels are highest in adenoma but decrease with increasing degrees of dysplasia, and the positive rates for MUC5AC expression were lower in CRC than in adenoma[11,25-27]. These results suggest that MUC5AC may play a role in early carcinogenesis and its expression status can be used to classify CRC from the viewpoint of pathogenesis.

Biemer-Hüttmann *et al*[28] and Losi *et al*[29] reported associations between MUC5AC expression and MSI-H, which is linked with histological subtypes like PDA and MUA. Kocer *et al*[12] analyzed MUC5AC expression in 41 CRCs that included 33 adenocarcinomas, 5 mucinous carcinomas, and 3 neuroendocrine carcinomas. They reported that MUC5AC-negative CRCs had lower rates

of disease-free status and of overall survival, but thy did not investigate associations between MSI and MUC5AC expression.

It has come to be well recognized that CRC comprises various carcinomas that originate from distinct pathogenetic pathways. Owing to this CRC heterogeneity, the significance of mucin core protein expression in CRC remains controversial. Thus, we performed these analyses for each histological CRC subtype.

In the present study, the expression profiles of MUC2 and MUC5AC in conventional adenocarcinoma (WMDA and PDA), MUA, and SRCC were similar to those in previous reports. High MUC2 expression levels in MUA and SRCC suggested distinct histogenetic pathways for these cancer cells, which maintained the feature of mucin-producing goblet cells, from conventional adenocarcinoma. In addition, the expression of the both mucin core proteins tended to decrease during the course of disease progression, from adenoma to carcinoma or from WMDA to PDA. Disease progression is usually accompanied by a decrease in cells of the goblet lineage.

These results are consistent with the hypothesis that the levels of the mucin core proteins may be a marker of malignancy potential in the adenoma-carcinoma sequence in both non-mucinous and mucinous carcinomas. We found that the prognostic significance of the mucin core proteins was different between MUC2 and MUC5AC. In our survival curve analysis, a tendency for better prognosis with MUC2-positive cases than for negative cases was observed in WMDA. A difference in prognosis was not evident from the MUC2 status in PDA.

However, MUC5AC expression in PDA was significantly associated with a better RFS and marginally associated with a better OS. MUC5AC was a significant factor for RFS by univariate Cox regression analysis. Although no significant effects of MUC5AC expression on RFS or OS were found by multivariate analysis, these results may have been because of the limited number of cases

or other unknown factors associated with this patient population. A larger study will be needed to clarify these points.

In comparison, MUC5AC expression was not a factor associated with better prognosis in WMDA and MUA. Aberrant MUC5AC expression is considered to be an early event in carcinogenesis. In addition, MUC5AC expression was significantly associated with right-sided location, absence of nodal metastasis, and a lower TNM stage and was marginally associated with older age in our PDA series. These clinical features as well as poor differentiation are characteristic of MSI-high tumors. MUC5AC expression in PDA was significantly associated with dMMR as shown by a loss of MMR protein expression (16 of 47 MUC5AC-positive cases *vs* 3 of 44 negative cases; $P = 0.003$). Unlike the report by Biemer-Hüttmann *et al*[28] stating that MUC2 expression was also associated with MSI-H, our PDA cases did not exhibit an association between the two. These results suggest that PDA also consists of heterogenous groups of cancers and that MUC5AC expression status may be one of the classification hallmarks.

To date, the mechanisms underlining aberrant MU-C5AC expression in the colon have not been determined. During colon carcinogenesis, MUC5AC expression may be regarded as the re-expression of this fetal mucin. MUC5AC mucin is detected from the fourth month of gestation and is maximum during the sixth month[30]. On the other hand, the MUC5AC promoter was shown to be activated by various inflammation mediators[31]. It was also reported that tumor necrosis factor-α stimulated colon cancer HT-29 cells, which are a goblet cell line, to secrete MUC5AC mucin in a dose-dependent manner[32]. Forgue-Lafitte *et al*[33] reported that MUC5AC mucin was detectable in the mucus of ulcerative colitis patients who underwent surgery. In their series, 10 patients suffering from ulcerative colitis tested were positive for MUC5AC, which suggested that long-term chronic inflammation may induce the production of this mucin in the colonic epithelium. In addition, MUC5AC expression in the regenerating areas close to ulcerations in Crohn's disease suggests its involvement in tissue repair mechanisms[34]. CRC with MUC5AC expression may originate from precancerous lesions owing to long-standing inflammation caused by bacterial infection, inflammatory bowel disease, or other reasons.

In our study, we sometimes observed aberrant MU-C5AC expression in normally-looking colonic mucosa at the interface between non-tumor and tumor tissue, where a strong anti-tumor inflammatory reaction had been observed. We speculate that this may be suggestive of the origin of a neoplasm with aberrant MUC5AC expression. Furthermore, it was previously reported that long-standing inflammation due to ulcerative colitis resulted in CRC with dMMR[35,36]. Taken together, a hypothesis of inflammation-related carcinogenesis may explain the pathogenesis of CRC from MUC5AC-positive precancerous lesions and an association between dMMR and

MUC5AC expression.

In conclusion, we investigated MUC2 and MUC5AC expression status in each of the histological CRC subtypes. MUC2 levels were decreased and MUC5AC levels were increased from WMDA to PDA. MUA and SRCC maintained high MUC2 levels. The expressions of these mucins in PDA and MUA decreased during disease progression in the adenoma-carcinoma sequence. MUC5AC expression was closely associated with MMR deficiency in PDA. MUC2 and MUC5AC expression tended to be associated with a better prognosis in WMDA and PDA, respectively, although these were not statistically significant. Thus, the mucin proteins show distinct clinical significance according to the histological subtypes, and this may also suggest different pathogeneses for these tumors.

COMMENTS

Background
Mucin (MUC)2 mucin is predominantly found in colorectal goblet cells and MU-C5AC mucin is primarily expressed in gastric mucosa. Altered mucin expression may be correlated with the biological behavior of and, possibly, the prognosis for colorectal carcinoma (CRC).

Research frontiers
To date, MUC2 and MUC5AC expressions in CRC have been investigated for their association with clinicopathologic characteristics and prognosis. However, many contradictory results make it difficult to interpret the clinical significance of altered mucin expression, possibly owing to various CRC subtypes. The authors investigated mucin expression by immunohistochemistry in each of the histological CRC subtypes individually and assessed its significance.

Innovations and breakthroughs
MUC2 levels may be associated with malignant potential in conventional adenocarcinoma, whereas mucinous adenocarcinoma and signet-ring cell carcinoma retain high MUC2 levels, which suggests distinct pathogenesis. In comparison, MUC5AC expression may be an early event in tumorigenesis. MUC5AC expression in poorly differentiated adenocarcinoma (PDA) was closely associated with mismatch repair deficiency. MUC2 and MUC5AC expression tended to be associated with a better prognosis in well-to-moderately differentiated adenocarcinoma and PDA, respectively. Thus, the mucin proteins show different clinical significance according to the histological subtypes, and this may also suggest different pathogeneses of these tumors.

Applications
These results could be the basis for further studies to understand the pathogenesis of CRC. Immunohistochemical detection of MUC5AC may be useful for further subclassifications and for predicting a favorable prognosis for PDA.

Terminology
MUC2 and MUC5AC are the backbone proteins of secreted mucins. MUC2 is synthesized in goblet cells in the gastrointestinal tract and MUC5AC is normally expressed in gastric foveolar epithelium. The *MUC2* and *MUC5AC* genes encode gel-forming mucins and are located in a cluster on chromosome 11p15.5.

Peer review
This study is important and interesting. Although there are a large number of publications on the roles of MUC2 and MUC5AC expression in CRC, the results are conflicting. This study adds some refinements to these issues, such as MUC2 and MUC5AC expressions in terms of CRC patient survival, adenoma-

carcinoma sequence, and expression of MMR proteins, with regard to associations with clinicopathologic variables by offering its own evidence.

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