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Relationship Between Immunophenotypes, Genetic Profiles, and Clinicopathologic Characteristics in Small Bowel Adenocarcinoma

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Abstract: Small bowel adenocarcinoma (SBA) is rare, and scant data exist regarding its molecular and clinicopathologic characteristics. This study aimed to clarify the correlation between immunophenotypes, DNA mismatch repair status, genomic profiling, and clinicopathologic characteristics in patients with SBA. We examined 68 surgical resections from patients with primary SBA for immunohistochemical analyses of CK7, CK20, CD10, CDX2, MUC1, MUC2, MUC4, MUC5AC, and MUC6 expression as well as mismatch repair status. Genomic profiling was performed on 30 cases using targeted next-generation sequencing. Tumor mucin phenotypes were classified as gastric, intestinal, gastrointestinal, or null based on MUC2, MUC5AC, MUC6, and CD10 immunostaining. The expression of these proteins was categorized into 3 classifications according to their relationship to: (1) tumor location: CK7/CK20, MUC4, and MUC6; (2) histologic type: mucinous adenocarcinoma was positive for MUC2 and negative for MUC6; and (3) TNM stage: CD10 was downregulated, whereas MUC1 was upregulated in advanced TNM stages. CDX2 was a specific marker for SBA generally expressed in the small intestine. MUC1 and MUC4 expression was significantly associated with worse prognosis. MUC2 expression correlated with better prognosis, except for mucinous adenocarcinoma. Although the difference was not statistically significant, gastric-type tumors were more frequently located in the duodenum and were absent in the ileum. *APC* and *CTNNB1* mutations were not found in the gastric-type tumors. The SBA immunophenotype correlated with tumor location, biological behavior, and genomic alterations. Our results suggest that the molecular pathway involved in carcinogenesis of gastric-type SBA differs from that of intestinal-type SBA.

Key Words: cytokeratin, DNA mismatch repair status, genomic profile, mucin phenotype, small bowel adenocarcinoma

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This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and Japanese regulations, and all experimental protocols were approved by the Ethics Committee of Nippon Medical School (approval no. B-2020-164). All the patients provided written informed consent to participate in the study.

A.H. participated in the study design, carried out the immunohistochemical studies, performed the statistical analysis, wrote the main manuscript, and prepared the figures. A.T. designed the study and drafted the manuscript. T.Y. performed genetic profiling studies and drafted the manuscript. S.K. performed genetic profiling studies. R.H., K.M., T.N., J.O., N.A., and T.H. collected the specimens and clinical data, and interpreted the data. K.G. and S.T. revised the manuscript. S.F., A.S., and K.I. supervised and controlled this study. All the authors have reviewed the manuscript.

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Small bowel cancers are rare, accounting for <5% of gastrointestinal cancers, but their incidence is increasing.[1,2] Small bowel adenocarcinoma (SBA) accounts for one third of small bowel cancers, and due to its rarity, scarce data exist on its molecular and clinicopathologic features.[1,3–5] The genomic profiling of SBA has been shown to be distinct from colorectal cancer and gastric cancer through comprehensive analysis of genomic alterations.[6] Furthermore, although SBA originates anywhere along the duodenum, jejunum, or ileum, and differences in clinicopathologic and molecular features exist among small bowel subsites.[3,4,7–10] Proximal (duodenal) adenocarcinomas have been shown to be associated with worse prognosis compared with distal (jejunoileal) adenocarcinomas.[3,4,7–9] Proximal adenocarcinoma has

higher *CDKN2A* and *ERBB2* alteration rates and conversely fewer *BRAF*, *PTEN*, and *PIK3R1* alteration rates, along with lower overall tumor mutational burden compared with distal adenocarcinomas.[6,11] However, it remains to be determined whether clinicopathologic factors other than tumor location within the small intestine affect genomic alterations.

Studies have found an association between certain mucin phenotypes and biological behavior in both gastric and colorectal cancer.[12–14] Gastrointestinal cancers can be classified into 4 types according to immunostaining for MUC2, MUC5AC, MUC6, and CD10. It is recognized that gastric-type gastric cancer and intestinal-type gastric cancer each have their own clinical, pathologic, and molecular characteristics, as seen in gastric-type and intestinal-type colorectal cancer.[13,14] Our own studies have shown that mutations of *APC* and *CTNNB1* have an inverse relationship with gastric-type markers, but it remains to be determined whether gastric-type and intestinal-type SBAs have different clinicopathologic characteristics.[15] Only a few studies have been reported that classified SBA into gastric and intestinal types and that examined clinicopathologic differences using immunostaining for phenotype markers.[16–19] Although immunostaining for CK7/20, CD10, CDX2, and various mucins has already been investigated on their own in a number of SBA studies, there has been no investigation to date that has brought all of these markers under one study for comprehensive analyses.[16,20–27] More studies are warranted to evaluate these issues through comprehensive analyses of immunohistochemical phenotype markers.

In this study, we performed immunostaining for CK7/20, the classic mucin proteins MUC1, MUC2, MUC4, MUC5AC, and MUC6, and CDX2 and CD10, to examine the relationship between mucin phenotype and tumor biological behavior in patients with SBA.

MATERIALS AND METHODS

Patients and Tissue Samples

We obtained 68 duodenal, jejunal, and ileal adenocarcinoma tissue samples from the archives of the Department of Pathology at Nippon Medical School Hospital and the Department of Pathology at Nippon Medical School Chiba Hokusoh Hospital for the immunohistochemical analysis of CK7, CK20, CD10, CDX2, and mucin proteins expression. Patients with predisposing conditions, including Lynch syndrome, familial adenomatous polyposis, celiac disease, and Crohn disease, were excluded from this study to focus on sporadic SBA. Patients with ampullary adenocarcinoma or metastatic cancer were excluded. Cancer-specific survival (CSS) was defined as the interval from the date of the first surgery until death due to SBA other than from other causes. Relapse-free survival (RFS) was defined as the interval from the date of the first surgery to relapse. All subjects provided informed consent, and the project was approved by the Ethics Committee of Nippon Medical School. All staging criteria were defined according to the International Union for Cancer TNM Classifications.

Immunohistochemical Analysis

Specimens were fixed in 10% formalin, embedded in paraffin wax, and immersed in 0.5% H_2O_2-methanol for 10 minutes to block endogenous peroxidase activity. The sections were then microwaved in 0.01 mol/l citrate phosphate buffer (pH 6.0) or EDTA (pH9.0) for antigen retrieval and incubated with 10% normal horse or goat serum for 10 minutes at 37°C to block nonspecific immunoglobulin (Ig) G binding. Thereafter, the sections were incubated for 18 hours at 4°C with primary antibodies, as listed in Supplemental Table S1 (Supplemental Digital Content 1, http://links.lww.com/PAS/B701). They were then treated with their respective biotinylated antibodies, namely anti-mouse IgG or anti-rabbit IgG (1:200; Vector) for 30 minutes at 25°C, followed by treatment with avidin-biotin peroxidase complex for 30 minutes at 25°C. The reaction products were developed by immersing the sections in a 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.03% H_2O_2.

Evaluation of Immunohistochemical Staining

Each case was blindly evaluated by 2 independent observers (A.H. and A.T.). Any disagreements were resolved using a multiheaded microscope.

DNA mismatch repair (MMR) status was defined by immunostaining for all MMR proteins; MLH1, MSH2, MLH6, and PMS2. Tumors were considered negative when there was complete absence of nuclear staining of neoplastic cells in the presence of an internal positive control assessed on a whole slide. Tumors with negative staining of one of the MMR proteins were considered deficient mismatch repair (dMMR), and all others were considered proficient MMR.

Cases showing immunoreactivity for MUC1, MUC2, MUC4, MUC5AC, MUC6, CK7, CK20, and CDX2 were scored in accordance with the percentage of unequivocally positive epithelial cells and classified according to a score range of 0 to 4: 0, <10% positively stained cells; 1, 10% to 25%; 2, 26% to 50%; 3, 51% to 75%; and 4, >75%. Cases showing immunoreactivity in ≥10% of the cancer cells were considered positive. CDX2 is usually diffusely stained in cancer cells, and most positive cases fall within ranges above 0 (≥10% of cancer cells); therefore, >75% was regarded as the positive cutoff rate. Mucin immunophenotypes were classified into gastric, intestinal, gastrointestinal, and null types, based on MUC2, MUC5AC, MUC6, and CD10 staining. Both MUC2 and CD10 are markers of intestinal-type tumors, whereas MUC5AC and MUC6 are markers of gastric-type tumors. Tissue samples showing both gastric and intestinal phenotypes were classified as gastrointestinal-type tumors, whereas those showing neither gastric nor intestinal phenotype expression were classified as null-type tumors.

Next-generation Sequencing

We performed comprehensive gene mutational analysis of SBA in 30 patients using next-generation sequencing (NGS). DNA extraction, NGS, and mutation calling were performed as described previously.[15,28] In brief, ~10 ng of DNA per sample was amplified using multiplex polymerase chain reaction (PCR) with the Ion AmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher Scientific), which was designed to amplify 207 amplicons covering the 2849 Catalogue of Somatic Mutations in Cancer (COSMIC; http://cancer.sanger.ac.uk/cosmic) mutations in hotspot regions for the 50 most commonly reported oncogenes and tumor suppressor genes. Pooled and barcoded libraries were clonally amplified on Ion Sphere particles using emulsion PCR with the Ion Chef system and Ion PGM HI-Q View Chef Kit (Thermo Fisher Scientific). The subsequently enriched template-positive Ion Sphere particles were loaded onto an Ion 318 chip and sequenced using an Ion Torrent PGM system (Thermo Fisher Scientific). Data from sequencing runs on the Ion Torrent PGM system were automatically transferred to the Torrent Server hosting Torrent Suite Software, v5.2.2 (Thermo Fisher Scientific). Torrent Suite Software uses the Torrent Browser, which includes the Torrent Mapping Alignment Program and Torrent Variant Caller for alignment and variant detection. Variant calling was performed with the CHP2 Panel Somatic PGM using low stringency settings. Only pathogenic or likely pathogenic variants according to CinVar were included. All detected variants were confirmed by droplet digital PCR using a QX200 Droplet Digital PCR system (Bio-Rad Laboratories).

Statistical Analysis

Immunostaining results for each protein were compared with clinicopathologic factors using the χ^2 test or Fisher exact test, as appropriate. The association between immunostaining for each protein was also assessed using the χ^2 test or Fisher exact test, as appropriate. The distribution of CSS and RFS was estimated using the Kaplan-Meier method, and the log-rank test was used to test for significant differences in CSS and RFS. A Cox proportional hazard model was used to assess the effect of tumor variables on CSS. In the multivariate analysis, variables with P-value <0.05 in the univariate analysis were included. Statistical significance was set at P-value <0.05.

RESULTS

Demographic Data of Patients

The patients included 49 men and 19 women, ranging in age from 32 to 84 years (mean: 65 y; median: 68 y). At the time of analysis, 21 patients had died. The overall 5-year survival rate was 64%. The median follow-up duration for the entire series was 36 months (mean: 51.4 mo; range: 5 to 124 mo).

CK7, CK20, CD10, CDX2, MUC1, MUC2, MUC4, MUC5AC, and MUC6 Localization in SBA

Immunostaining for CD10, CDX2, MUC1, MUC2, MUC4, MUC5AC, and MUC6 is shown in Figure 1. The immunoreactivities of the mucin proteins, CD10 and CDX2, scored in accordance with the percentage of unequivocally positive epithelial cells, are shown in Supplemental Table S2 (Supplemental Digital Content 2, http://links.lww.com/PAS/B702). The relationship between immunohistochemical results and clinicopathologic factors is shown in Table 1.

Normal small bowel epithelial cells showed $CK7^-$, $CK20^+$, $CD10^+$, $CDX2^+$, $MUC1^-$, $MUC2^+$, $MUC4^-$, $MUC5AC^-$, and $MUC6^-$ expression. MUC4 was occasionally positive for the reactive epithelial cells adjacent to cancer cells. This tendency was more pronounced in the ileum than in the duodenum. MUC6 was negative in normal small bowel mucosa except for Brunner's gland cells. The positivity rates in cancer cells were: CK7, 43%; CK20, 72%; CD10, 37%; CDX2, 71%; MUC1, 37%; MUC2, 60%; MUC4, 46%; MUC5AC, 37%; and MUC6, 44%, respectively. CDX2 was commonly diffusely stained in cancer cells and its immunoreactivity rate was >10% in almost all cases examined (Supplemental Table S2, Supplemental Digital Content 2, http://links.lww.com/PAS/B702). Even in positive cases, MUC6 was focally stained in cancer cells, except in 2 cases where it was stained diffusely in cancer cells (Supplemental Table S2, Supplemental Digital Content 2, http://links.lww.com/PAS/B702).

The positivity rate of CK7 tended to decrease, whereas that of CK20 tended to increase toward the distal end from the duodenum to the ileum. CK20 was positive in all cases of the ileum. Approximately half of the combination patterns of CK7 and CK20 consisted of $CK7^-/CK20^+$, corresponding to those of normal small bowel mucosa or colorectal cancer (Supplemental Table S3, Supplemental Digital Content 3, http://links.lww.com/PAS/B703). This pattern was the most common at all sites, especially in the ileum, in 4 of the 5 cases. In contrast, the $CK7^+/CK20^-$ pattern was found at the lowest rate in all sections of the small intestine. All 4 patterns were observed in varying degrees in the duodenum and jejunum. MUC4 expression was also associated with tumor location and the positivity rate tended to increase toward the distal end from the duodenum to the ileum. In contrast, the MUC6 positivity rate was highest in the duodenum, decreased in the jejunum, and was absent in the ileum.

MUC2 expression was strongly associated with histologic type, and mucinous carcinoma was positive in all 4 cases. In contrast, MUC6 expression was negative in all the mucinous carcinomas.

The positivity rate of CD10 was significantly lower in tumors with vessel invasion and perineural invasion and tended to be lower in tumors with lymph node metastasis. In contrast, the positivity rate of MUC1 was significantly higher in tumors with vessel invasion, perineural invasion, and lymph node metastasis.

MUC5AC expression was not associated with tumor location, histologic type, or TNM stage.

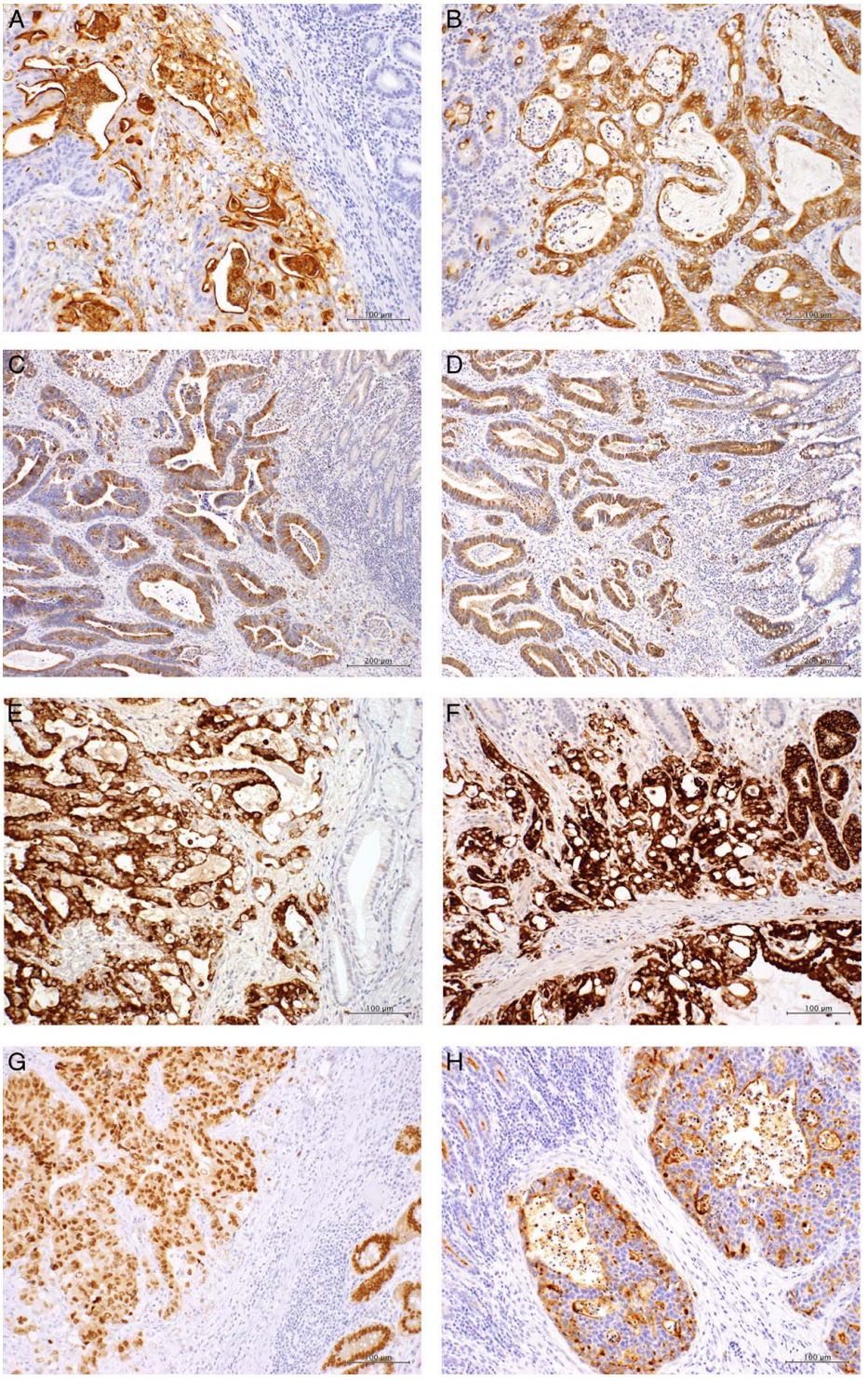

FIGURE 1. Immunohistochemical localization of MUC1 (A), MUC2 (B), MUC4 (C, D), MUC5AC (E), MUC6 (F), CDX2 (G), and CD10 (H) in SBA. A, MUC1 is stained in the apical membrane and cytoplasm of adenocarcinoma cells, but not in normal small bowel epithelial cells. B, MUC2 is stained in both cancer cells and normal small bowel epithelial cells. MUC2 immunostaining showed supranuclear expression in normal epithelial cells, but cytoplasmic expression in cancer cells. C, MUC4 is stained in the cytoplasm of adenocarcinoma cells, but not in normal duodenal epithelial cells. D, MUC4 is stained in both cancer cells and normal ileal epithelial cells. E, MUC5AC is stained in the cytoplasm of adenocarcinoma cells, but not in normal small bowel epithelial cells. F, MUC6 is stained in the cytoplasm of adenocarcinoma cells and Brunner's gland cells, but not in normal small bowel epithelial cells. G, CDX2 is stained in the nucleus in both cancer cells and normal small bowel epithelial cells. H, CD10 is stained in the apical membrane in both cancer cells and normal small bowel epithelial cells.

TABLE 1. Relationship Between the Mucin Proteins, CD10, CDX2, CK7, CK20, and Clinicopathologic Factors (N = 68)

		MUC1		MUC2		MUC4		MUC5AC		MUC6		CD10		CDX2		CK7		CK20	
	N	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*
Age (y)																			
< 68	35	14 (40)	NS	22 (63)	NS	16 (46)	NS	13 (37)	NS	15 (43)	NS	11 (31)	NS	22 (63)	NS	14 (40)	NS	26 (74)	NS
≥ 68	33	11 (33)		19 (58)		15 (46)		12 (36)		15 (46)		14 (42)		26 (79)		15 (46)		23 (70)	
Sex																			
Female	19	7 (37)	NS	13 (68)	NS	11 (58)	NS	8 (42)	NS	8 (42)	NS	7 (37)	NS	12 (63)	NS	10 (53)	NS	12 (63)	NS
Male	49	18 (37)		28 (57)		20 (41)		17 (34)		22 (45)		18 (37)		36 (74)		19 (39)		37 (76)	
Site																			
Duodenum	36	8 (22)	0.001	20 (56)	NS	10 (28)	0.020	13 (36)	NS	25 (69)	< 0.001	20 (56)	0.001	25 (69)	NS	20 (56)	NS	24 (67)	NS
Jejunum	27	17 (63)		17 (63)		16 (59)		11 (41)		5 (19)		3 (11)		18 (67)		8 (30)		20 (74)	
Ileum	5	0		4 (80)		5 (100)		1 (20)		0		2 (40)		5 (100)		1 (20)		5 (100)	
Histology																			
WD, MD	54	20 (37)	NS	31 (57)	NS	25 (46)	NS	19 (35)	NS	27 (50)	NS	21 (37)	NS	37 (69)	NS	25 (46)	NS	39 (72)	NS
PD	9	3 (33)		5 (56)		3 (33)		4 (44)		3 (33)		1 (11)		6 (67)		2 (22)		6 (67)	
Muc	5	2 (40)		5 (100)		3 (60)		2 (40)		0		3 (60)		5 (100)		2 (40)		4 (80)	
pT factor																			
pT1-3	56	18 (32)	NS	36 (64)	NS	25 (45)	NS	21 (38)	NS	26 (46)	NS	21 (38)	NS	40 (71)	NS	23 (41)	NS	40 (71)	NS
pT4	12	7 (58)		5 (42)		6 (50)		4 (33)		4 (33)		4 (33)		8 (67)		6 (50)		9 (75)	
Lymphatic invasion																			
Negative	29	2 (7)	< 0.001	20 (69)	NS	7 (24)	0.003	10 (35)	NS	18 (62)	0.014	17 (59)	0.002	23 (79)	NS	14 (48)	NS	21 (70)	NS
Positive	39	23 (59)		21 (54)		24 (62)		15 (39)		12 (31)		8 (21)		25 (64)		15 (39)		28 (74)	
Venous invasion																			
Negative	32	5 (16)	0.001	22 (69)	NS	8 (25)	0.001	11 (34)	NS	19 (59)	0.017	17 (53)	0.008	24 (75)	NS	15 (7)	NS	23 (72)	NS
Positive	36	20 (56)		19 (53)		23 (64)		14 (39)		11 (31)		8 (22)		24 (67)		14 (39)		26 (72)	
Perineural invasion																			
Negative	29	2 (7)	< 0.001	20 (69)	NS	6 (21)	0.001	9 (31)	NS	18 (62)	0.014	17 (59)	0.002	23 (79)	NS	14 (48)	NS	21 (72)	NS
Positive	39	23 (59)		21 (54)		25 (64)		16 (41)		12 (31)		8 (21)		25 (64)		15 (39)		28 (72)	
Lymph node status																			
N0	41	7 (17)	< 0.001	27 (66)	NS	15 (37)	NS	13 (32)	NS	21 (51)	NS	19 (46)	0.071	31 (76)	NS	18 (44)	NS	29 (71)	NS
Nx	27	18 (67)		14 (52)		16 (59)		12 (44)		9 (33)		6 (22)		17 (63)		11 (41)		20 (74)	
M factor																			
M0	53	17 (32)	NS	33 (62)	NS	21 (40)	NS	18 (34)	NS	27 (51)	0.042	22 (42)	NS	37 (70)	NS	24 (45)	NS	39 (74)	NS
Mx	15	8 (53)		8 (53)		10 (67)		7 (47)		3 (20)		3 (20)		11 (73)		5 (33)		10 (67)	
TNM stage																			
I	25	0	< 0.001	17 (68)	NS	5 (20)	0.011	8 (32)	NS	18 (72)	0.001	16 (64)	0.005	20 (80)	NS	13 (52)	NS	17 (68)	NS
II	16	7 (44)		10 (63)		10 (63)		5 (32)		3 (19)		3 (19)		11 (69)		5 (31)		12 (75)	
III	12	10 (83)		6 (50)		6 (50)		5 (42)		6 (50)		3 (25)		6 (50)		6 (50)		10 (83)	
IV	15	8 (53)		8 (53)		10 (67)		7 (47)		3 (20)		3 (20)		11 (73)		5 (33)		10 (67)	
MMR status																			
Proficient	59	22 (37)	NS	33 (56)	NS	25 (42)	NS	21 (36)	NS	26 (44)	NS	25 (42)	0.021	40 (68)	NS	27 (46)	NS	42 (71)	NS
Deficient	9	3 (33)		8 (89)		6 (67)		4 (44)		4 (44)		0		8 (89)		2 (22)		7 (78)	

MD indicates moderately differentiated; Muc, mucinous adenocarcinoma; NS, nonsignificant; PD, poorly differentiated; WD, well differentiated.

TABLE 2. Classifications of Tumor Mucin Phenotypes and Clinicopathologic Factors of SBA Patients (N = 68)

		n (%)				
	N	Gastric type (N = 12)	Gastrointestinal type (N = 27)	Intestinal type (N = 24)	Null type (N = 5)	*P*
Site						
Duodenum	36	8 (22)	19 (53)	7 (19)	2 (6)	0.052
Jejunum	27	4 (15)	7 (26)	13 (48)	3 (11)	
Ileum	5	0	1 (20)	4 (80)	0	
Histology						
WD, MD	54	10 (19)	22 (41)	18 (33)	4 (7)	NS
PD	9	2 (22)	3 (33)	3 (33)	1 (11)	
Muc	5	0	2 (40)	3 (60)	0	
pT factor						
pT1-3	56	9 (16)	23 (41)	21 (38)	3 (5)	NS
pT4	12	3 (25)	4 (33)	3 (25)	2 (17)	
LN status						
N0	41	6 (15)	18 (44)	15 (37)	2 (5)	NS
Nx	27	6 (22)	9 (33)	9 (33)	3 (11)	
M factor						
M0	53	8 (15)	23 (43)	18 (34)	4 (8)	NS
Mx	15	4 (27)	4 (27)	6 (40)	1 (7)	
TNM stage						
I	25	3 (12)	16 (64)	6 (24)	0	NS
II	16	3 (19)	2 (13)	9 (56)	2 (13)	
III	12	2 (17)	5 (42)	3 (25)	2 (17)	
IV	15	4 (27)	4 (27)	6 (40)	1 (7)	
MMR status						
Proficient	59	11 (19)	24 (41)	19 (32)	5 (9)	NS
Deficient	9	1 (11)	3 (33)	5 (56)	0	
CK7						
Negative	39	4 (10)	13 (33)	19 (49)	3 (8)	0.038
Positive	29	8 (28)	14 (48)	5 (17)	2 (7)	
CK20						
Negative	19	9 (47)	7 (37)	3 (16)	0	< 0.001
Positive	49	3 (6)	20 (41)	21 (43)	5 (10)	

LN indicates lymph node; MD, moderately differentiated; Muc, mucinous adenocarcinoma; NS, nonsignificant; PD, poorly differentiated; WD, well differentiated.

The Mutual Relationship Between the Mucin Proteins, CD10, CDX2, CK7, and CK20 Expression

The mutual interrelationships between mucin proteins, CD10, CDX2, CK7, and CK20 expression are shown in Supplemental Table S4 (Supplemental Digital Content 4, http://links.lww.com/PAS/B704). A significant positive relationship was observed between MUC2 and CDX2 expression levels. There was a significant positive relationship between MUC5AC and MUC6 expression, which was expected, given that both are gastric markers. MUC5AC was negatively associated with CD10 and CDX2, which are intestinal markers. An inverse relationship was observed between gastric markers (MUC5AC, MUC6, and CK7) and intestinal markers (MUC2, CD10, CDX2, and CK20).

Tumor Mucin Phenotypes Based on Immunostaining for CD10, MUC2, MUC5AC, and MUC6

The classification of tumor mucin phenotypes and clinicopathologic factors in the patients is summarized in Table 2. Tumor mucin phenotypes included gastric type (n = 12), intestinal type (n = 24), gastrointestinal type (n = 27) and null type (n = 5) based on combined immunostaining for CD10, MUC2, MUC5AC, and MUC6. Histologically, gastric-type SBA was characterized by tubular epithelial glands lined by cuboidal to low columnar cells with pale eosinophilic cytoplasm. Intestinal-type SBA consisted of tubular structures lined by tall columnar cells with variable eosinophilic cytoplasm and goblet cell differentiation (Fig. 2). Although not statistically significant, gastric-type tumors were more frequently located in the duodenum and absent in the ileum. Histologically, no gastric-type tumors were found in mucinous adenocarcinomas. The incidence of intestinal-type tumors tended to increase toward the distal end, from the duodenum (19%), through the jejunum (48%) and to the ileum (80%). Gastrointestinal-type tumors did not correlate with either the tumor location or histologic type. CK7 positivity rates were higher in gastric type and lower in intestinal-type tumors. CK20 positivity rates were lower in gastric-type and higher in intestinal-type tumors. Therefore, most intestinal-type tumors were $CK7^{-}/CK20^{+}$ (Supplemental Table S3, Supplemental Digital Content 3, http://links.lww.com/PAS/B703).

No significant association was found between tumor mucin phenotypes and MMR status, except for a tendency for more dMMR cases found in intestinal-type tumors.

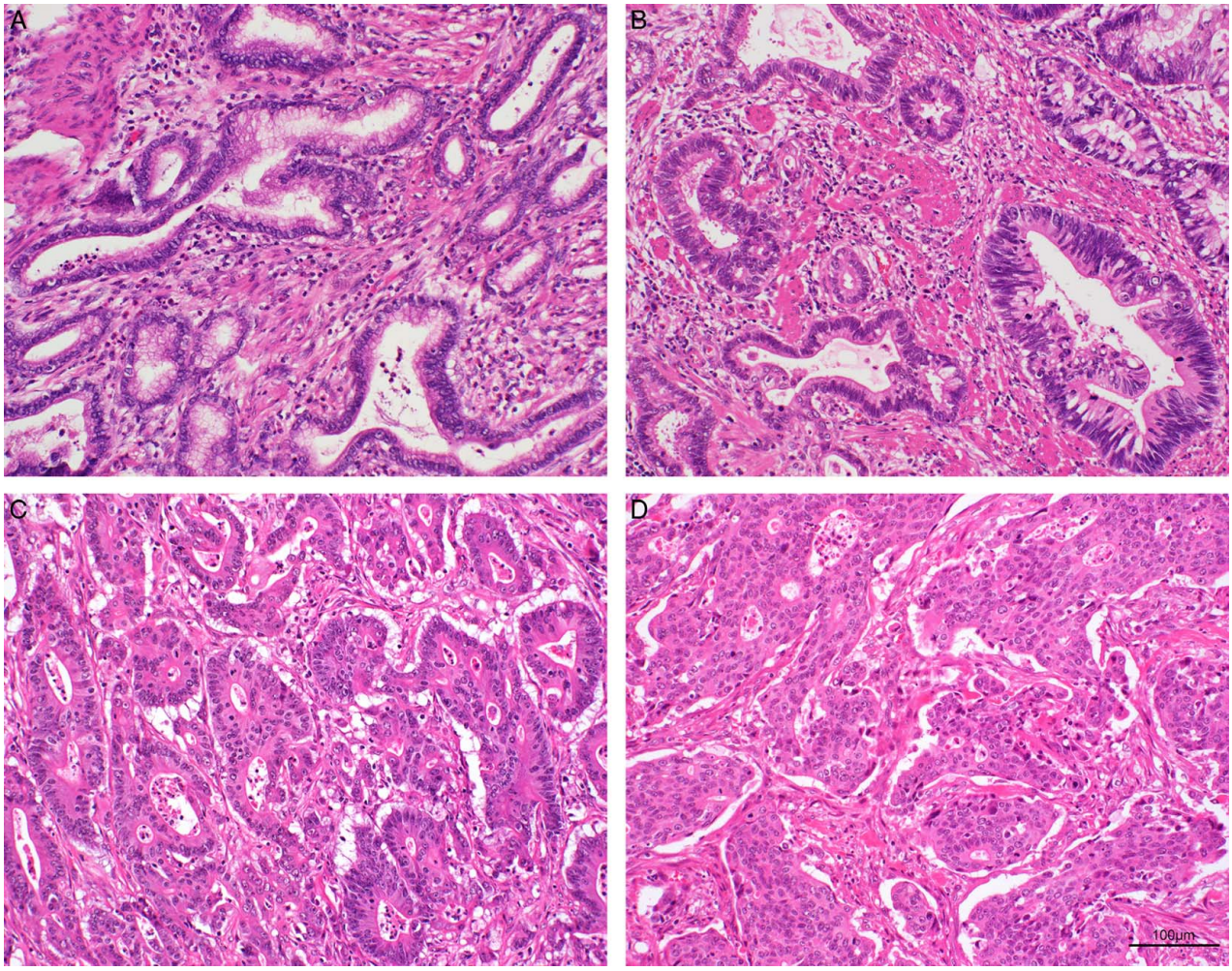

FIGURE 2. Representative hematoxylin and eosin stain images of mucin phenotypes of gastric-type (A), intestinal-type (B), gastrointestinal-type (C), and null-type (D) SBA. A, Gastric-type SBA is characterized by tubular epithelial glands lined by cuboidal to low columnar cells with pale eosinophilic cytoplasm. B, Intestinal-type SBA consists of tubular structures lined by tall columnar cells with variable eosinophilic cytoplasm and goblet cell differentiation. C, Gastrointestinal-type SBA consists of a mixture of epithelial cells with gastric and intestinal types. D, Null-type SBA consists of epithelial cells with eosinophilic cytoplasm and without mucous.

Relationship Between Gene Mutations and Mucin Phenotypes

The raw NGS data are presented in Supplemental Table S5 (Supplemental Digital Content 5, http://links.lww.com/PAS/B705). Overall, at least 1 genomic alteration was found in 27 of the 30 cases (Fig. 3). The most common genomic alterations were *TP53* (n = 17, 56.7%), followed by *KRAS* (n = 7, 33.3%), *APC* (n = 5, 16.7%), *PIK3CA* (n = 5, 16.7%), *GNSA* (n = 4, 13.3%), *CTNNB1* (n = 3, 10%), *KIT* (n = 3, 10%); then 2 (6.7%) each for *BRAF*, *CDKN2A*, and *PTEN*; and 1 (3.3%) each for *FBXW7*, *EGFR*, *ERBB2*, *ERBB4*, *STK11*, *VHL*, and *NOTCH1*.

The classification of tumor mucin phenotypes and gene mutations in the patients is summarized in Table 3. *APC* and *CTNNB1* mutations were not found in gastric-type tumors. *GNAS* mutations were found more frequently in gastric-type tumors than in intestinal-type tumors, although the difference was not significant.

Comparative Survival Analysis

Patients with dMMR tumors had significantly better CSS than patients with proficient MMR tumors, with a mortality rate of 0 (log-rank test, $P = 0.043$). MUC1 expression was significantly associated with worse CSS and RFS (log-rank test, $P = 0.002$, <0.001, respectively) (Figs. 4A, E). MUC2 expression was significantly associated with better CSS, except for mucinous adenocarcinoma (log-rank test, $P = 0.025$), and was significantly associated with better RFS (log-rank test, $P = 0.015$) (Figs. 4B, F). MUC4 expression was significantly associated with worse CSS and RFS (log-rank test, $P = 0.038$, 0.021, respectively) (Figs. 4C, G). MUC6 expression was significantly associated with better CSS (log-rank test, $P = 0.034$).

When differences in survival rates were compared against mucin phenotype classifications, patients with gastrointestinal-type tumors showed the best prognosis,

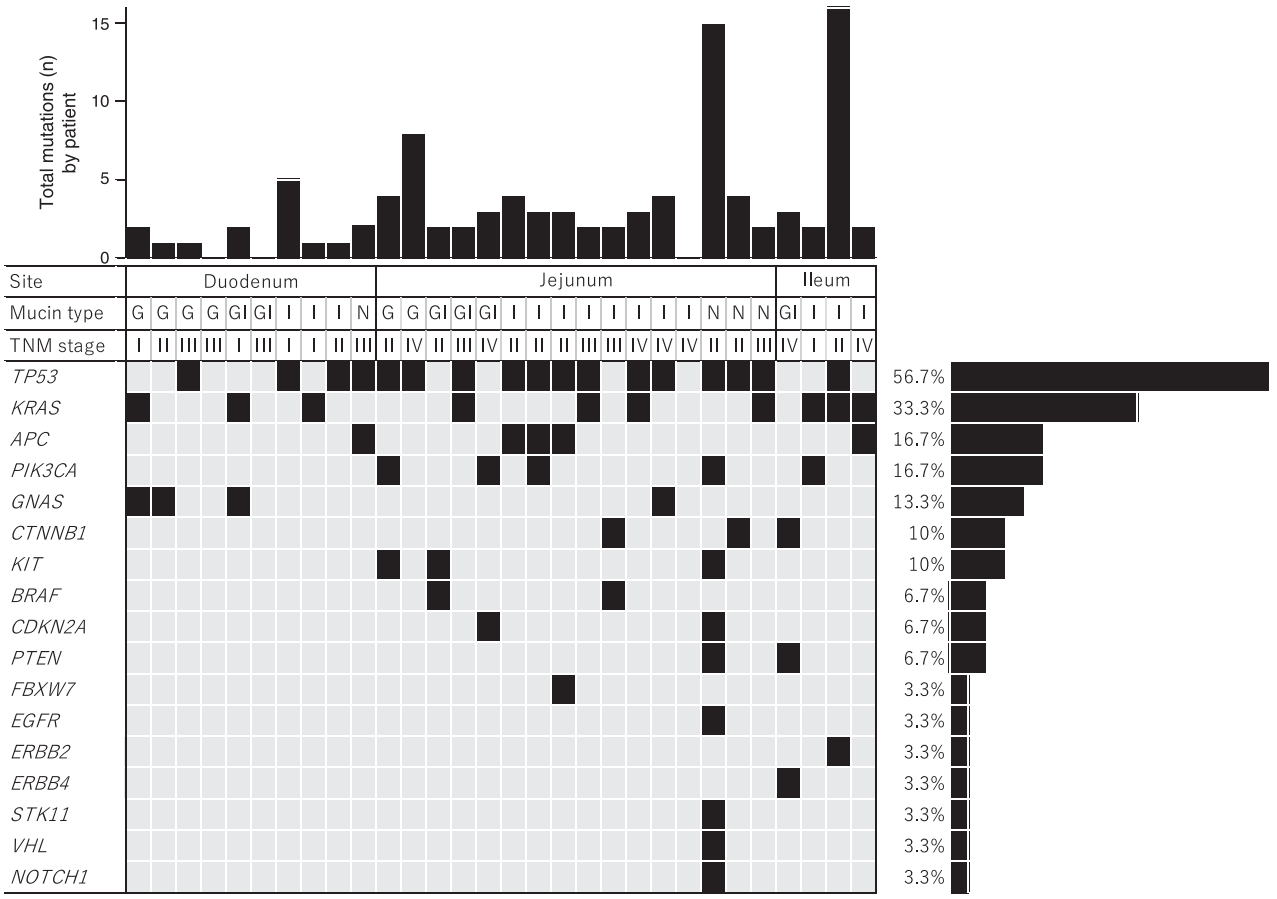

FIGURE 3. Mutational landscape of 30 patients with SBA. The somatic mutational profiles of all cases. The upper panel shows the number of total mutations in each tumor. The central plot shows the key clinical parameters, below which the recurrent mutated genes for each case are shown. The figure includes 25 genes identified in SBA patients (n = 30). The most common genomic alterations found were *TP53* (n = 17, 56.7%).

whereas patients with null-type tumors showed the worst prognosis (Fig. 4D).

In the univariate analysis using the Cox proportional hazards model for CSS, histologic type, pT factor, lymph node status, MUC1 expression, MUC4 expression, and MUC6 expression had significant prognostic value (Table 4). In the multivariate analysis performed by introducing all the above variables in the Cox proportional hazards model, only lymph node status retained independent prognostic significance.

DISCUSSION

We examined the expression, localization, and clinical significance of CK7, CK20, MUC1, MUC2, MUC4, MUC5AC, MUC6, CD10, and CDX2 in SBA. Although there have been a number of reports regarding the expression of mucin series and cytokeratin in SBA, their clinical significance remains controversial.[16,20–27] Such discrepancies have been explained by differences in scoring methods, cutoff value of immunostaining, heterogeneities of SBA, and any bias originating from the inclusion of an insufficient number of cases.

Frist, since SBA can originate anywhere throughout the duodenum, jejunum, and ileum, we should consider whether differences in the expression of any of the proteins found in normal epithelial cells exist among small bowel subsites. Normal small bowel epithelial cells showed $CK7^{-}$, $CK20^{+}$, $CD10^{+}$, $CDX2^{+}$, $MUC1^{-}$, $MUC2^{+}$, $MUC5AC^{-}$, and $MUC6^{-}$ expression regardless of small bowel subsites. In SBA, CK7 expression decreased from the duodenum to the ileum, whereas CK20 expression increased. Therefore, the expression of proteins in duodenal adenocarcinoma tends to resemble that of the normal stomach, and that in ileal adenocarcinoma parallels that of the normal colon in terms of CK7/CK20 staining pattern. This is considered logical from an anatomic and embryological standpoint. Although MUC4 is basically negative in normal small bowel epithelial cells, it is sometimes positive in the ileum, which may be related to the fact that its expression was higher in ileal adenocarcinoma than in duodenal adenocarcinoma. Shibahara et al[24] showed that the positivity rate for MUC4 expression was 29% in the duodenum and 65% in the jejunum and ileum, consistent with our results. MUC6 is

TABLE 3. Relationship Between Common Genetic Alterations and Clinicopathologic Factors of Patients With SBA (N = 30)

		TP53		KRAS		APC		PIK3CA		GNAS		CTNNB1	
	N	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*	n (%)	*P*
Site													
Duodenum	10	4 (40)	NS	3 (30)	NS	1 (10)	NS	0	NS	3 (30)	NS	0	NS
Jejunum	16	12 (75)		4 (25)		3 (19)		4 (25)		1 (6)		2 (13)	
Ileum	4	1 (25)		3 (75)		1 (25)		1 (25)		0		1 (25)	
Histology													
WD, MD	23	13 (57)	NS	9 (39)	NS	5 (22)	NS	4 (17)	NS	3 (13)	NS	1 (4)	NS
PD	5	3 (60)		1 (20)		0		1 (20)		1 (20)		1 (20)	
Muc	2	1 (50)		0		0		0		0		1 (50)	
Mucin phenotype													
Gastric type	6	3 (50)	NS	1 (17)	NS	0	NS	1 (17)	NS	2 (33)	NS	0	NS
Gastrointestinal type	6	1 (17)		2 (33)		0		1 (17)		1 (17)		1 (17)	
Intestinal type	14	9 (64)		6 (43)		4 (29)		2 (14)		1 (7)		1 (7)	
Null type	4	4 (100)		1 (25)		1 (25)		1 (25)		0		1 (25)	
TNM stage													
I	5	1 (20)	NS	4 (80)	NS	0	NS	1 (20)	NS	2 (40)	NS	0	NS
II	10	8 (80)		1 (10)		3 (30)		3 (30)		1 (10)		1 (10)	
III	8	5 (63)		3 (38)		1 (13)		0		0		1 (13)	
IV	7	3 (43)		2 (29)		1 (14)		1 (14)		1 (14)		1 (14)	
MMR status													
Proficient	24	12 (50)	NS	8 (33)	NS	2 (8)	0.041	3 (13)	NS	4 (17)	NS	3 (13)	NS
Deficient	6	5 (83)		2 (33)		3 (50)		2 (33)		0		0	

MD indicates moderately differentiated; Muc, mucinous adenocarcinoma; NS, nonsignificant; PD, poorly differentiated; WD, well differentiated.

negative in normal small bowel epithelium but positive in Brunner's gland cells; therefore, it makes sense that it is found exclusively in duodenal adenocarcinoma. It has been postulated that MUC6 positive duodenal cancer originates in Brunner's glands.[29,30] MUC6 was positive in 40% of cases examined in our cohort of patients, but focally stained in the majority of cases. Only 2 cases showed a diffuse staining pattern for MUC6. Therefore, we believe that MUC6 appears ectopically and aberrantly during tumor development in common, although we cannot rule out the possibility of Brunner's gland-origin theory for tumors accompanied by Brunner's gland hyperplasia and diffusely stained MUC6. Considering the above results, CK7, CK20, MUC4, and MUC6 expression may be related to the staining properties of normal gastrointestinal epithelial cells.

Next, the proteins associated with histologic type were MUC2 and MUC6, both of which correlated with mucinous adenocarcinoma, with the former being positive in all cases and the latter being negative in all cases. Although Kumagai et al[25] reported that mucinous adenocarcinoma was negative for CD10, an association between CD10 expression and histologic type seems unlikely.

The proteins associated with tumor progression were MUC1 and CD10, the former upregulated and the latter downregulated with progression of TNM stage. Kumagai et al[25] suggested that since CD10 is expressed in normal small intestinal epithelial cells, loss of CD10 expression is associated with depolarization of the immunophenotype, which in turn leads to aggressive behavior in tumors. The CD10 positivity rate was the highest in the duodenum and lowest in the jejunum, although this may be explained in part by the high prevalence of stage I cancer in the duodenum, suggesting that the correlation between CD10 expression and TNM stage is stronger than its association with tumor location.

The positivity rate of MUC4 was lower in stage I than in stage II to IV cancers; but when limited to the jejunum and ileum, it was not statistically associated with stage, suggesting that MUC4 expression may be influenced by tumor location rather than stage. CDX2 expression did not correlate with tumor location, histologic type, or TNM stage in our cohort of patients. The CDX2 positivity rate was 73%, even for stage IV, suggesting that CDX2 expression was retained in malignant transformation from normal cells and did not change throughout disease progression.

The relationship between protein expression and prognosis cannot be established until it is taken into account that expression of these proteins differs depending on tumor histologic type and small bowel subsites. MUC2 expressed markedly in mucinous carcinoma and distal side of the small intestine, consistent with a previous report.[24] When survival analysis was performed excluding mucinous carcinoma, patients with tumors negative for MUC2 had worse prognosis than patients with tumors positive for MUC2. Previous reports also suggest that decreased expression of MUC2 may be associated with higher tumor grade and aggressive behavior, except for mucinous carcinoma.[31] It has been reported that $Muc2^{-/-}$ mice developed adenoma in the small intestine that progressed to invasive adenocarcinoma, thereby indicating the protective role of MUC2 in colorectal cancer.[32] These results suggest that MUC2 expression in SBA is strongly associated with mucinous carcinoma, as has been seen in

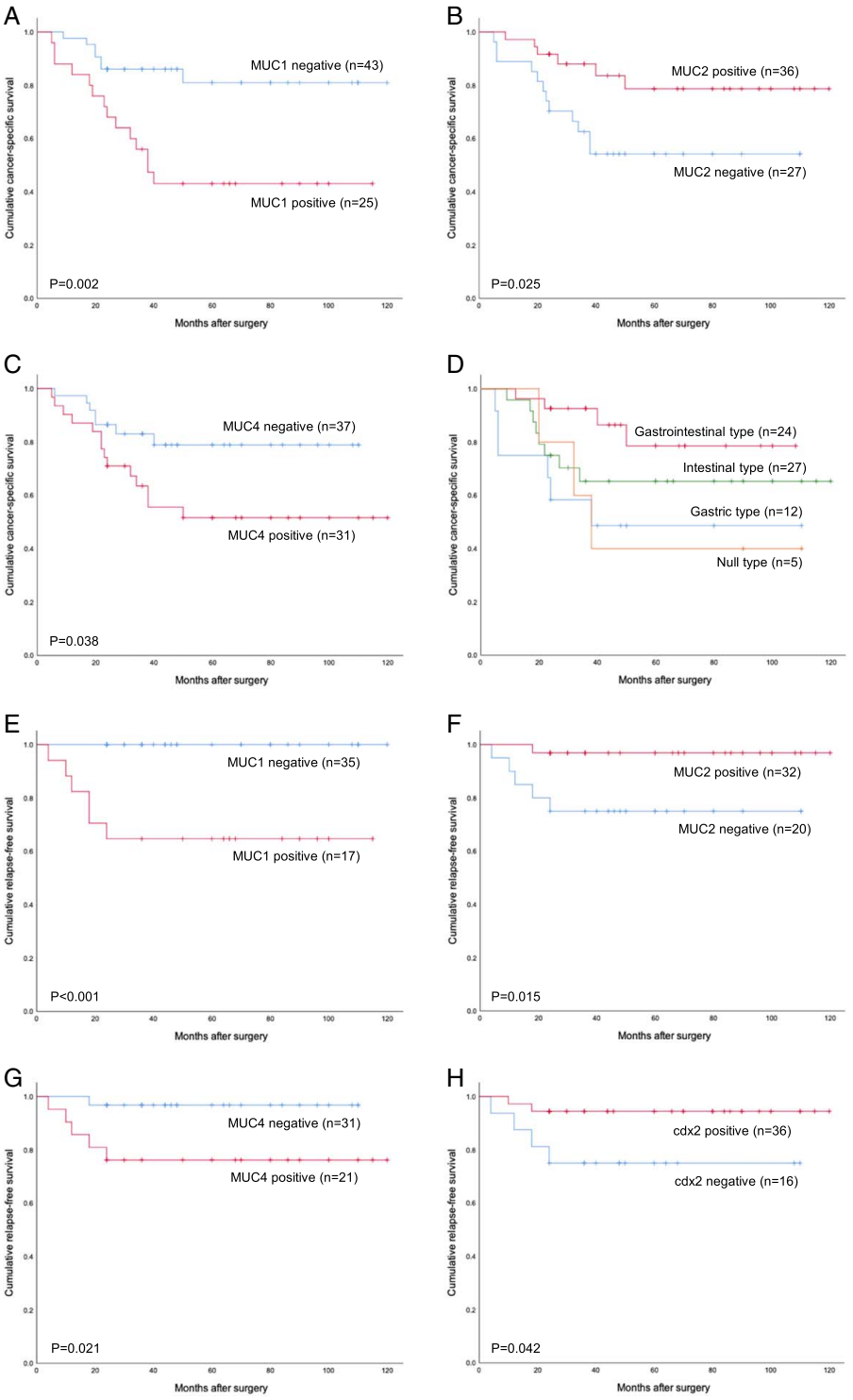

FIGURE 4. Kaplan-Meier CSS (A–D) and RFS (E–H) curve for immunostaining patterns in SBA patients evaluated by the log-rank test. A, CSS rates were significantly worse in MUC1-positive SBA patients. B, CSS rates were significantly better in patients with MUC2-positive SBA except for mucinous adenocarcinoma. C, CSS rates were significantly worse in MUC4-positive SBA patients. D, CSS differences were observed among 4 SBA patient groups classified according to immunophenotype. E, RFS rates were significantly worse in MUC1-positive SBA patients. F, RFS rates were significantly better in MUC2-positive SBA patients. G, RFS rates were significantly worse in MUC4-positive SBA patients. H, RFS rates were significantly better in CDX2-positive SBA patients.

TABLE 4. Univariate and Multivariate Cox Proportional Hazards Analyses for CSS (Stage I to IV) (N = 68)

		Univariate analysis		Multivariate analysis	
Variables	**Categories**	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Histologic type	PD, Muc vs. WD, MD	2.494 (1.003-6.200)	0.049	1.962 (0.534-7.203)	NS
pT factor	pT4 vs. pT1-3	4.069 (1.678-9.868)	0.002	1.262 (0.446-3.575)	NS
LN metastasis	Positive vs. negative	49.19 (6.58-367.92)	< 0.001	36.92 (4.091-333.2)	0.001
MUC1	Positive vs. negative	3.793 (1.530-9.405)	0.004	1.276 (0.360-4.517)	NS
MUC2	Positive vs. negative	0.472 (0.199-1.121)	0.089	0.475 (0.162-1.390)	NS
MUC4	Positive vs. negative	2.516 (1.015-6.238)	0.046	1.050 (0.338-3.262)	NS
MUC5AC	Positive vs. negative	1.063 (0.440-2.564)	NS		
MUC6	Positive vs. negative	0.363 (0.133-0.993)	0.048	0.398 (0.126-1.253)	NS
CD10	Positive vs. negative	0.502 (0.184-1.371)	NS		
CDX2	Positive vs. negative	0.666 (0.276-1.609)	NS		

HR indicates hazard ratio; LN, lymph node; MD, moderately differentiated; Muc, mucinous adenocarcinoma; NS, nonsignificant; PD, poorly differentiated; WD, well differentiated.

colorectal cancer, and could be a hallmark of good prognosis in patients with nonmucinous carcinoma. Our study showed a significant association between MUC4 expression and poorer prognosis using univariate analysis and log-rank test. MUC4 expression has been significantly associated with worse overall survival in patients with biliary tract carcinoma, pancreatic cancer, and colorectal cancer.[33] However, as mentioned above, MUC4 is related to localization rather than progression, so results may vary depending on the small bowel subsite ratio of cases examined. MUC6 expression was significantly associated with better CSS, although this may be explained in part by the high prevalence of stage I cancer in the duodenum, suggesting that the correlation between MUC6 expression and tumor location is stronger than its association with prognosis.

Studies have also correlated MUC1 with the aggressive behavior of tumors.[23,24] Shiba et al[34] characterized the functional expression of MUC1 in human duodenal adenocarcinoma. Silencing of MUC1 significantly reduced proliferation, migration, and invasion in duodenal cancer cell lines. They also found that MUC1 expression was associated with worse prognosis in patients with duodenal adenocarcinoma. MUC1 overexpression may play a role in the neoplastic transformation and metastatic process of colorectal cancer.[35] MUC1 has been shown to be a prognostic factor in gastric cancer by meta-analysis.[36] These findings suggest that MUC1 expression may be associated with worse outcomes throughout the gastrointestinal tract, regardless of the small intestinal subsite.

We found that CDX2 expression was significantly associated with better RFS, but not CSS. Jun et al[23] linked CDX2 to better prognosis in patients with SBA, somewhat consistent with our results. If the cutoff of CDX2 immunostaining is set to 10% like other proteins, most cases will be regarded as positive, so the relationship with prognosis may depend on the cutoff value. In addition, it has been reported that CDX2 activates MUC2 expression in gastric cancer cell lines.[37] In fact, MUC2 expression has been found to be associated with CDX2 expression. This is consistent with the fact that loss of expression of CDX2 and MUC2 correlates with poor prognosis.

We classified SBA into 4 types using gastric and intestinal markers. Gastric-type SBAs had more cases originating from the duodenum and jejunum, with differentiated type histology, but with no correlation to MMR status. In contrast, intestinal-type SBAs were more common in the ileum, with no correlation to histologic type but with a tendency for more dMMR cases. Considering tumor location, all 4 types of tumors were found in both the duodenum and jejunum, but gastric-type tumors were not seen in the ileum. Considering the staining patterns of CK7 and CK20, most tumors in the ileum showed a $CK7^{-}/CK20^{+}$ pattern, as has been observed in colorectal cancer. In contrast, a variety of combination patterns of gastric and intestinal markers were observed in duodenal and jejunal tumors. These results suggest that duodenal and jejunal tumors share common immunophenotypes distinct from those of the ileum. Genetic profiles indicate that the mutation rates of *KRAS*, *APC*, and *CTNNB1* are lower in gastric-type SBA, although these are more commonly observed in intestinal-type SBA, suggesting that the characteristics of intestinal-type SBA resembles those of colorectal cancer.[15] In contrast, gastric-type SBA had fewer mutations, with the exception of *GNAS* mutations, implying that its characteristics are distinguishable from those of colorectal cancer. It has been reported that gastric phenotype is inversely associated with Wnt-signaling abnormalities, which is consistent with our results.[13,19] From the above results, we conclude that the molecular pathways of carcinogenesis differ between the gastric and intestinal types of SBAs.

In our cohort of patients, patients with null-type tumors had the worst prognosis, whereas patients with gastrointestinal-type tumors had the best prognosis. Although results may vary depending on cohort bias, our results paralleled those reported for colorectal cancer, where patients with unclassified-type (null-type) tumors had the worst prognosis among all 4 tumor types.[14]

This study has several limitations. First, the sample size is small. In addition, there is a bias in tumor localization. Second, MMR status was determined based

solely on the results of immunohistochemical staining for MMR proteins. Third, it takes time for SBA to be discovered. This is supported by the fact that one third of patients with SBA are diagnosed with stage IV at a higher rate than patients with colorectal cancer. This is thought to originate from the nonspecificity of symptoms and the lack of population-based screening, which suggests that the staging of SBA by TNM classification is biased.

In conclusion, the relationship between each protein and any clinicopathologic factor is as follows: CK7/CK20 correlates with tumor location and mucin phenotypes; CD10 is downregulated as TNM stage advances; MUC1 upregulated as TNM stages advanced; MUC2 correlates with histologic type, MUC4 with tumor location, and MUC6 with histologic type and tumor location. CDX2 is a specific marker of SBA generally expressed in the small intestine with no connection to clinicopathologic factors. The significance of mucin staining results cannot be fully interpreted unless at least the above correlations with clinicopathologic factors are considered, whereas prognosis cannot be predicted unless adjustments are made for these factors. Considering all these elements, it appears that tumors of the duodenum and jejunum share common immunophenotypes that are distinct from tumors of the ileum. Furthermore, the results of this study indicate different molecular pathways of carcinogenesis in gastric-type and intestinal-type SBAs.

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