Expression of survivin, MUC2 and MUC5 in colorectal cancer and their association with clinicopathological characteristics

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Abstract. Survivin is a bifunctional protein that suppresses apoptosis and regulates cell division, and is highly expressed in various cancer types. Mucins are high-molecular-weight, heavily glycosylated proteins. In the present study, the association between survivin, mucin 2 (MUC2) and MUC5 expression, and the clinicopathological features of colorectal cancer (CRC) were investigated. The immunohistochemistry and western blotting results demonstrated that survivin was highly expressed in CRC tissues and rarely expressed in normal colon tissues. Moreover, the overexpression of survivin and MUC5 was strongly associated with lymph node metastasis, poor cellular differentiation, advanced tumor stage and a poor prognosis in CRC. By contrast, low expression of MUC2 was significantly associated with lymph node metastasis, poor cellular differentiation and an advanced tumor stage in CRC. The results of the present study suggest that survivin, MUC2 and MUC5 levels may be associated with tumor progression and could be used to aid the early diagnosis and clinical characterization of CRC.

Introduction

Despite recent advances in detection and treatment, colorectal cancer (CRC) remains the third most common type of cancer and a major cause of cancer-related mortality worldwide (1,2). There have been a number of recent advances in CRC screening. Probing a combination of sensitive and specific molecular markers could be particularly promising for early diagnosis, prediction of drug response and other clinical applications (3). Survivin is a member of the inhibitor of apoptosis protein family. High survivin expression levels are associated with poor outcomes in the majority of cancer types (4-9). There is also evidence of survivin expression in specific adult tissues, including healthy oral epithelium, colonic epithelium, placenta and healthy endometrium (4,10,11). A recent study reported that survivin can also act as a subunit of the chromosomal passenger complex (CPC), and direct the other subunits of CPC such as Aurora-B, Borealin and the inner centromere protein to regulate chromosome separation and cell division (4,12).

Mucins are high-molecular-weight, heavily glycosylated proteins (13). At present, >20 mucin types have been identified and classified into two separate classes according to their structure and function (14). The two structurally and functionally distinct classes are: i) Secreted gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) and ii) transmembrane mucins (MUC1, MUC3A, MUC3B, MUC4, MUC12 and MUC17). The MUC2 glycoprotein is a secreted mucin that consists of two distinct regions with a high degree of internal homology (15). MUC2 is commonly expressed in the healthy colonic epithelium and expression is decreased in non-mucinous colon adenocarcinomas (16-18). MUC2 and MUC5AC are clustered at the same chromosomal locus (11p15.5), and their expression and function may be regulated by a common mechanism (19). The MUC5AC gene is primarily expressed in the gastric and tracheobronchial mucosa; however, MUC5AC is not expressed in the healthy colonic epithelium (20). Although the expression of MUC5AC increases in differentiated CRC, the absence of MUC5AC expression in tumors can be a prognostic factor for more aggressive colon adenocarcinomas (21). Moreover, the expression of MUC2 and MUC5 is regulated by an extracellular signal-regulated kinase pathway in epithelial growth factor (EGF)/RAS proto-oncogene, GTPase (Ras)/Raf proto-oncogene, serine/threonine kinase (Raf)-positive cells (22). A study have indicated that EGF-mutant cancer cell lines express high levels of survivin (23). At present, however, to the best of our knowledge, no studies have investigated the link between

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survivin expression and MUC2/MUC5 expression in CRC. In the present study, the expression of survivin and its association with MUC2, MUC5 and the clinicopathological features of CRC were examined.

Materials and methods

Patients and tissue samples. CRC and normal tissue samples were obtained from 6 patients who underwent surgery at the Affiliated Hospital of Guilin Medical University (Guilin, China). A total of 20 normal colon mucosa samples and 139 advanced carcinomas (76 men and 63 women) were obtained from the Affiliated Hospital of Guilin Medical University and the archive of Hiroshima University Hospital (Hiroshima, Japan). All samples were obtained following approval by the Ethics Committees of Guilin Medical University and Hiroshima University. All patient records were complete, and each diagnosis was obtained by attending clinicians. Histologically, 117 carcinoma cases were classified as well/moderately differentiated and 22 as poorly differentiated according to the criteria of the Japanese Society for colorectal cancer (10,11). Tissues from each patient were fixed in formalin, cut into parallel 4-5-mm sections and embedded in paraffin. Tissue sections 4-μm thick were stained with hematoxylin and eosin for immunohistochemical examination. Informed consent was obtained from all subjects.

Immunohistochemistry. For immunohistochemical examination, tissue sections (4 μm) were incubated with the following primary antibodies: MUC2 (catalog no. NCL-MUC2; mouse monoclonal antibody, dilution, 1:100; Novocastra; Leica Microsystems GmbH, Wetzlar, Germany), MUC5 (catalog no. NCL-MUC5; mouse monoclonal antibody, dilution 1:100; Novocastra; Leica Microsystems GmbH), survivin (cat no. NB500-201, dilution, 1:1,000; Novus Biologicals, LLC, Littleton, CO, USA) and Ki-67 (cat no. M7240, MIB-1, mouse monoclonal antibody, dilution, 1:100; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). All were incubated at 4˚C overnight following antigen retrieval by microwave treatment in citrate buffer (pH 6.0; ZSGB-Bio, Beijing, China) and detection by the avidin-biotin peroxidase complex system using a labeled streptavidin-biotin kit (Dako; Agilent Technologies, Inc.) according to manufacturers's protocol. For MUC2, MUC5, survivin and Ki-67, immunoreactivity was graded according to the percentage of positive tumor cells as follows: Strong, >60% of tumor cells intensely stained; moderate, >20% intensely stained; mild, 5-20% intensely stained; or negative, <5% intensely stained. The expression levels of Survivin MUC2, MUC5 and Ki-67 were also graded as high (>20% of positive cells) or low (<20% of positive cells).

Western blot analysis. Colorectal tissues were lysed in radioimmunoprecipitation lysis buffer (cat no. R0020; Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) according to manufacturer's protocol. Protein concentrations were detected by the Bradford method, using bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) as the standard. Equal amounts of tissue extract (40 μg) underwent 10% SDS-PAGE separation and were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for antibody blotting. The membrane was then blocked with 5% nonfat dried milk (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 1 h, and incubated with primary antibodies at 4˚C overnight and secondary antibodies (cat no. sc-2004, sc-2005, dilution, 1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature and then an enhanced chemiluminescence kit (cat no. 170-5061; Clarity™ Western ECL Substrate; Bio-Rad Laboratories, Inc.) was used for visualization of specific protein antigens. Then, images of the membrane were captured in a darkroom, and the results were analyzed. The primary antibodies were anti-MUC2 (cat no. NCL-MUC2; mouse monoclonal antibody; dilution 1:1,000; Novocastra; Leica Microsystems GmbH), anti-MUC5 (cat no. NCL-MUC5; mouse monoclonal antibody, dilution, 1:1,000; Novocastra; Leica Microsystems GmbH), anti-survivin (cat no. NB500-201, dilution, 1:2,000; Novus Biologicals, LLC) and anti-β-actin (cat no. TA-09, dilution, 1:5,000; ZSGB-Bio).

Statistical analysis. The SPSS software package v.17.0 (SPSS, Inc., Chicago, IL, USA) was used for analysis. A χ^2 test was used for comparison of data between groups. Survival analyses were conducted using the Kaplan-Meier method and survival characteristics were compared using log-rank tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of survivin, MUC2 and MUC5 in healthy and cancerous colon mucosa. Expression levels of survivin, MUC2 and MUC5 were measured by immunohistochemical methods in 20 normal colon mucosa samples and 139 carcinoma cases. High survivin expression rates (nucleus, cytoplasmic staining) in healthy colon mucosa and CRC cases were 0% (0/20) and 39.57% (55/139), respectively (Table I and Fig. 1A). Rates of high MUC2 expression (cytoplasmic staining) in the healthy colon mucosa and in CRC tissue were 100% (20/20) and 48.20% (67/139), respectively (Table I and Fig. 1A). Rates of high MUC5 expression (cytoplasmic staining) in the healthy colon mucosa and CRC tissue were 0% (0/20) and 28.06% (39/139), respectively (Table I and Fig. 1A). In conclusion, the expression and staining intensity of survivin and MUC5 were significantly increased in CRC tissues ($P<0.01$), whereas those of MUC2 were significantly decreased in CRC tissues ($P<0.01$).

Western blot analysis revealed that the expression levels of survivin and MUC5 in healthy colon mucosa tissues were lower than levels in CRC tissues (Fig. 1B). However, expression levels of MUC2 in the healthy colon mucosa were higher than levels in CRC tissues (Fig. 1B). These findings support the immunohistochemical data, as high expression of MUC2 was observed in healthy colon mucosa, whereas high expression of survivin and MUC5 was observed in CRC cells (Fig. 1B).

Survivin expression and correlation with MUC2, MUC5, Ki-67 and clinicopathological features in CRC. The present study examined the correlation between survivin, MUC2, MUC5 and Ki-67 in CRC cases. A total of 55/139 patients with CRC exhibited high survivin expression levels (Table II). Moreover, of the 55 patients with high survivin expression, 28 exhibited high expression of MUC5 and 41 exhibited high expression of Ki-67, whereas 40 patients exhibited low expression of MUC2.

Table I. Survivin, MUC2 and MUC5 expression in normal colon mucosa and cancer.

		Survivin expression, n			MUC2 expression, n			MUC5 expression, n		
Tissue type	Total	Low	High	P-value	Low	High	P-value	Low	High	P-value
Normal	20	20	0	<0.01[a]	0	20	<0.01[a]	20	0	<0.01[a]
Cancer	139	84	55		72	67		100	39	

[a]Normal tissue vs. cancer tissue. MUC, mucin.

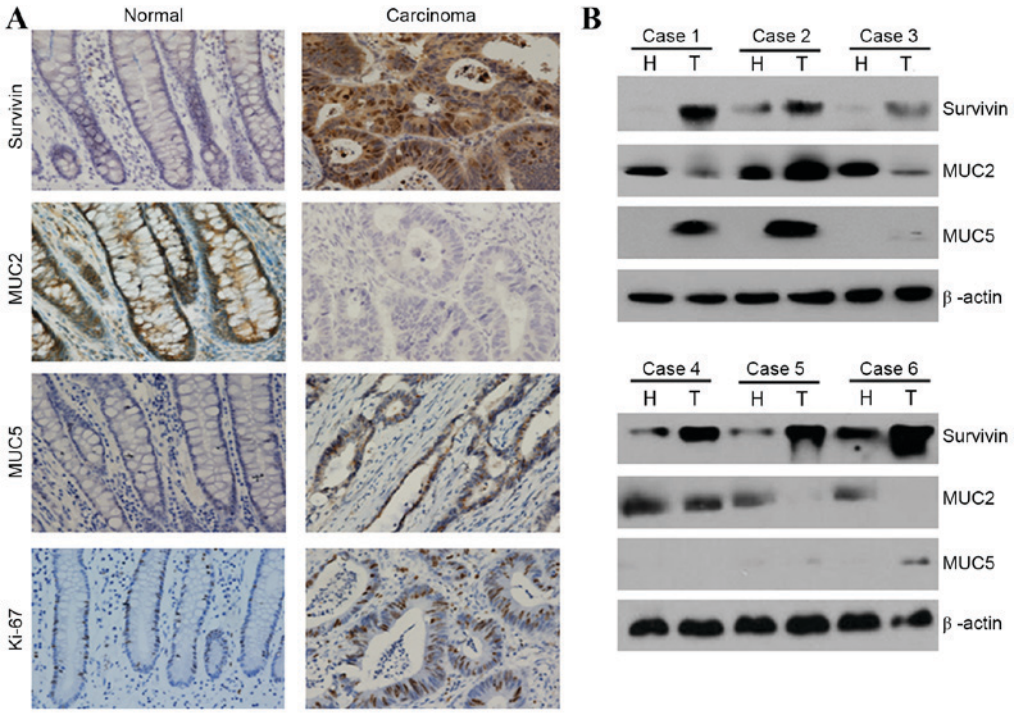

Figure 1. Examples and quantification of survivin and mucin expression in healthy and CRC cells. (A) Immunohistochemical staining for MUC2, MUC5 and survivin in healthy colorectal tissues and adjacent CRC tissues. Representative case of MUC2, MUC5 and survivin expression in normal adjacent tissues and CRC tissues are shown (magnification, x200). (B) Expression of MUC2, MUC5 and survivin in healthy and cancerous colorectal tissues was examined by western blot analysis. β-actin expression was used as a control. MUC, mucin; CRC, colorectal cancer; H, healthy tissue; T, tumor tissue.

This demonstrated that survivin expression was directly associated with MUC5 and Ki-67 expression, and inversely correlated with MUC2 expression in CRC (P<0.01).

Patients with high survivin expression levels demonstrated significantly increased incidences of lymph node metastasis (P<0.01) and stage-D tumors (P<0.01) (8 Edition, Japanese Classification of Colorectal Carcinoma) (10,11) compared with cases exhibiting low survivin expression levels (Table III). However, no evidence of association between survivin expression level and tumor size, gender or histological differentiation was found.

Patients with low MUC2 expression levels demonstrated significantly lower cell differentiation (P<0.01) and higher incidences of lymph node metastasis (P<0.05) and stage-D tumors (P<0.01) when compared with cases displaying high expression of MUC2 (Table IV). The association between clinicopathological factors and MUC5 expression in CRC was also examined. In comparison to MUC2, patients expressing high levels of MUC5 tended to exhibit poor CRC cell differentiation (P<0.05), higher rates of lymph node metastasis (P<0.01) and higher tumor stage (P<0.01) (Table IV).

Survival analysis. The Kaplan-Meier method was used to assess the survival rate of 139 patients who expressed survivin, MUC2 and MUC5 (Fig. 2). The 5-year survival rate of those whose tumors expressed low levels of survivin and MUC5 was higher than that of patients with high expression levels of survivin and MUC5. By comparison, the 5-year survival rate of those with tumors expressing high levels of MUC2 was higher than that of patients whose tumors expressed low levels of MUC2.

Discussion

Survivin is a bifunctional protein that suppresses apoptosis and regulates cell division, which is highly expressed in various cancer types (12). Additionally, >20 mucins are classified as

Table II. Survivin expression and its correlation with MUC2 and MUC5 expression in colorectal cancer.

	Survivin expression		
Clinicopathological factor	Low (n=84)	High (n=55)	P-value
MUC2 expression, n			<0.01
Low	32	40	
High	52	15	
MUC5 expression, n			<0.01
Low	73	27	
High	11	28	
Ki-67 expression, n			<0.01
Low	57	14	
High	27	41	

MUC, mucin.

Table III. Survivin expression and its correlation with clinicopathological findings in colorectal cancer.

	Survivin expression		
Clinicopathological factor	Low (n=84)	High (n=55)	P-value
Tumor size (mm), n			0.56
≥50	37	27	
<50	47	28	
Histological differentiation, n			<0.01
Poor	10	12	
Well/moderate	74	43	
Lymph node metastasis, n			<0.01
Negative	60	16	
Positive	24	39	
Sex, n			0.31
Male	43	33	
Female	41	22	
Tumor stage, n			<0.01
B/C	76	39	
D	8	16	

either secreted mucins or transmembrane mucins according to their structure and function (13). MUC2 and MUC5 are secreted mucins. However, to the best of our knowledge, there have been no studies to date investigating the correlation of survivin expression with MUC2 and MUC5 expression in CRC. The present study focused on the expression levels of survivin, MUC2 and MUC5 in healthy and CRC tissues using immunohistochemical analysis. Additionally, the present study aimed to investigate the potential of these biomarkers to aid in the early diagnosis of CRCs, as well as other clinical applications.

In this study, survivin was revealed to be expressed at high levels in CRC tissues, but at low levels in healthy colon tissues (Fig. 1 and Table I). Moreover, patients with high expression of survivin demonstrated significantly poorer cellular differentiation, higher rates of lymph node metastasis and a higher incidence of stage-D tumors than did cases with low expression of survivin. Furthermore, overexpression of survivin has previously been found to be associated with poor prognosis in CRC, HCC, and head and neck cancer (4,10-12).

The present study also examined the expression levels of MUC2 and MUC5 in healthy colon mucosa and in CRC patients. MUC2 was found to be expressed at high levels in normal colon tissue and at lower levels in CRC tissue (Fig. 1 and Table I). Moreover, cases with low expression of MUC2 demonstrated significantly poorer cell differentiation, higher rates of lymph node metastasis and a higher incidence of stage-D tumors compared with cases with high levels of expressed MUC2. Similarly, several studies have shown that loss of MUC2 expression is correlated with poor prognosis in CRC (24-26). Notably, however, another study found that overexpression of MUC2 is associated with poorer overall survival (27). In a further previous study, decreased expression of MUC2 was found to be associated with colon carcinogenesis, decreased apoptosis and increased migration of intestinal epithelial cells (20).

A number of studies have shown that low expression of MUC2 is associated with poorly differentiated adenocarcinoma of the colon and rectum (28,29). By contrast, in the present study, MUC5 was expressed at high levels in CRC tissues but at low levels in normal colon tissues (Fig. 1 and Table I). Similarly, other studies have shown that MUC5AC is not detected in the normal colon, but is frequently found in adenomas and carcinomas (18,30-32). Other prior studies reported that an increase in expression of MUC5AC was observed in sporadic cancer with high microsatellite instability (33) and that MUC5AC expression in intrahepatic cholangiocarcinoma was found to be an independent prognostic factor by multivariate survival analysis (34). The presence of MUC2 and/or MUC5AC in colorectal mucinous adenocarcinoma has been shown to be associated with proximal (right-sided) CRC location (32,33). There was no statistically significant association between gender and expression of MUC2 and/or MUC5 in the present study. Expression of survivin was also compared with the expression of MUC2, MUC5 and Ki-67 (Fig. 1; Table II). Survivin expression was found to be directly correlated with MUC5 and Ki-67 expression, and inversely correlated with MUC2 expression (Fig. 1 and Table II). These findings led to the hypothesis that survivin and MUC5 are expressed at high levels in CRC, whereas low MUC2 expression levels confer a poor prognosis in CRC.

In conclusion, the present study revealed that the normal-to-carcinoma sequence was significantly associated with the high expression and staining intensity of survivin and MUC5 (P<0.01), and the low expression of MUC2. Additionally, cases with high expression of survivin and MUC5 and/or low expression of MUC2 demonstrated significantly increased rates of lymph node metastasis and incidences of advanced tumor stage. Therefore, increased survivin and MUC5 or decreased MUC2 expression levels are associated with the malignant potential of colon carcinoma. Further investigations

Table IV. MUC2 and MUC5 expression and its correlation with clinicopathological findings in colorectal cancer.

	MUC2 expression			MUC5 expression		
Clinicopathological factor	Low (n=72)	High (n=67)	P-value	Low (n=100)	High (n=39)	P-value
Tumor size (mm), n			0.099			0.99
≥50	38	26		46	18	
<50	34	41		54	21	
Histological differentiation, n			<0.01			<0.05
Poor	17	5		12	10	
Well/moderate	55	62		88	29	
Lymph node metastasis, n			<0.05			<0.01
Negative	34	43		66	11	
Positive	38	24		34	28	
Sex, n						0.21
Male	38	38		58	18	
Female	34	29		42	21	
Tumor stage, n			<0.01			<0.01
B/C	54	61		90	25	
D	18	6		10	14	

MUC, mucin.

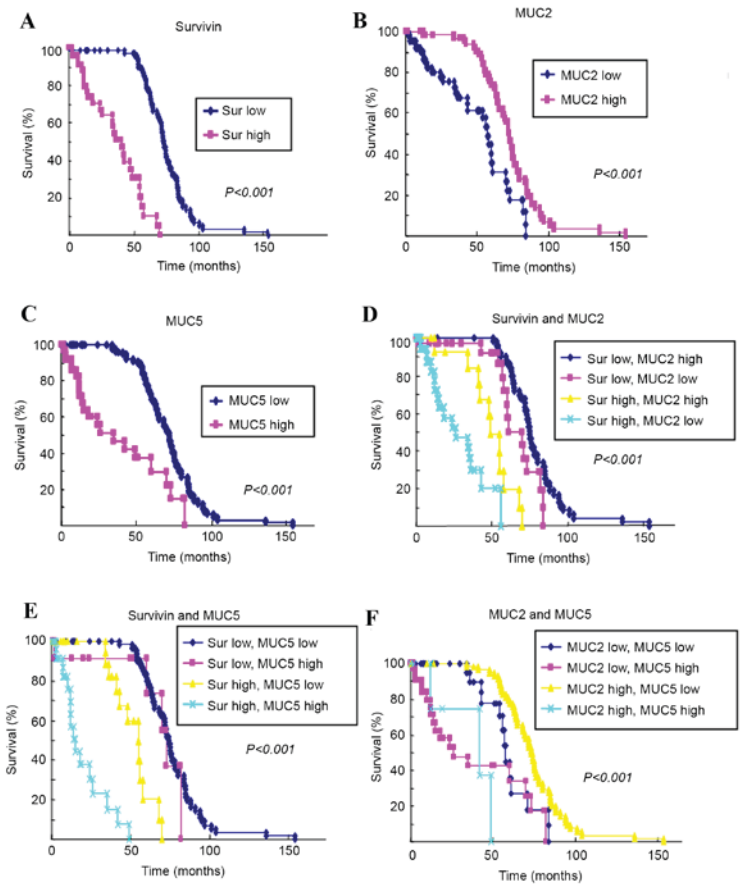

Figure 2. Kaplan-Meier survival curves of patients with colorectal cancer according to MUC2, MUC5 and survivin expression levels. (A) The 5-year survival rate of patients expressing high survivin levels was 10.3 vs. 80.7% for those expressing low survivin levels. (B) The 5-year survival of patients with high MUC2 expression was 40.2 vs. 73.9% for those expressing low MUC2 levels. (C) The 5-year survival rate of patients expressing high MUC5 levels was 29.8 vs. 69.9% for those expressing low survivin levels. (D) Comparison of the 5-year survival rate of patients with the indicated expression levels of (D) survivin and MUC2, (E) 5 survivin and MUC5, and (F) MUC2 and MUC5. MUC, mucin.

using appropriate techniques based on clinical data are required to confirm the present findings.

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