

# Chemoradiotherapy-induced Changes in Mucinous Components in Rectal Cancer Tissue: Evaluation on High Iron Diamine-alcian Blue and Mucin 1 Staining

HIROSHI MIYAKITA<sup>1</sup>, SOTARO SADAHIRO<sup>1</sup>, YUTARO KAMEI<sup>1</sup>, TAKASHI OGIMI<sup>1</sup>, GOTA SAITO<sup>1</sup>, KAZUTAKE OKADA<sup>1</sup>, AKIRA TANAKA<sup>1</sup>, TOSHIYUKI SUZUKI<sup>1</sup>, HIROSHI KAJIWARA<sup>2</sup> and TAKESHI AKIBA<sup>3</sup>

<sup>1</sup>Department of Surgery, Tokai University, Kanagawa, Japan;

<sup>2</sup>Department of Pathology, Tokai University, Kanagawa, Japan;

<sup>3</sup>Department of Radiology, Tokai University, Kanagawa, Japan

**Abstract.** *Background/Aim:* The standard treatment for rectal cancer is neoadjuvant chemoradiotherapy (nCRT) followed by surgery. Mucinous carcinoma responds poorly to nCRT. In some patients, mucin lakes (MLs) are induced by nCRT. Identifying whether MLs are induced or originally present would be of great importance. *Patients and Methods:* We studied 20 patients with MLs (CRT-MC group) among 205 patients who received nCRT. Among 88 patients who did not receive nCRT, we studied 9 patients with mucinous carcinoma (non-CRT-MC group) and 18 patients with MLs in differentiated adenocarcinoma (non-CRT-AC group). Tumors were stained with high iron diamine-Alcian blue (HID-AB) and MUC1 staining. *Results:* Rate of AB>HID staining of cancer cells was significantly higher in the CRT-MC group than in non-CRT-MC group ( $p=0.0004$ ). Rate of MUC1 staining in MLs was significantly higher in the CRT-MC group ( $p=0.0254$ ). *Conclusion:* nCRT can induce qualitative changes in mucinous components, however, other methods are required to distinguish induced components from originally existing components.

Total mesorectal excision (TME) after neoadjuvant chemoradiotherapy (nCRT) is the standard treatment for rectal cancer (1-4). Patients with mucinous carcinoma have been reported to have a poor response to nCRT, as well as poor outcomes. In some patients, however, nCRT may induce the formation of mucin lakes (MLs) in rectal cancer

tissue (5-12). The ability to determine whether MLs are induced or originally exist in resected specimens would be of great clinical significance.

Sialomucin and sulphomucin are included in intracytoplasmic mucin of cancer cells and MLs in patients with colorectal cancer and can be identified by high iron diamine-Alcian blue (HID-AB) staining (13). Mucin 1 (MUC1), a transmembrane mucin and a membrane-bound glycoprotein, exists in cancer cells and in MLs in colorectal cancer tissue and has been reported to be related to outcomes (14-17).

HID-AB staining and MUC1 immunohistochemical staining was, therefore, performed in resected specimens of rectal cancer obtained from patients who received nCRT and those who did not, to evaluate mucinous components in cancer cells and MLs in cancer tissue and examine whether nCRT induces changes in mucinous components.

## Patients and Methods

*Subjects.* From 2005 through 2015, a total of 293 patients with rectal cancer underwent curative surgery. Among the 205 patients who received nCRT, the ML formation which occupied at least 20% of the area of the maximum cut surface of the resected specimens was found in 20 patients. These patients were designated as the CRT-MC group. Among the 88 patients who did not receive CRT, 9 patients were histologically diagnosed with mucinous carcinoma on the resected specimens in which ML formation was remarkable (non-CRT-MC group), and 18 patients were diagnosed with well- or moderately differentiated adenocarcinoma but had apparent ML formation in less than 50% of the area ratios of the tumors. These patients were designated as the non-CRT-AC group. A total of 47 patients were studied. This study was approved by the institutional review board of our University (17R-238), and all patients gave their written informed consent.

*HID-AB staining, MUC1 staining, and evaluations.* To perform HID-AB staining, the sections were deparaffinized and then stained with HID solution, prepared using N, N-dimethyl-m-phenylenediamine dihydrochloride (120 mg) and N,N-dimethyl-p-phenylenediamine

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*Correspondence to:* Sotaro Sadahiro, MD, Ph.D., Department of Surgery, Tokai University, 143 Shimokasuya Isehara, Kanagawa, 259-1193, Japan. Tel: +81 463931121, Fax: +81 463965577, e-mail: Sadahiro@is.icc.u-tokai.ac.jp

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monohydrochloride (20 mg) (Sigma-Aldrich Japan Inc., Osaka, Japan), and pH 2.5 Alcian blue solution (Muto Pure Chemicals, Tokyo, Japan). For MUC1 staining, the sections were deparaffinized in xylene, and antigen activation was performed at 95°C for 76 min, using pH 6.0 ULTRA CC1 buffer (Ventana Medical Systems Inc., Tucson, AZ, USA). After antigen activation, the sections were allowed to react with anti-MUC1 H23 (Ventana Medical Systems Inc.) for 40 min. After HID-AB staining, the tumor cells and MLs were separately evaluated. Tumor cells or MLs predominantly stained by AB were classified as AB>HID, and those predominantly stained by HID were classified as HID>AB. The results of MUC1 staining were classified as positive or negative. The results of staining were evaluated separately by 2 physicians (HM and HK). If different evaluations were obtained, microscopic examinations were repeated by the 2 physicians (HM, HK) to determine the results.

*Statistical analysis.* Chi-square tests were used to evaluate differences between the 2 groups. *p*-Values of less than 0.05 were considered to indicate statistical significance. Statistical analysis was performed with software package JMP10 (SAS institute Inc., Cary, NC, USA).

**Results**

*Patient characteristics.* Table I shows the patient characteristics. The proportion of women was higher in the subgroup of patients with MLs in differentiated adenocarcinoma (non-CRT-AC group) than in the other 2 groups. In the CRT-MC group, the proportion of patients with lower rectal cancer was significantly higher than that in the 2 non-CRT groups.

*HID-AB staining (Table II, Figure 1).* As for the results of staining for tumor cells, the percentage of tumor cells predominantly stained with AB was significantly higher in the CRT-MC group than in the non-CRT group (*p*=0.0004). The results of staining for MLs did not differ between the CRT-MC group and the non-CRT group (*p*=0.6889). The results of HID-AB staining for tumor cells and MLs did not differ between the CRT-MC group and the non-CRT group (*p*=0.1939 and *p*=0.1661).

*MUC1 staining (Table III, Figure 2).* The rate of positive staining for tumor cells did not differ between the CRT-MC group and the non-CRT group (*p*=0.4039). On comparing the non-CRT-AC group with the non-CRT-MC group, the rate of positive staining for tumor cells was significantly higher in the non-CRT-AC group (*p*=0.0377). The rate of positive staining for MLs was significantly higher in the CRT-MC group (70%) than in the non-CRT-MC group (37%, *p*=0.0254).

**Discussion**

Patients with mucinous carcinoma have been reported to poorly respond to nCRT, resulting in poor outcomes. In some of the patients, however, MLs are induced by nCRT in rectal

Table I. Patient characteristics.

Variable	CRT-MC n (%)	MC n (%)	AC n (%)	<i>p</i> -Value
Gender				
Male	17 (85.0)	7 (77.8)	8 (44.4)	0.0218
Female	3 (15.0)	2 (22.2)	10 (55.6)	
Age (years)				
Range	35-82	36-81	40-86	0.6663
Median	61.9	63.4	65.8	
Location of tumor				
Upper rectum	7 (35.0)	6 (66.7)	11 (61.1)	0.0069
Lower rectum	13 (65.0)	3 (33.3)	7 (38.9)	
Surgical Procedure				
LAR	13 (65.0)	6 (66.7)	16 (88.9)	0.1082
Hartmann	1 (5.0)	0	2 (11.1)	
APR	5 (25.0)	2 (22.2)	0	
Total colectomy	0	1 (11.1)	0	
Local excision	1 (5.0)	0	0	
T classification				
0	0	0	0	0.2106
1	2 (10.0)	0	1 (5.6)	
2	3 (15.0)	0	0	
3	12 (60.0)	7 (77.8)	17 (94.4)	
4	3 (15.0)	2 (22.2)	0	
N classification				
+	7 (35.0)	3 (33.3)	10 (55.6)	0.3634
-	13 (65.0)	6 (66.7)	8 (44.4)	
ly				
+	10 (50.0)	6 (66.7)	12 (66.7)	0.5155
-	10 (50.0)	3 (33.3)	6 (33.3)	
v				
+	11 (55.0)	5 (55.6)	13 (72.2)	0.5048
-	9 (45.0)	4 (44.4)	5 (27.8)	
(yp) Stage				
1	2 (10.0)	0	0	0.5271
2	12 (60.0)	6 (66.7)	8 (44.4)	
3	7 (35.0)	3 (33.3)	10 (55.6)	

LAR: Low anterior resection; APR: abdominoperineal resection; Ly: lymphatic invasion; v: venous invasion.

cancer tissue (5-12). In patients who receive nCRT, the incidence of mucinous carcinoma is relatively high, ranging from 13% to 24% (6, 8, 11). Patients in whom MLs are induced have been reported to have better outcomes than patients who originally have MLs (12). However, the nCRT-induced qualitative changes in the components of MLs remain to be confirmed. We therefore examined whether nCRT induces qualitative changes in the mucinous components of cancer cells and cancer stromal tissue.

HID-AB staining is a dual staining technique for identifying intracytoplasmic mucin of cancer cells. Sialomucin is stained blue, and sulphomucin is stained black. A mixture of neutral and acidic mucins is present in most intracytoplasmic mucin of cancer cells. Acidic mucin is a mixture of sialomucin and sulphomucin. MLs are usually

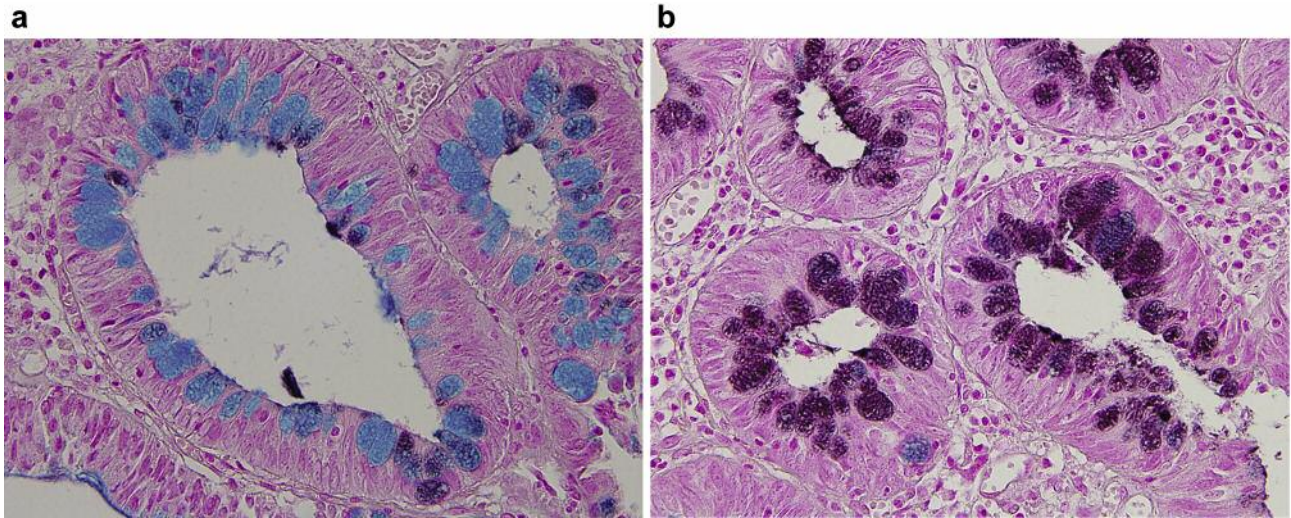


Figure 1. High iron diamine-alcian blue staining for tumor cells. (a) Example of AB>HID staining of intracytoplasmic mucin of cancer cells. Sialomucin was stained as blue. (b) Example of HID>AB staining of intracytoplasmic mucin of cancer cells. Sulphomucin was stained as black.

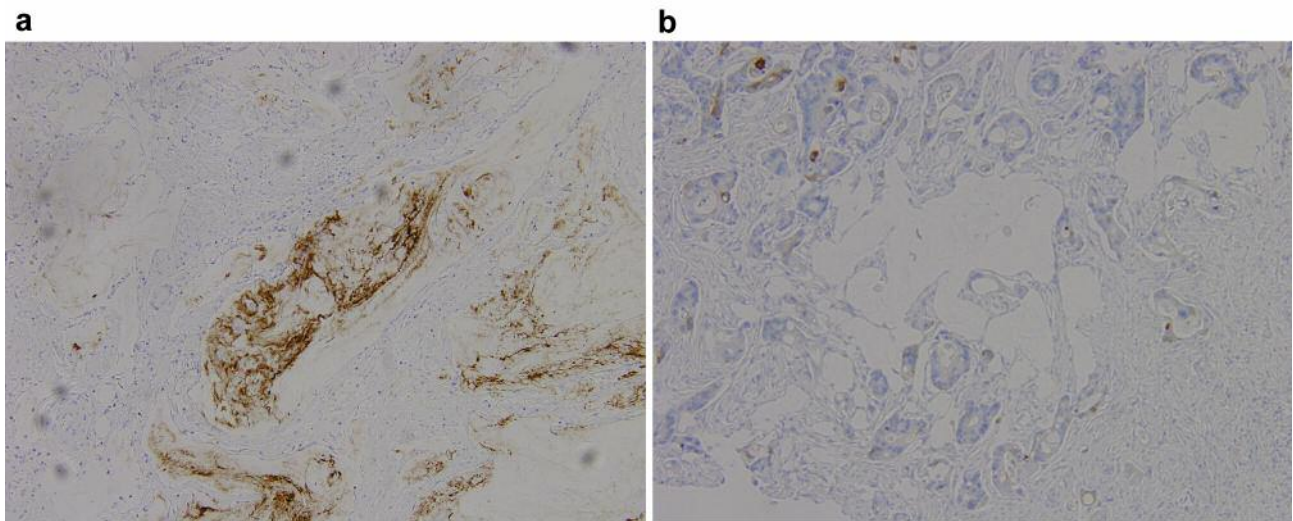


Figure 2. MUC1 Staining for Mucin Lakes. (a) Example of positive MUC1 staining. (b) Example of negative MUC1 staining.

positive for acidic mucin and are characterized by a mixture of sialomucin and sulphomucin. However, sialomucin is more abundant in MLs (18).

Ionila *et al.* reported that the presence of poorly differentiated cancer in mucinous carcinoma is associated with a decrease in sulphomucin and an increase in a mixture of sialomucin and sulphomucin (13). In our study, mucinous components in tumor cells were predominantly stained with AB in patients who received nCRT ( $p=0.007$ ). However, HID-AB staining of MLs did not differ between the groups.

Bergonie *et al.* reported that the presence of poorly differentiated cancer is associated with a high nuclear/cytoplasmic ratio, high proliferative activity, and high radiosensitivity (19). In our study, mucinous components in tumor cells were predominantly stained by AB rather than HID in patients who received nCRT. This is attributed to the fact that nCRT decreased the number of poorly differentiated cancer cells, thereby decreasing sulphomucin and increasing mixtures of sialomucin and sulphomucin.

Table II. *HID-AB staining and chemoradiotherapy.*

Group	Tumor cells			MLs		
	AB>HID	HID>AB	<i>p</i> -Value	AB>HID	HID>AB	<i>p</i> -Value
CRT-MC	12 (60)	8 (40)	0.0004*	13 (65)	7 (35)	0.6889*
Non-CRT	3 (11.1)	24 (88.8)		16 (59.3)	11 (40.7)	
Non-CRT-AC	1 (5.6)	17 (94.4)	0.1939†	9 (50.0)	9 (50.0)	0.1661†
Non-CRT-MC	2 (22.2)	7 (77.8)		7 (77.8)	2 (22.2)	

MLs: Mucin lakes; CRT: chemoradiotherapy; \*CRT vs. non-CRT; †non-CRT-AC vs. non-CRT-MC.

Table III. *MUC1 staining and chemoradiotherapy.*

Group	Tumor cells			MLs		
	MUC1-	MUC1+	<i>p</i> -Value	MUC1-	MUC1+	<i>p</i> -Value
CRT-MC	3 (15)	17 (85)	0.4039*	6 (30)	14 (70)	0.0254*
Non-CRT	2 (7.4)	25 (92.6)		17 (63)	10 (37.0)	
Non-CRT-AC	0 (0)	18 (100)	0.0377†	12 (66.7)	6 (33.3)	0.5730†
Non-CRT-MC	2 (22.2)	7 (77.8)		5 (55.6)	4 (44.4)	

MLs: Mucin lakes; CRT: chemoradiotherapy; \*CRT vs. non-CRT; †non-CRT-AC vs. non-CRT-MC.

Transmembrane mucins are stained by MUC1. MUC1 is expressed in colorectal cancer, and patients with MUC1 expression have been reported to have poor outcomes (14-16). MUC1 is a molecule that is induced by irradiation. Irradiation of the human colorectal cancer cell line HT29 has been reported to increase the rate of positive staining for MUC1 in cancer cells in a radiation-dose-related manner (20).

In our study, the rate of positive staining for MUC1 in MLs was 70% in patients who received CRT and 37% in those who did not, suggesting that nCRT induced MUC1 expression in MLs. The rate of positive staining for MUC1 in cancer cells was higher in the non-CRT-AC group than in the non-CRT-MC group. The difference between the groups was the quantitative difference in mucinous nodules in the maximum cut surface of tumor sections. Therefore, the reasons for the difference in the rate of positive staining for MUC1 in cancer cells remain unclear.

In our previous study, we measured the area ratio of MLs in rectal cancer tissue by magnetic resonance imaging and reported that the area ratio of MLs did not largely differ before and after nCRT and that the amount of MLs was largely unchanged after nCRT, compared to that before nCRT (21).

In the present study, we examined whether nCRT can induce qualitative changes in mucinous components in cancer tissue. However, because our study had a small sample size, it was difficult to evaluate whether nCRT induced mucinous components in cancer cells and MLs in cancer stromal tissue based only on the results of staining by

HID-AB and MUC1. Further studies are needed to confirm our results by using other diagnostic methods in larger numbers of patients.

### Conclusion

In patients with rectal cancer who received nCRT, mucinous components in cancer cells were predominantly stained with AB, and the rate of positive MUC1 staining for MLs was higher than that in patients with rectal cancer who did not receive nCRT. These findings suggested that nCRT induced quantitative changes in mucinous components. However, based only on the results of two staining methods, it is difficult to distinguish mucinous components induced by nCRT from mucinous components that were originally present.

### Conflicts of Interest

H. Miyakita, S. Sadahiro, Y. Kamei, T. Ogimi, G. Saito, K. Okada, A. Tanaka, T. Suzuki, H. Kajihara and T. Akiba have no potential conflicts of interest to report. None of the Authors received any funding support for this work.

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