Table 3 Rates of complication ≥ Gr IIIa and anastomotic leakage according to the site of primary colorectal resection and extent of hepatectomy

Primary colorectal resection	Hepatectomy	Complication ≥ Gr IIIa	Anastomotic leakage
Colectomy	<Lobectomy	4/40 (10%)	5/39[a] (13%)
	≥Lobectomy	0/7 (0%)	1/7 (14%)
Rectal resection	<Lobectomy	11/32 (34%)	11/28[b] (39%)
	≥Lobectomy	2/7 (29%)	1/7 (14%)

[a] One patient who underwent Hartmann's operation was excluded from the analysis

[b] Four patients who underwent abdomino-perineal resection were excluded from the analysis

This study evaluated morbidity, especially anastomotic leakage, after simultaneous resection for SCLM in order to assess the safety of simultaneous resection. Anastomotic leakage is sometimes fatal and can cause a difficult situation with physical and mental discomfort or pain. The morbidity rate of patients who underwent simultaneous resection for SCLM seemed to be higher than that of patients with resected metachronous colorectal hepatic metastasis or that of patients who underwent only resection for colorectal primary cancer. Predictive factors for postoperative morbidity and for anastomotic leakage were intraoperative blood loss and operation time greater than 8 h, respectively. The overall morbidity rate and the rate of anastomotic leakage were 91% and 50%, respectively, in patients with operation time greater than 8 h, and 54% and 13%, respectively, in patients with operation time less than or equal to 8 h. Blood loss and operation time usually represent the amount of surgical stress. Excessive surgical stress was possibly correlated with postoperative morbidity. Hospitalization of patients with complications was significantly longer than that of patients without complications. In particular, the average hospitalization of the 18 patients with anastomotic leakage was more than 43 days. Retrospective studies have also indicated that the occurrence of anastomotic leakage is associated with increased morbidity, mortality, and prolonged hospital stay. Additionally, anastomotic leakage may be associated with an increased risk of local recurrence.[19]

Various risk factors for anastomotic leakage have been analyzed by several investigators. Age, sex, obesity, level of anastomosis, smoking, blood transfusion, tumor diameter, preoperative (chemo) radiotherapy, physical status, obstruction, and coronary heart disease have been shown to be significant risk factors for leakage.[20–24] In simultaneous resection for SCLM, not only the factors related to the tumor, the patient, or the colorectal operation, but factors related to the hepatectomy could affect the occurrence of anastomotic leakage. However, the extent of hepatic resection, sequence of colectomy, hepatectomy, anastomosis, use of the Pringle maneuver, and total time of the Pringle maneuver were not predictive factors for anastomotic leakage or postoperative complications in patients with resected SCLM.

Recently, a diverting stoma has been often used to prevent anastomotic leakage in patients who undergo low anterior resection by diverting the fecal stream and keeping the anastomosis free of material.[19,25,26] In this study, the presence of a diverting stoma was not a predictive factor for absence of postoperative anastomotic leakage. However, the analysis estimating efficacy of a diverting stoma in this study was not accurate, because a diverting stoma was basically used in patients whose risk for anastomotic leakage was considered to be high by the surgeons. The site of primary tumor that has been reported as a strong predictive factor in previous studies was not a predictive factor for anastomotic leakage in this series. Use of diverting stoma might affect the result of analyses of predictive factors for anastomotic leakage. A randomized, controlled trial is needed to elucidate the efficacy of a temporary diverting stoma.

Although several rationales for the simultaneous resection for SCLM are clear, staged resections should be selected to prevent anastomotic leakage or serious complications when the scheduled operation would result in considerable surgical stress, i.e., predicted operation time greater than 8 h according to the results of the present study. Predicted operation time should be calculated by considering various factors, such as characteristics of the patient, primary and metastatic tumor, extent of operation, difficulty of the procedure, and so on. Based on the results of this study, we now select staged resections when operation time is expected to be greater than 8 h; otherwise, we select simultaneous resection. A prospective study of SCLM to evaluate the efficacy and safety of the operation time-based decision model is in progress.

Currently, adjuvant chemotherapy is one of the key factors which could affect prognosis. Then, comparison of ratio of patients who could receive adjuvant chemotherapy will be essential when comparing the efficacy of simultaneous resection and that of staged resections in a future study of SCLM. Furthermore, in staged resections, there is a risk that some patients could not undergo a second resection after the first resection due to tumor progression or complication of first surgery. Resection rate of patients who could undergo both primary and hepatic resections

should be assessed when comparing simultaneous resection and staged resections in SCLM.

The limitations of our study are its retrospective design and the relatively small number of patients studied.

Conclusion

The morbidity rate and the frequency of anastomotic leakage were high with simultaneous resection for SCLM, especially in patients with greater intraoperative blood loss or operation time greater than 8 h. For patients with SCLM, staged resections should be considered when simultaneous resection would involve excessive surgical stress.

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Gastrointestinal Cancer: Targets and Therapy Dovepress

ORIGINAL RESEARCH

Application of miRNA expression analysis on exfoliated colonocytes for diagnosis of colorectal cancer

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Background: Several methods for the early detection of colorectal cancer to reduce its mortality rate have been reported. Here, we investigated the potential of a fecal micro RNA test for the early detection of colorectal cancer.

Methods: Patients with colorectal cancer (n = 299) and healthy volunteers (n = 116) with no abnormalities detected by screening colonoscopy were enrolled in this case-control study. Micro RNA expression in the colonocytes of patients with colorectal cancer (n = 47) and in healthy volunteers (n = 35) were analyzed in the training set, and the micro RNA expression in the colonocytes of patients with colorectal cancer (n = 252) and healthy volunteers (n = 81) was validated in the validation set.

Results: In the training study, significant differences in the relative expression level of miR-17-92 cluster, -106a, -135, and -146a were observed between patients with colorectal cancer and healthy volunteers ($P < 0.01$). The area under the receiver operating characteristic curve using miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a was more than 0.7. The overall sensitivity and specificity in the training study using these micro RNAs was 70.2% (33/47) and 74.3% (26/35), respectively. The overall sensitivity and specificity in the validation study was 67.5% (170/252) and 75.3% (61/81), respectively.

Conclusion: We have developed a fecal micro RNA test for exfoliated colonocytes for colorectal cancer screening. Further comparative study of this test for colorectal cancer screening is needed.

Keywords: colorectal cancer, fecal micro RNA, colonocytes, cancer screening, fecal RNA test

Introduction

The early stage of colorectal cancer is curable by surgical resection, thus a suitable colorectal cancer screening test is necessary to reduce its mortality rate. The fecal occult blood test has been used widely as a screening test for colorectal cancer.[1–3] However, large-scale studies have shown that the sensitivity of the fecal occult blood test is not very high using total colonoscopy as a reference standard in all subjects.[4–7] Therefore, several attempts for the early detection of colorectal cancer have been reported. In fecal DNA-based analysis, the stool DNA test[6] was recommended as a colorectal cancer screening method.[8] Further, we have reported several DNA-based methods for the detection of early-stage colorectal cancer using direct sequence analysis[9] and single-strand conformation polymorphism analysis[10] in exfoliated colonocytes. However, the sensitivity and specificity of the stool DNA test were insufficient compared with that of the fecal occult blood test.[11] Another technical issue was that several mutation sites of adenomatous polyposis coli (*APC*), *Kras*, and *p53* genes in colorectal cancer

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tissue were not always identical in those genes.[12] In addition, the DNA mutation analysis was complicated and expensive. This may make the use of fecal DNA analysis for colorectal cancer screening unrealistic.

Gene expression analysis based on real-time reverse transcription polymerase chain reaction (RT-PCR) has been shown to be relatively simple and cost-effective. Several attempts to detect colorectal cancer by RT-PCR in fecal samples have been reported.[13–15] In those reports, the expression analyses of *COX2* and *MMP7* in fecal RNA, and *COX2*, *MMP7*, *MYBL2*, and *TP53* in colonocyte RNA were conducted.[13,15,16]

MicroRNAs (miRNAs) are small (18–25 nucleotide) noncoding RNA molecules that regulate the activity of specific mRNA targets and play a major role in development of cancer. miRNA downregulates multiple target gene expressions by degrading mRNA or blocking its translation into protein through RNA interference.[17,18] Several miRNAs, such as miRNA-21 (miR-21), the miR-17-92 cluster and miR-135, were found to be highly expressed in colorectal cancer tissue.[19–22] Several recent studies have clarified that the circulating miRNA in plasma is a potential marker for detection of colorectal cancer,[23,24] and is remarkably stable in plasma and protected from endogenous RNase activity.[25]

We have developed a fecal miRNA test using colonocyte RNA.[26] In the present study, we analyzed several miRNAs using an optimal internal control to improve the accuracy of the fecal miRNA test. Following selection of a suitable target and threshold in the training study, the fecal miRNA test was evaluated in a validation study to determine its potential for early detection of colorectal cancer.

Materials and methods

Fecal samples and isolation of exfoliated cells

Naturally evacuated fecal samples were obtained from patients with colorectal cancer before surgical resection. Fecal samples were also obtained from healthy volunteers a few weeks after screening colonoscopy. All patients with colorectal cancer and healthy volunteers were instructed to evacuate at home into a disposable 5 × 10 cm polystyrene tray (AsOne, Osaka, Japan) and then bring the sample to the reception counter at the outpatient clinic or the Cancer Prevention and Screening Center of the National Cancer Center. The fecal samples were processed immediately after they were brought to our laboratory.

For the isolation of colonocytes from naturally evacuated feces, we used two kinds of immunomagnetic beads tagged with antihuman EpCAM monoclonal antibodies, ie, Dynabeads Epithelial Enrich (Dynal, Oslo, Norway) and JSR beads (JSR, Tsukuba, Japan).[27] The ability to isolate cells from feces using Dynal beads and JSR beads was almost same. The samples were processed as described previously.[9] Briefly, the fecal sample was homogenized with a buffer (40 mL) consisting of Hanks' solution, 10% fetal bovine serum, and 25 mM HEPES buffer (pH 7.35) at 200 rpm for one minute using a Stomacher system (Seward, Thetford, UK). The homogenate was filtered through a nylon filter (pore size, 512 μm), and following the addition of 80 μL of the immunomagnetic beads, the sample mixture was incubated for 30 minutes under gentle rolling conditions at room temperature. The mixture on the magnet was incubated on a shaking platform for 15 minutes at room temperature. The supernatant was then removed and the colonocytes in the pellet were stored at –80°C until RNA extraction.

miRNA array for selection of internal control and target miRNA

To determine the internal control for miRNA analysis and the suitable target of miRNA, the colonocyte RNA of five patients with colorectal cancer and five healthy volunteers was analyzed using the TaqMan MicroRNA Array v3.0 (Applied Biosystems, Foster, CA), in accordance with the manufacturer's instructions. RT-PCR was performed using an Applied Biosystems 7900HT fast real-time PCR system. Next, the target miRNAs were validated using total RNA extracted from both the cancer tissue and the normal mucosa of 31 patients with colorectal cancer.

Fecal miRNA analysis in patients with colorectal cancer and healthy volunteers

From August 2003 to November 2003 and from June 2004 to July 2004, 47 patients with colorectal cancer and 35 healthy volunteers were enrolled into the training study, respectively. From November 2003 to November 2009 and from July 2004 to March 2005, 252 patients with colorectal cancer and 81 healthy volunteers were enrolled in the validation study, respectively. The characteristics of these patients and volunteers are summarized in Table 1. All the patients with colorectal cancer had undergone surgical resection of their primary cancer at the National Cancer Center Hospital, Tokyo, Japan. No remarkable changes were observed except Dukes' stage classification between the training study and the validation study. All the healthy volunteers were confirmed to have no symptoms and evident abnormalities (eg, adenoma or carcinoma, including hyperplastic polyps) by screening

Table 1 Characteristics of CRC patients and healthy volunteers

Characteristics	Training set		Validation set	
	CRC patients (n = 47)	Healthy volunteers (n = 35)	CRC patients (n = 252)	Healthy volunteers (n = 81)
Age, years				
Median	62	60	63	59
Range	35–83	40–69	32–86	41–70
Sex, no (%)				
Male	33 (70.2)	19 (54.3)	162 (64.3)	33 (40.7)
Female	14 (29.8)	16 (45.7)	90 (35.7)	48 (59.3)
Tumor location, no (%)				
Cecum	2 (4.3)		17 (6.7)	
Ascending colon	7 (14.9)		39 (15.5)	
Transverse colon	2 (4.3)		15 (6.0)	
Descending colon	2 (4.3)		10 (4.0)	
Sigmoid colon	9 (19.1)		51 (20.2)	
Rectum	25 (53.2)		120 (47.6)	
Tumor size, mm				
Median	38		37	
Range	7–76		9–160	
Histology, no (%)				
W/D	21 (44.7)		143 (56.7)	
M/D	23 (48.9)		93 (36.9)	
P/D	2 (4.3)		7 (2.8)	
Mucinous carcinoma	1 (2.1)		8 (3.2)	
Carcinoid tumor			1 (0.4)	
Tumor depth, no (%)				
T1	5 (10.6)		34 (13.5)	
T2	8 (17.0)		60 (23.8)	
T3	33 (70.2)		154 (61.1)	
T4	1 (2.1)		4 (1.6)	
Dukes' stage, no (%)				
A	10 (21.3)		78 (31.0)	
B	9 (19.1)		69 (27.4)	
C	21 (44.7)		88 (34.9)	
D	7 (14.9)		17 (6.7)	

Abbreviations: CRC, colorectal cancer; W/D, well-differantiated adenocarcinoma; M/D, moderately differentiated adenocarcinoma; P/D, poorly differentiated adenocarcinoma.

colonoscopy performed at the Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo. The median age of the healthy volunteers was relatively younger than that of the patients with colorectal cancer. Regarding gender, the number of women was relatively higher among the healthy volunteers than among the patients with colorectal cancer. All participants were provided with detailed information about the study, and each gave written informed consent to participate in the study, which was approved by the institutional review board of National Cancer Center, Japan.

miRNA expression analysis using real-time PCR

Total RNA was extracted from the colonocytes isolated from the fecal samples using an miRNeasy Mini Kit (QIAGEN, Valencia, CA), and cDNA was synthesized using a TaqMan MicroRNA RT Kit (Applied Biosystems), in accordance with the manufacturer's instructions. RT-PCR was performed with precycling heat activation at 95°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 3 seconds, and annealing/extension at 60°C for 30 seconds, using an Applied Biosystems 7500 fast RT-PCR system. For the analysis of all miRNAs, we used the TaqMan microRNA assay (Applied Biosystems). miRNA expression analysis was conducted using the comparative Ct (threshold cycle) method. In this analysis, the formulae for the relative quantification of each gene were as follows: (dCt of each miRNA) = (Ct of each miRNA) – (Ct of miR-24), and (relative quantification of each miRNA) = $2^{-(\text{dCt of each miRNA})}$.

Statistical analysis

Differences in relative quantification of the miRNAs were analyzed using the two-sided Mann–Whitney *U*-test.

Statistical analyses were performed using SPSS Statistics version 19 for Windows (IBM, Tokyo, Japan). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Suitable internal control of miRNA analysis

Of 749 miRNAs, the average number of PCR-successful miRNA was 180 (range 90–295) in patients with colorectal cancer and 157 (53–242) in healthy volunteers, respectively. Forty miRNAs could be detected in all five patients with colorectal cancer and five healthy volunteers using the TaqMan MicroRNA Array, and these miRNAs served as candidates for internal control (Figure 1). Average Ct values of these miRNAs in the patients with colorectal cancer and healthy volunteers were 27.72 (23.81–31.16) and 28.78 (25.04–32.94), respectively. Mean differences in Ct values of miR-16, 24, -200c, and U6 from the average Ct values of these miRNAs were -0.12 ± 0.99, -1.48 ± 0.48, -2.57 ± 1.04, and 1.18 ± 3.19, respectively. miR-24 expression was the most stable and constant from among all miRNAs.

Selection of target miRNAs for colorectal cancer detection

According to the results of miRNA array, 20 miRNAs were selected as candidates for miRNA analysis (Table 2). Using tissue RNA, miR-17, -18a, -19a, -19b, -20a, -21, -92a, -106a, -135a, -135b, -146a, -183, -223, and -454* in cancer tissue were expressed at significantly higher levels than those in normal tissue ($P < 0.05$). On the other hand, there was no significant difference of expression for miR-34a, -155, -191, -206, -564, and -1208 between cancer tissue and normal tissue ($P > 0.1$). These 14 miRNAs were selected to target miRNAs for detection of colorectal cancer.

Relative quantification of each miRNA in colonocytes

The relative expression level of each miRNA was calculated using that of miR-24 as an internal control for 47 patients with colorectal cancer and 35 healthy volunteers in the training set (Table 1). We observed significant differences in the relative expression level of miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135a, -135b, and -146a between the patients

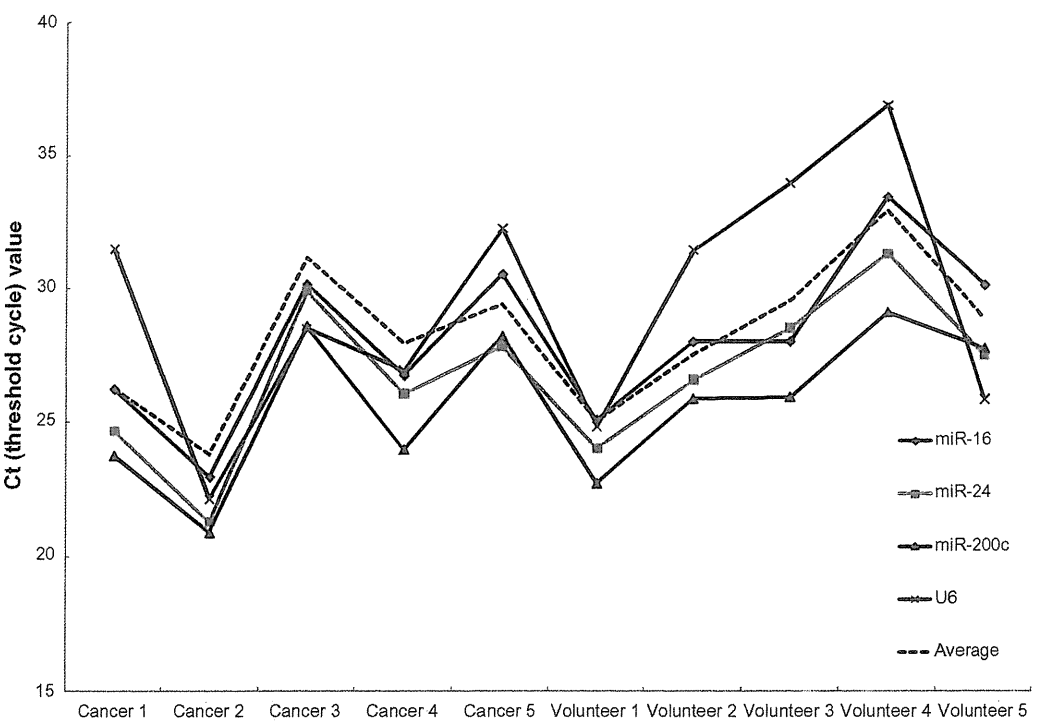

Figure 1 Ct values of candidates for internal control. Of 749 miRNAs, 40 could be detected in all of five patients with colorectal cancer and five healthy volunteers using the TaqMan MicroRNA Array.

Notes: The average Ct values of these miRNAs in patients with colorectal cancer and healthy volunteers were 27.72 (23.81–31.16) and 28.78 (25.04–32.94). The differences in the Ct values of miR-16, 24, -200c, and U6 from the average Ct values of these miRNAs were -0.12 ± 0.99 (average ± standard deviation), -1.48 ± 0.48, -2.57 ± 1.04, and 1.18 ± 3.19. The average Ct value of 40 miRNAs is indicated by the dotted line.

Abbreviations: Ct, threshold cycle; miRNA, micro RNA.

Table 2 Mean values of relative quantifications of target miRNA in tissue samples

	Colorectal cancer (n = 31)	Normal mucosa (n = 31)	*P* value
	Mean RQ (range)	Mean RQ (range)	
miR-17	1.50 (0–4.56)	0.44 (0.20–0.95)	<0.001
miR-18a	0.037 (0.002–0.135)	0.007 (0.001–0.020)	<0.001
miR-19a	0.007 (0.001–0.041)	0.002 (0–0.005)	<0.001
miR-19b	0.040 (0.002–0.164)	0.012 (0.002–0.040)	0.001
miR-20a	0.472 (0.047–1.462)	0.119 (0.026–0.284)	<0.001
miR-21	0.850 (0.190–2.239)	0.216 (0.065–0.757)	<0.001
miR-34a	0.024 (0.005–0.047)	0.023 (0.010–0.039)	0.8
miR-92a	5.117 (0.434–27.569)	1.893 (0.728–3.779)	<0.001
miR-106a	0.311 (0.092–1.187)	0.120 (0.054–0.286)	<0.001
miR-135a	0.008 (0.001–0.028)	0.001 (0–0.002)	<0.001
miR-135b	0.092 (0.014–0.330)	0.006 (0.001–0.024)	<0.001
miR-146a	0.216 (0.050–0.641)	0.139 (0.033–0.387)	0.001
miR-155	0.144 (0.038–0.431)	0.153 (0.059–0.437)	0.4
miR-183	0.012 (0.004–0.030)	0.004 (0.001–0.009)	<0.001
miR-191	0.515 (0.106–1.335)	0.485 (0.117–1.250)	0.5
miR-206	0.002 (0–0.016)	0.002 (0–0.010)	0.6
miR-223	0.416 (0.072–2.144)	0.205 (0.044–0.754)	0.006
miR-454*	0.0001 (0–0.0003)	0.0001 (0–0.0002)	0.03
miR-564	0.0003 (0–0.0025)	0.0003 (0–0.0022)	0.2
miR-1208	0.0001 (0–0.0008)	0.0002 (0–0.0028)	0.6

Notes: *P* value was analyzed by the Mann–Whitney *U*-test and $P < 0.05$ was considered to indicate a statistically significant difference.
Abbreviation: RQ, relative quantification.

Table 3 Mean values of relative quantifications of target miRNA compared with an internal control, miR-24

	CRC patients (n = 47)	Healthy volunteers (n = 35)	*P* value
	Mean RQ (range)	Mean RQ (range)	
miR-17	1.34 (0–3.76)	0.94 (0–11.85)	<0.001
miR-18a	0.12 (0–0.96)	0.04 (0–0.80)	<0.001
miR-19a	0.30 (0–1.55)	0.12 (0–1.66)	<0.001
miR-19b	1.35 (0–7.89)	0.71 (0–5.38)	<0.001
miR-20a	0.84 (0–3.56)	0.33 (0–2.13)	<0.001
miR-21	16.90 (0.28–66.49)	12.02 (0–64.94)	0.2
miR-92a	7.45 (0.38–35.02)	2.74 (0–14.05)	<0.001
miR-106a	1.26 (0–4.08)	0.78 (0–6.07)	<0.001
miR-135a	0.004 (0–0.043)	0.00002 (0–0.0006)	0.01
miR-135b	0.16 (0–2.21)	0.02 (0–0.28)	<0.001
miR-146a	0.53 (0–3.05)	0.13 (0–1.95)	<0.001
miR-183	0.010 (0–0.202)	0.009 (0–0.104)	0.5
miR-223	14.41 (1.59–49.90)	16.33 (0.03–53.63)	0.9
miR-454*	0.013 (0–0.560)	0.003 (0–0.097)	1

Notes: *P* value was analyzed by the Mann–Whitney *U*-test and $P < 0.05$ was considered to indicate a statistically significant difference.
Abbreviations: CRC, colorectal cancer; RQ, relative quantification.

with colorectal cancer and the healthy volunteers ($P < 0.01$). On the other hand, there was no significant difference in the relative expression level of miR-21, -183, -223, and -454* between the colorectal cancer patients and the healthy volunteers ($P > 0.1$, Table 3).

Area under ROC curve

The data for sensitivity and specificity calculated using relative quantifications of miRNA in patients with colorectal cancer and healthy volunteers were blotted into a receiver operating characteristic (ROC) curve (Figure 2). Areas under the ROC curve using miR-21, -135a, -183, -223, and -454* were less than 0.6. On the other hand, areas under the ROC curve using miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a were more than 0.7.

Sensitivity and specificity of miRNA expression analysis in training study

From the abovementioned results, we set miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a as a new miRNA set for detection of colorectal cancer. The thresholds of miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a were 2.1, 0.16, 0.57, 2.5, 1.4, 8.2, 3.2, 0.13, and 0.61, respectively (Table 4). The specificity of the healthy volunteers using each miRNA was set at 94.3% (33/35). The overall sensitivity of patients with colorectal cancer and the specificity of healthy volunteers were 70.2% (33/47, 95% confidence interval [CI] 55.1–82.7) and 74.3% (26/35, 95% CI 56.8–87.5), respectively.

Sensitivity and specificity of miRNA expression analysis in validation study

After the training study, 252 patients with colorectal cancer and 81 healthy volunteers were validated in the validation study (Table 1). The thresholds of all miRNAs for the validation study were the same as those for the training study. The overall sensitivity of the patients with colorectal cancer and the specificity of the healthy volunteers were 67.5% (170/252, 95% CI 61.3–73.2) and 75.3% (61/81, 95% CI 64.5–84.2), respectively (Table 5). There was no remarkable difference between the training study and the validation study.

Discussion

In our recent preliminary study, we analyzed the expression of miRNA in exfoliated colonocytes using oncogenic miRNAs, such as the miR-17-92 cluster, miR-21, and miR-135 normalized by U6.[26] We found that the expression analysis on the miRNA extracted from exfoliated colonocytes was feasible. In the present study, we adopted a more suitable internal control for miRNA expression and the optimal miRNA set for detecting colorectal cancer using TaqMan MicroRNA

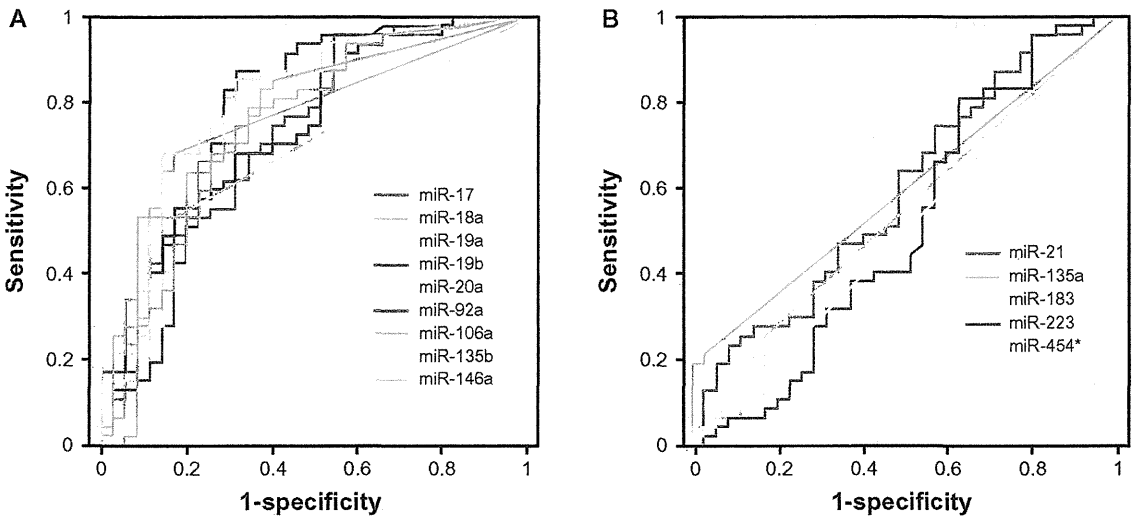

Figure 2 Areas under the ROC curve. (A) ROC curve using miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a. Area under the ROC using these miRNAs were more than 0.7. (B) ROC curve using miR-21, -135a, -183, -223, and -454*.
Note: Areas under the ROC curve using these miRNAs were less than 0.6.
Abbreviations: miRNA, micro RNA; ROC, receiver operating characteristic.

Array. The highly stable expression in both patients with colorectal cancer and in healthy volunteers was necessary for the internal control. Because expression of miR-24 in the colonocytes of patients with colorectal cancer and healthy volunteers was more stable and constant than that of the miR-200 family or U6 that are sometimes used as a provisional internal control, miR-24 was adopted as an internal control in the present study. However, miR-24 was not used as an internal control in previous studies. Therefore, we believe that establishment of a universal internal control for miRNA analysis is urgently needed.

The miR-17-92 cluster, -21, -34a, -106a, -135, -146a, -155, -183, -191, -206, -223, -454*, -564, and -1208 were selected from 749 miRNAs as candidates for miRNA analysis using TaqMan MicroRNA Array. Among those, the miR-17-92 cluster, -21, -106a, -135, -146a, -183, -223, and -454* were highly expressed in colorectal cancer tissue compared with the normal mucosa in our preliminary results. To date, various reports have shown that the miR-17-92 cluster, -21, -106a, -135, and -223 were expressed more strongly in colorectal cancer tissue than in normal colorectal tissue.[19–22,28–31] Though it has been shown that miR-146a is highly expressed in several types of cancer tissue,[32,33] it has been reported that miR-146a is tumor suppressor miRNA.[34] These results are controversial; however, miR-146a was expressed to a significantly greater extent in colorectal cancer tissue than in normal mucosa in our study. Thus, we decided to use miR-17-92 cluster, -21, -106a, -135, -146a, -183, -223,

Table 4 Sensitivity and specificity of each miRNA expression using optimal threshold in training set

	Threshold	CRC patients (n = 47)		Healthy volunteers (n = 35)	
		No	Sensitivity (%) (95% CI)	No	Specificity (%) (95% CI)
Combined markers		33	70.2 (55.1–82.7)	26	74.3 (56.8–87.5)
miR-17	2.1	8	17.0 (7.6–30.8)	33	94.3 (80.9–99.3)
miR-18a	0.16	11	23.4 (12.3–38.0)	33	94.3 (80.9–99.3)
miR-19a	0.57	7	14.9 (6.2–28.3)	33	94.3 (80.9–99.3)
miR-19b	2.5	6	12.8 (4.8–25.7)	33	94.3 (80.9–99.3)
miR-20a	1.4	10	21.3 (10.7–35.7)	33	94.3 (80.9–99.3)
miR-92a	8.2	15	31.9 (19.1–47.2)	33	94.3 (80.9–99.3)
miR-106a	3.2	1	2.1 (0.1–11.3)	33	94.3 (80.9–99.3)
miR-135b	0.13	13	27.7 (15.6–42.7)	33	94.3 (80.9–99.3)
miR-146a	0.61	13	27.7 (15.6–42.7)	33	94.3 (80.9–99.3)

Abbreviations: CRC, colorectal cancer; 95% CI, 95% confidence interval.

Table 5 Sensitivity and specificity of miRNA expression (validation set)

microRNA	CRC patients (n = 252)		Healthy volunteers (n = 81)	
	No	Sensitivity (%) (95% CI)	No	Specificity (%) (95% CI)
Combined markers	170	67.5 (61.3–73.2)	61	75.3 (64.5–84.2)
miR-17	26	10.3 (6.8–14.8)	77	95.1 (87.9–98.6)
miR-18a	42	16.7 (12.3–21.8)	76	93.8 (86.2–98.0)
miR-19a	3	1.2 (0.2–3.4)	81	100 (95.5–100)
miR-19b	7	2.8 (1.1–5.6)	80	98.8 (93.3–100)
miR-20a	18	7.1 (4.3–11.0)	79	97.5 (91.4–99.7)
miR-92a	124	49.2 (42.9–55.5)	78	96.3 (89.5–99.2)
miR-106a	6	2.4 (0.9–5.1)	80	98.8 (93.3–100)
miR-135b	51	20.2 (15.5–25.7)	77	95.1 (87.9–98.6)
miR-146a	27	10.7 (7.2–15.2)	77	95.1 (87.9–98.6)

Abbreviations: CRC, colorectal cancer; 95% CI, 95% confidence interval.

and -454* for colorectal cancer detection using colonocytes in the present study.

In the training study, the expressions of miR-21, -183, -223, and -454* in exfoliated colonocytes of patients with colorectal cancer were not significantly different from those of healthy volunteers. Because relative expression of miR-135a was low in both patients with colorectal cancer and healthy volunteers, the area under the ROC curve using miR-135a was under 0.6. From the present results, we determined that miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a were useful for detection of colorectal cancer. The sensitivity and specificity of the miRNA assay in colonocytes was 70.2% and 74.3%, respectively. These results are almost the same as those of our previous studies,[9,10,16] and we have subsequently validated the miRNA set in the validation study.

Although the rate of patients with early-stage colorectal cancer was slightly high in the validation study compared with the training study, there were no remarkable changes between the characteristics of the training study and those of the validation study. The sensitivity and specificity of the miRNA assay in the validation study was 67.56% and 75.3%, respectively. The sensitivity and specificity of the fecal miRNA test were almost the same between the training study and the validation study. Furthermore, we could not find any specific difference between miRNA expression and the clinicopathological characteristics of colorectal cancer.

In summary, the fecal miRNA test using miR-17, -18a, -19a, -19b, -20a, -92a, -106a, -135b, and -146a was found to be useful for the detection of colorectal cancer in both the training study and the validation study. The present data may warrant further comparative study between fecal occult blood test and the fecal miRNA test for colorectal cancer screening in terms of sensitivity, specificity, and cost-effectiveness.

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Disclosure

The authors report no conflicts of interest in this work.

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ORIGINAL ARTICLE

Postoperative chylous ascites after colorectal cancer surgery

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Abstract
Purpose To evaluate the diagnosis, epidemiology, risk factors, and treatment of chylous ascites after colorectal cancer surgery.
Methods Among 907 patients who underwent colorectal cancer resection at our institution between 2006 and 2009, chylous ascites developed in 9. We analyzed the clinical data for these 9 patients.
Results Five of the nine patients with chylous ascites had undergone right hemicolectomy and seven had undergone D3 lymph node dissection. In all patients, chylous ascites began to develop the day after commencement of oral intake or the next day. Two patients had no change in diet, one was started on a high-protein and low-fat diet, and six were put on intestinal fasting. Drainage tubes were removed within 5 days after treatment in seven patients. The hospital stay was about 2 weeks after surgery and 1 week after treatment. We found that the tumor area, tumors fed by the superior mesenteric artery, and D3 lymph node dissection were significantly associated with chylous ascites.
Conclusions Chylous ascites after colorectal cancer surgery occurred at an incidence of 1.0%, but was significantly more frequent after surgery for tumors fed by the superior mesenteric artery and after D3 lymph node dissection. Conservative treatment was effective in all cases.

Keywords Chylous ascites · Colorectal cancer · Postoperative · Risk factors

Introduction

Chylous ascites is defined as the extravasation of milky or creamy peritoneal fluid rich in triglycerides [1]. This condition is caused by interruption of the thoracic duct, cisterna chyli, or their major tributaries. Postoperative chylous ascites is most frequently reported after abdominal aortic surgery, resection and replacement of the inferior vena cava, lymphadenectomy for testicular and renal cancers, pelvic surgery for advanced gynecologic malignancies, and spinal surgery [2–5]. Conversely, it is an unusual complication of colorectal cancer surgery, and its diagnosis and treatment in this setting are not well established. Thus, we conducted this study to investigate chylous ascites following colorectal cancer surgery in a large patient population, examining the risk factors for its onset, and determining the appropriate treatment.

Patients and methods

Patients

Between January, 2006 and May 2009, 907 patients underwent resection of colorectal cancer at the National Cancer Center Hospital East (NCCHE), Chiba, Japan. Chylous ascites developed postoperatively in nine of these patients, whose detailed clinical characteristics were analyzed.

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Definition

Chylous ascites is defined as the extravasation of milky or creamy peritoneal fluid, rich in triglycerides from the thoracic or intestinal lymph in the abdominal cavity [1, 5, 6]. In this study, we defined chylous ascites as non-infectious milky fluid in the drainage tubes. We defined lack of infection as the absence of fever and peritonitis at the time of development of chylous ascites, or low grade fever without elevation of the white blood cell (WBC) count.

Surgical procedures

All operations were performed by or with the assistance of one of five colorectal surgeons, and based on the guidelines approved by the Japanese Society for Cancer of the Colon and Rectum (JSCCR) [7]. In the JSCCR guidelines, D3 lymph node dissection is defined as dissection of all regional lymph nodes with cutting of the root of the inferior mesenteric artery, and D2 lymph node dissection is defined as dissection of the paracolic and intermediate lymph nodes. D3 dissection was performed for patients with Stage II and Stage III disease, and D2 dissection was performed for patients with Stage I disease. Tissue was decorticated and cut using an electrosurgical knife and an ultrasonically activated scalpel, and major vessels and lymph ducts were ligated. The basic procedure for rectal cancer was total mesorectal excision (TME) or tumor-specific mesorectal excision (TSME) with a distal resection margin >3 cm (RS or Ra) or >2 cm (Rb). Lateral lymph node dissection was performed for Rb tumors, based on preoperative staging (T3–T4, or lymph node-positive cases).

Treatment

There are few studies on the treatment regimens for chylous ascites, but the standard initial treatment is a high-protein and low-fat diet or fasting to reduce chyle formation [2, 5]. Somatostatin and octreotide can be given to patients who do not respond to the initial therapy [8–10]. If conservative treatment fails, patients can be treated surgically by direct ligation of the source of chylous leakage [2, 5, 11]. The initial treatment for most of our patients was fasting, with the addition of somatostatin if they did not respond to fasting. None of our patients required surgical treatment.

Statistical analysis

We analyzed the influence of operative time, operative blood loss, tumor diameter, operative procedure, laparoscopic surgery, range of lymph node dissection, and tumor area at the time of occurrence of chylous ascites, by the Chi-square test using SPSS v. 17. A p value <0.05 was considered significant.

Results

Chylous leakage was detected in 9 (1.0%) of the 907 patients who underwent colorectal cancer resection. Table 1 summarizes the characteristics and postoperative clinical course of these nine patients. Right hemicolectomy was performed in five patients, for ileocecal cancer in three, ascending colon cancer in one, and transverse colon cancer in one, and was the most common surgical procedure preceding postoperative chylous leakage. Seven of the nine patients underwent open surgery, and seven underwent D3 lymph node dissection.

Chylous ascites developed either on the day of, or the day after recommencement of food intake. The largest volume of chylous drainage was 700 ml/day, in patient 6 (100–700 ml/day). Two patients received no therapy, one was treated only with a high-protein and low-fat diet to reduce chyle formation, and six were treated with intestinal fasting. The amount of chylous ascites diminished gradually after treatment, and drainage tubes were removed within 5 days of the start of treatment in seven patients. The hospital stay was about 2 weeks after surgery and about 1 more week after the development of chylous ascites.

Table 2 shows the surgical features of the patients with chylous ascites. Univariate analysis revealed that the tumor area and tumors fed by the superior mesenteric artery (vs. the inferior mesenteric artery; $p < 0.01$) were significantly associated with chylous ascites. Operative time, blood loss, tumor diameter, laparoscopic surgery, and the dissection range of lymph nodes were not associated with chylous ascites. Chylous ascites occurred at a significantly higher frequency in patients with a tumor fed by the superior mesenteric artery and when D3 lymph node dissection was performed (Table 3).

All nine patients with chylous ascites improved after conservative treatment and eight responded to dietary restriction only. The chylous ascites persisted in one patient, who was then commenced on intravenous somatostatin, 300 μg/day, which reduced the chylous discharge (Fig. 1). The median time of onset of chylous ascites was the day after food intake commenced and drains were removed at a median time of 4 days after the beginning of treatment. The median hospital stay was 8 days after the beginning of treatment.

Discussion

Our PubMed search for all relevant literature on chylous ascites after colorectal surgery revealed several case reports [8, 9, 12–14], but extensive information is not available. Hence, the diagnostic criteria, epidemiology,

Table 1 Characteristics and postoperative courses of the nine patients with chylous ascites as a complication after colorectal cancer surgery

Patient	Age	Sex	BMI	Cancer location	Procedure	Open/ lap	Lymph node dissection	Time (min)	Blood loss (cc)	POD of initiation of food intake	POD of onset of chylous ascites	Daily amount of drainage (ml)	Management	Days with no food	POD of removal of drain	Hospital stay
1	51	F	18.9	Ileocecum	RHC	Open	D3	180	391	4	5	100	No meal	1	10	13
2	80	F	24.1	Rectum	HAR	Open	D2	151	474	4	5	220	No meal	3	10	13
3	58	M	28.9	Ileocecum	RHC	Open	D3	162	360	3	4	150	No meal	3	8	21
4	60	F	26.1	Ileocecum	RHC	Open	D3	152	244	5	5	100	–	0	7	9
5	49	F	20.3	Rectum	LAR	Lap	D3	285	69	4	4	200	No meal	3	8	9
6	58	F	19.5	Rectum	HAR	Lap	D2	235	35	4	4	700	No meal	10	16	20
7	89	M	24.4	Ascending colon	RHC	Open	D3	198	898	7	8	430	–	0	11	15
8	72	M	22.1	Sigmoid colon	SR	Open	D3	145	126	6	7	350	MCT	0	11	13
9	63	M	24	Transverse colon	RHC	Open	D3	255	172	4	5	230	No meal	4	12	14

onset features, and treatment of chylous ascites after colorectal cancer remain unclear. The mechanism of the formation of chylous ascites is related to failure of the lymphatic system, which may also occur after traumatic injury, obstruction, or with congenital factors [15]. Postoperative chylous ascites is likely to result from both traumatic injury and obstruction of the lymphatic system. To our knowledge, this is the first study on the risk factors for chylous ascites after colorectal cancer surgery and provides the first indication of the incidence of this condition.

The epidemiology of chylous ascites after colorectal surgery is unclear. We found an incidence of chylous ascites of 1.0% after 907 colorectal cancer operations, with a higher frequency in patients with a tumor fed by the superior mesenteric artery versus the inferior mesenteric artery. Expanded lymph node dissection may have caused the higher frequency of chylous ascites in our patient population. We found one published case each of chylous ascites after right hemicolectomy with D3 lymphadenectomy for colorectal cancer, after low anterior resection with D3 lymphadenectomy for colorectal cancer, and after paraaortic lymphadenectomy for recurrent colorectal cancer [8, 9, 12, 13]. A connection between the range of lymph node dissection and the frequency of chylous ascites following colorectal cancer surgery has not been established. However, there are several reports of chylous ascites following D3 dissection for gastric cancer surgery. Yol et al. reported one (3.9%) case of chylous ascites among 34 patients who underwent gastric cancer surgery. In the JCOG 9501 study, lymphorrhea was observed in 10 of 260 D3 surgery cases (3.8%) [16], while Maeta et al. [17] reported four (5.6%) cases of lymphorrhea among 70 patients who underwent D3 lymphadenectomy. These articles suggest that the incidence of chylous ascites following gastric cancer with D3 dissection is 3–5%. Hence, the frequency of chylous ascites following colorectal cancer surgery with D3 dissection is lower than that in gastric cases, and also seems to be lower than the 6.6% reported by Neyer et al. after surgery for urological cancer with retroperitoneal lymph node dissection [15, 16, 18–21]. The degree of chyle leakage after colorectal surgery may be milder than that after lung or upper abdominal surgery because peripheral lymph systems tend to be injured in colorectal surgery, whereas central lymph systems (thoracic duct or cisterna chyli) may be injured in lung or upper abdominal surgery [6]. Thus, chylous ascites after colorectal surgery is probably more likely to resolve with conservative therapy, than a chylous complication after surgery on other organs.

Few studies have focused on the treatment regimen for chylous ascites. The most effective treatment is intraoperative identification of the source of chylous leakage and

Table 2 Surgical features of the nine patients with chylous ascites after colorectal cancer surgery

Variable	None	Chylous ascites	*p* value
Number of patients	898	9 (1.0%)	
Median operative time (min)	210 (50–723)	180 (145–285)	0.38
Median blood loss (ml)	177 (0–10440)	244 (35–898)	0.72
Tumor diameter (mm)	35 (0–230)	40 (20–90)	0.21
Surgical procedure			
Open	583	7 (1.2%)	0.36
Laparoscopy	315	2 (0.6%)	
Lymph node dissection range			
D0	10	0	
D1	24	0	
D2	366	2 (0.5%)	0.22
D3	498	7 (1.4%)	
Surgical region			
SMA	204	5 (2.4%)	<0.01
IMA	694	4 (0.6%)	

Table 3 Surgical features of the nine patients with chylous ascites after colorectal cancer surgery with D3 lymph node dissection

	SMA	IMA	*p* value
Chylous ascites (−)	84	414	<0.01
Chylous ascites (+)	5 (5.60%)	2 (0.50%)	

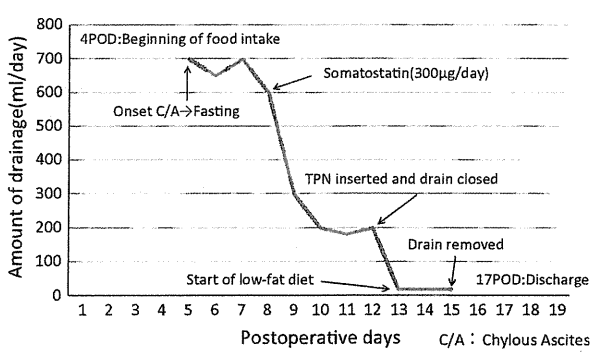

Fig. 1 Postoperative course in patient no. 6

subsequent suturing or ligation. Postoperatively, dietary restriction with a high-protein and low-fat diet with medium-chain triglycerides or fasting can reduce lymph flow. Patients who do not respond to this treatment may be responsive to somatostatin, which can reduce lymph fluid excretion through specific receptors found in the intestinal wall of lymphatic vessels [2]. Somatostatin has been shown to reduce the output of lymphatic leakage after 24–72 h [12]. In some case reports, concomitant fasting and treatment with subcutaneous octreotide, a synthetic analog of somatostatin, were effective and the patient did not require surgery [8–10].

Surgical exploration and direct ligation of the source of chylous leakage may be required for patients who do not respond to conservative treatment [22]. However, it may be difficult to detect the source of chylous leakage in the reoperation [11]. None of our patients required surgery, but it should be considered if the patient is unresponsive to conservative treatment. The treatment strategy is almost the same as that for other organs; however, in patients with upper abdominal cancer or lung cancer, surgical exploration and direct ligation of the source of chylous leakage tend to be performed when conservative therapy is not effective because the amount of chylous ascites is greater and it is easier to detect the source of chylous leakage than after surgery for colon cancer [1, 5, 13, 15, 16, 19, 23].

The current definition of chylous ascites is not based on established criteria. Chylous leakage is generally defined as milky fluid in the drainage tubes, with proof of the absence of infection, and the presence of chylomicrons in Sudan staining. A triglyceride level >110 mg/dl in the milky fluid has been proposed to be highly suggestive of chyle [4, 5, 15]. In our retrospective study, the diagnosis of chylous effusion was based on findings of a milk-like appearance and no infection, confirmed by the absence of fever and peritonitis, or patients with low grade fever but no elevation in the WBC count.

In conclusion, the incidence of chylous ascites was 1.0% in this series of 907 colorectal cancer surgery patients. Chylous ascites developed significantly more frequently in patients with tumors fed by the superior mesenteric artery and those who underwent D3 lymph node dissection. Conservative treatment was effective in all cases.

Conflict of interest None of the authors have a conflict of interest to declare.

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ORIGINAL ARTICLE

Differences in tissue degeneration between preoperative chemotherapy and preoperative chemoradiotherapy for colorectal cancer

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Abstract
Purpose Preoperative chemoradiotherapy (CRT) for rectal cancer is administered to improve local control, but can also induce severe anal dysfunction after surgery, while preoperative chemotherapy that significantly reduces the primary lesion in rectal cancer has recently been developed. The aim of the study was to examine differences in the effects of preoperative CRT and chemotherapy on tissue degeneration of patients with colorectal cancer.
Methods The subjects were 91 patients, including 68 with rectal cancer who underwent internal sphincteric resection with (n=47, CRT group) or without (n=21, control group) preoperative CRT, and 23 with colorectal cancer who received preoperative FOLFOX treatment. Peripheral nerve degeneration was evaluated histopathologically using H&E-stained sections, based on karyopyknosis, disparity of the nucleus, denucleation, vacuolar or acidophilic degeneration of the cytoplasm, and adventitial neuronal changes.
Results The incidence of neural degeneration was significantly higher in the CRT group than in the control group and FOLFOX group. There were no differences in any items of neural degeneration between the FOLFOX and control groups.
Conclusion CRT induced marked neural degeneration around the rectal tumor. FOLFOX treatment produced mild neural degeneration similar to that in the control group.

Keywords Chemoradiotherapy · Internal sphincteric resection · Neural degeneration · Rectal cancer · FOLFOX

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Background

Innovative treatment for lower rectal cancer has recently tended toward preservation of the anus. Low anterior resection with coloanal anastomosis [1] and intersphincteric resection (ISR) [2] are advanced anus-preserving operations for treatment of low rectal cancer with avoidance of a colostomy. Anastomoses are made near to or under the dentate line in the anal canal, and the procedures have a tolerable and clinically acceptable local recurrence rate [3, 4]. Preoperative chemoradiotherapy (CRT) or radiotherapy is also thought to be necessary to decrease local recurrence following ISR [5–7].

Investigations of functional outcome after ISR [6, 8–11] have shown that satisfactory anal function is preserved in most patients, but that some have severe dysfunction [11, 12] and conversion to colostomy may be necessary as an additional treatment [8, 12]. Preoperative CRT is strongly associated with poor anal function after ISR, suggesting that

patients with rectal cancer who undergo ISR after preoperative CRT are likely to experience incontinence [13, 14]. Lim et al. [15] reported that a conventionally fractionated 45-Gy dose of preoperative CRT caused poor anorectal function due to damage to the pudendal nerve. Rectal function may also be worsened by radiation-induced proctitis and reduction of rectal compliance due to fibrosis of the rectal wall [16, 17]. Direct radiation injury to the internal anal sphincter muscles can also cause anal sphincter dysfunction [18].

FOLFOX therapy and other chemotherapies have contributed to extension of the survival period in patients with colorectal cancer of stage IV through significant reduction of the primary lesion [19–22]. In a Phase III study of induction XELOX chemotherapy, preoperative chemoradiotherapy, and surgery, the response rate of XELOX therapy was as high as 88% [23]. Thus, such chemotherapy may have an effect on local control that is equivalent to that of chemoradiotherapy. There have also been several recent reports of cases treated with preoperative chemotherapy and surgery, and positive effects on postoperative anal function are likely with omission of preoperative radiotherapy.

Pathological analysis of the anal sphincter muscle area may reveal an association of preoperative therapy with anal sphincter dysfunction, but this relationship has not been studied. Therefore, we examined the degree of tissue degeneration, with a particular focus on neural degeneration and tissue fibrosis, in surgical specimens resected from patients who underwent surgery with or without preoperative CRT or with preoperative chemotherapy only. The objective of the study was to determine differences in tissue damage caused by preoperative CRT and preoperative chemotherapy in patients with colorectal cancer.

Methods

Patients

Between 2001 and 2011, 91 patients underwent surgery for colorectal cancer at the National Cancer Center Hospital East, Chiba, Japan. Of these patients, 47 received CRT before ISR, 21 underwent ISR alone (control group), and 23 received preoperative chemotherapy (FOLFOX) before surgery. For ISR cases from 2002 to 2004, CRT was performed for all patients who gave consent. Patients treated before and after this period and ISR cases in which patients did not consent to CRT were examined as the surgery-only group. From 2007 to 2011, preoperative FOLFOX therapy was performed as a pilot study in patients who gave consent to this procedure. All cases in this pilot study were included in the analysis in the current study.

Our operative indications for ISR were a tumor edge 5 cm above the anal verge or 3 cm above the dentate line; adenocarcinoma confirmed histologically by preoperative biopsy; and age less than 76 years old [8]. Preoperative chemotherapy is not currently performed as standard therapy. However, preoperative FOLFOX therapy was performed as a pilot study in patients who gave informed consent to this procedure. The patients who received preoperative FOLFOX therapy were stage IV cases and those with colon cancer. Preoperative stage was determined based on the UICC classification [24].

Surgical procedure

ISR was performed as described previously [8]. First, dissection was performed by the abdominal approach until total mesorectal excision was complete. The outside layer of the internal sphincter muscle was then exposed and circumferentially divided from the puborectal muscle and the external sphincter. After the abdominal approach was completed, perianal resection was performed. The mucosa and the internal sphincter muscle were incised 1–2 cm distal to the tumor.

About preoperative chemotherapy patients with complete resection of the primary lesion who received preoperative chemotherapy were examined. They included patients diagnosed as R0 for the residual tumor, and those diagnosed as R1 based on metastasis to the liver or lungs.

Preoperative CRT

Forty-seven patients with clinical T3 tumors agreed to undergo CRT. Over a 5-week period, a dose of 45 Gy was administered along with intravenous infusion of 5-fluorouracil (250 mg/m^2/day) to increase the efficacy of radiotherapy. Nerve-sparing resection surgery was performed 2 weeks after completion of preoperative CRT [25].

Preoperative chemotherapy

Patients received preoperative FOLFOX-6 or FOLFOX-4 [26–28]. None of the subjects received Bevacizumab or other targeted therapies. The cycles of FOLFOX therapy differed for individual patients. Surgery was performed at least 28 days after completion of the therapy.

Pathological examination of nerves near the primary tumor

Prior to pathological evaluation, the number of nerves in the H&E-stained sections were counted in low-power magnification fields (10× 10). Ten nerves around the primary lesion were selected and photographed, and the consistency of features of the nerves in each photograph was evaluated. In this manner,

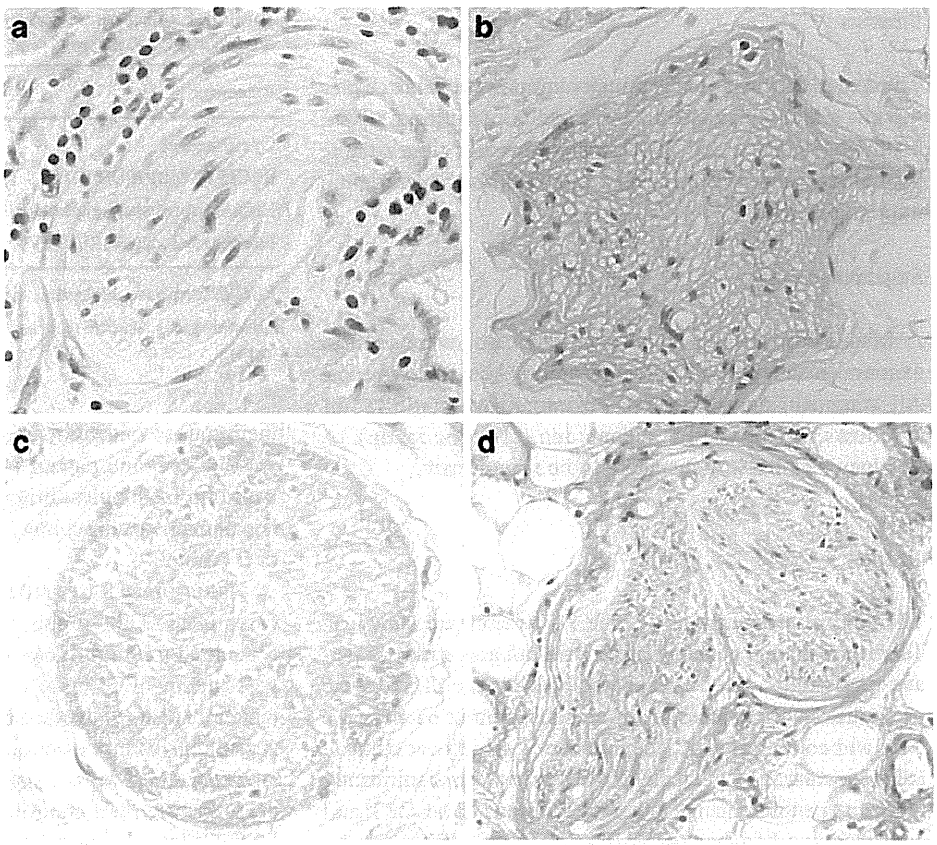

Fig. 1 Pathological evaluation. H&E sections were assessed under a standard light microscope at low-power magnification (×100). The evaluated items (**a–d**) were **a** Normal; **b** Vacuolar degeneration and adventitial neuron change, Grade 3; **c** Denucleation, acidophilic degeneration of cytoplasm and adventitial neuron change, Grade 2; and **d** Karyopyknosis , acidophilic degeneration of cytoplasm and adventitial neuron change, Grade 1

pathological tissue degeneration was evaluated for 10 nerves near to the tumor in each patient, based on the following features: karyopyknosis, vacuolar degeneration, acidophilic degeneration of cytoplasm, denucleation, and adventitial neuronal changes. The sections were evaluated by two authors (S.F. and Y.N.) who were blinded to the clinical information for the patients. Representative histopathological findings for neurons are shown in Fig. 1.

Table 1 Clinical characteristics of the patients (n=91)

	CRT group	Control group	FOLFOX group
Patients	47	21	23
Median age (range)	56 (27–77)	60 (39–72)	56 (34–77)
Gender, M:F	35:12	15:6	18:5
Median AV (cm)	3.5 (0–5.0)	4.0 (2.5–5.5)	3.5 (2.5–4.5)[a]
Operative procedure (%)			
ISR	47(100)	21(100)	16 (70)
APR	0(0)	0(0)	1 (4)
LAR	0(0)	0(0)	3 (13)
Sigmoidectomy	0(0)	0(0)	3 (13)
Clinical/Pathology Stage (%)			
I	9 (19)/25 (53)	4 (19)/4 (19)	0 (0)/3 (13)
II	16 (34)/6 (13)	8 (38)/5 (24)	3 (13)/6 (26)
IIIa	9 (19)/5 (11)	5 (24)/6 (29)	7 (30)/8 (35)
IIIb	11 (23)/8 (17)	3 (14)/6 (29)	8 (35)/1 (4)
IV	2 (4)/2 (4)	1 (5)/0 (0)	5 (22)/5 (22)
Postoperative complications (%)	14 (29)	9 (43)	8 (35)
Anastomotic leakage	5 (11)	3 (14)	3 (13)
Pelvic abscess	6 (12)	5 (24)	3 (13)

ISR intersphincteric resection, *CRT* preoperative radiochemotherapy, *APR* abdominoperineal resection, *LAR* low anterior resection

[a]AV data in the FOLFOX group consist of ISR cases only

Fibrosis

The degree of fibrosis of the primary tumor was evaluated on a 4-point scale, with grades 0, 1, 2, and 3 reflecting <10%, 10–30%, 30–50%, and ≥50% replacement of tumor tissue by fibrosis, respectively, in the section with the maximum tumor diameter [18].

Statistical analysis

A Mann–Whitney test and Fisher exact test were used to examine histological differences among the three groups. All statistical analyses were performed using SPSS for Windows, v.13.0J (SPSS-Japan Inc., Tokyo, Japan). A p value of <0.05 was considered to be significant.

Results

The clinical characteristics of the 91 patients are shown in Table 1, including patient number, median age, gender, operative procedure, and clinical/pathological stage. There were no significant differences in age and gender ratio between the CRT and control groups. The number of subjects at clinical/pathological stage IV in the FOLFOX group was significantly higher than those in the other two groups. The CRT and control groups had only ISR cases. The FOLFOX group included cases with other operative procedures, including abdominoperineal resection (APR), low anterior resection (LAR), and sigmoidectomy because preoperative FOLFOX therapy was performed as a pilot study and there were only a small number of cases (Table 1). Of the 23 cases in the FOLFOX group, three treated with sigmoidectomy were not cases of rectal cancer and the other 20 were rectal cancer.

For the 23 subjects who received preoperative FOLFOX, the therapy was performed for an average of 5.7 cycles and the response evaluation criteria in solid tumors response rate was 73.9%. One patient (4.3%) was diagnosed as grade 3 based on histological criteria for assessment of response to neoadjuvant therapy, but 17 patients (73.9%) were grade 1 or 2 (Table 2).

Table 2 Clinical parameters of patients (n=23) who received preoperative FOLFOX therapy

Parameter	Value
Cycle (range)	5.7 (2–12)
RECIST (CR/PR/SD/PD)	2/15/3/3
Histological response grade (0/1a/1b/2/3)[a]	1/13/4/4/1
Pathological stage (I/II/III/IV)	3/6/9/5

RECIST response evaluation criteria in solid tumors

[a] Histological criteria for assessment of response to neoadjuvant therapy

Tissue fibrosis of grade 2 or 3 was observed in 73% of cases in the CRT group, whereas fibrosis of grade 0 or 1 accounted for 86% of cases in the control group and 87% of cases in the FOLFOX group. There was a significantly higher incidence of severe fibrosis in the CRT group, with no severe fibrosis in the control and FOLFOX groups (Table 3). However, there was a significant difference in the fibrosis grade between the control and FOLFOX groups (p=0.024: Mann–Whitney test) because the FOLFOX group had more grade 1 cases than the control group (Table 3).

The incidence of neural degeneration was significantly higher in the CRT group than in the control group and FOLFOX group. None of the measures of neural degeneration differed between ISR and non-ISR (LAR, APR, and sigmoidectomy) cases or between rectal cancer (ISR, LAR, and APR) and non-rectal cancer (sigmoidectomy) cases in the FOLFOX group. None of the measures of neural degeneration differed between the FOLFOX and control groups

Table 3 Histological changes evaluated to assess neurodegeneration

Item	CRT Group (n=47)	FOLFOX group (n=23)	Control group (n=21)	P value		
				CRT vs. FOLFOX	CRT vs. Control	FOLFOX vs. Control
Fibrosis grade 0/1/2/3 (%)	2/11/13/21 (4/23/28/45)	8/12/1/2 (35/52/4/9)	16/2/2/1 (76/10/10/5)	<0.001*	<0.001*	0.024*
Karyopyknosis (%)	19 (40)	0 (0)	0 (0)	<0.001*	0.001*	1.0
Vacuolar degeneration (%)	32 (68)	4 (17)	4 (19)	<0.001*	<0.001*	0.887
Acidophilic degeneration of cytoplasm (%)	15 (32)	0 (0)	0 (0)	0.002*	0.002*	1.0
Adventitial neuron change 0/1/2/3 (%)	2/25/7/13 (4/53/15/28)	22/1/0/0 (96/4/0/0)	17/4/0/0 (81/19/0/0)	<0.001*	<0.001*	0.129
Denucleation (%)	26 (55)	0(0)	0 (0)	<0.001*	<0.001*	1.0

Karyopyknosis, vacuolar degeneration, acidophilic degeneration of cytoplasm, adventitial neuron change, and denucleation were evaluated to assess neurodegeneration

*p<0.05

(Table 3). Representative histopathological findings for neurons are shown in Fig. 1.

Tissue degeneration in resected samples from cases that underwent preoperative FOLFOX chemotherapy for colorectal cancer is shown in Fig. 2. In a case of histological response grade 1a (Fig. 2a), the tumor was reduced and a cavity was created between the gland duct structure of tumor tissues and the surrounding interstitial structure. Mild infiltration of inflammatory cells was observed in the interstitium, but fibrotic tissues were not observed. In a case with adventitial neuron changes of grade 1 (Fig. 2b), thickened perineuria were observed, but the neuronal degeneration did not reach the inside of nerves. This case was the only one of the 23 cases to show adventitial neuron changes of grade 1. Thus, neural degeneration was mild after preoperative FOLFOX for colorectal cancer, with almost no effects on tissues surrounding the primary lesion.

Discussion

The results of the study indicated that preoperative CRT for colorectal cancer caused high rates of tissue degeneration around the primary lesion, whereas almost no tissue degeneration was observed with preoperative chemotherapy. This is the first study of tissue degeneration in resected samples after preoperative FOLFOX chemotherapy for colorectal cancer. Neural degeneration after FOLFOX was similar to that in the control group (no preoperative CRT), which shows that the severity of tissue damage is mild in chemotherapy.

The negative effect of preoperative CRT on degeneration around the rectal tumor suggests that it is important to examine degeneration of neurons around the internal sphincter muscle for prediction of anal dysfunction. Moreover, preoperative CRT has been shown to have a major effect on anal dysfunction after ISR. Therefore, a negative effect of CRT on anal function occurs regardless of the extent of internal sphincter muscle preservation [13]. The cause of the negative effect of conventionally fractionated CRT on anorectal function remains unclear. Lim et al. [15] suggested that poor anorectal function after preoperative CRT was due to damage to the pudendal nerve, and rectal function may also be worsened by radiation-induced proctitis and reduced rectal compliance [16, 17]. Moreover, anal sphincter dysfunction may be caused by direct radiation injury to the internal anal sphincter muscles [18]. Our results showed a significantly higher incidence of neural degeneration and fibrosis in the CRT group. Since neural degeneration was mild after FOLFOX chemotherapy, preoperative radiotherapy seems to be the cause of the tissue degeneration. However, we also note that oxaliplatin is generally known to cause nerve damage. Acute neurotoxicity caused by oxaliplatin appears to be related with hyperexcitability of the peripheral nerves, which has been attributed to channelopathy. As a result, the channelopathy leads to disorder of nerve iron channel, which is characterized by an increase or decrease in excitability of a neuron. This damage has not been manifested as a pathological change by light microscope. Moreover, acute changes in axonal excitability seem to become less pronounced in later treatment cycles, possibly because chronic nerve dysfunction and sensory loss mask the acute effects at higher cumulative doses. Characteristically, this channelopathy is a cumulative, sensory, symmetric distal axonal neuropathy without motor involvement [29–32].

The tissue and nerves were evaluated in surgical tissue specimens. These specimens and the left internal and external sphincter muscles were similarly affected by CRT, which suggests that the histological changes in these specimens were present widely in the body. The nerve examined in the study is an autonomic nerve that is distributed longitudinally

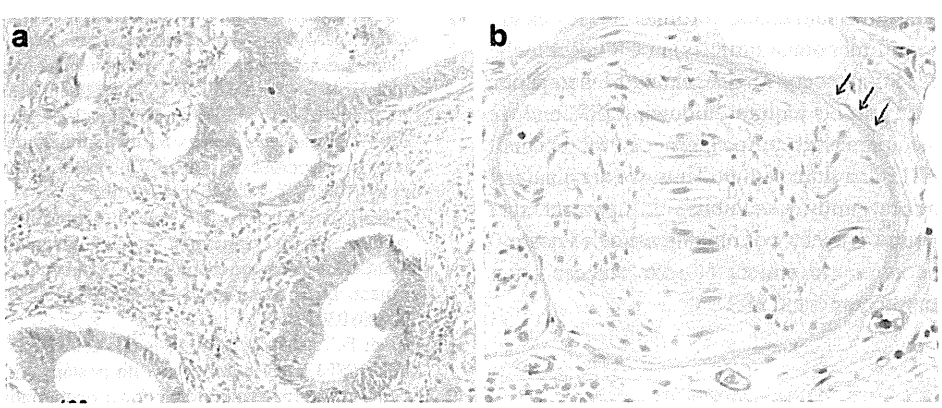

Fig. 2 Pathological evaluation of preoperative FOLFOX. **a** A case of histological response grade 1a. The tumor was reduced and a cavity was created between the gland duct structure of tumor tissues and surrounding interstitial structure. Mild infiltration of inflammatory cells was observed in the interstitium, but no fibrotic tissues were observed. **b** In evaluation of neural degeneration, a thickened perineuria was observed in a case of adventitial neuron change, Grade 1 (indicated by an *arrow*), but the neuronal changes did not reach the inside of nerves