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Effects of Irradiation Combined with Cis-diamminedichloroplatinum (CDDP) Suppository in Rabbit VX2 Rectal Tumors

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Abstract. **To decrease local recurrence and increase disease free survival, various preoperative therapies for patients with advanced rectal cancer have been studied. Cis-diamminedichloroplatinum (II) (CDDP) has become one of the most widely used cancer chemotherapeutic drugs. It has also been found to have radiosensitizing properties. In this experimental study, the efficacy of chemoradiotherapy using a novel CDDP suppository, and one with mixed micelles, was examined in a rabbit VX2 rectal tumor model. Rabbits were divided into four groups: control group, irradiation (R) group, CDDP suppository plus irradiation (CR) group, and mixed micelles plus CDDP suppository plus irradiation (CMR) group. Tumor growth ratios were reduced significantly in the CR and CMR groups as compared with the ratio in the control group. Microscopically, response rates of main tumors were 0%, 33.3%, 70.0%, and 91.7%, respectively. The number of metastatic lymph nodes in the CR and CMR groups decreased significantly compared to the control group and the R group. The microscopic response rates of metastatic lymph nodes were 0%, 11.1%, 40.0%, and 41.7%, respectively. Lung metastases were observed in three rabbits in the R group, and in one rabbit in the CMR group. Tissue platinum concentrations both in tumors and in regional lymph nodes increased significantly when mixed micelles were used. Chemoradiotherapy using the CDDP suppository and mixed micelles was effective for local control in the rabbit VX2 rectal tumor model.**

Preoperative chemoradiotherapy for the treatment of primary rectal cancers has been regarded as an effective adjuvant to surgery [1–8]. It is thought to reduce local recurrence, increase the feasibility of sphincter-preserving surgery, and improve survival. Autonomic nerve-sparing surgery combined with preoperative chemoradiotherapy has been provided for advanced rectal cancer patients, for the purpose of reducing local recurrence and preserving genitourinary function [9]. The widely used drugs in preoperative chemoradiotherapy were 5-fluorouracil, leucovorin, cisplatin, oxaliplatin, folinic acid, and tegafur suppository [1–9]. The purpose of this experimental study was to investigate the effects of chemoradiotherapy, using a novel cis-diamminedichloroplatinum II (CDDP), or cisplatin suppository and mixed micelles in rabbit VX2 rectal tumors.

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Materials and Methods

VX2 Tumor Inoculation into Rabbit Rectum

Female Japanese white rabbits, weighing approximately 3 kg each, were anesthetized with 25 mg/kg pentobarbital via the auricular vein. VX2 tumor cells (Funabashi Farm, Chiba, Japan) were maintained subcutaneously in the intact rabbits. Before inoculation, tumor tissue specimens were collected aseptically, and single cell suspensions were prepared in saline. VX2 cells (5×10^6 cells in 0.5 ml of saline) were inoculated into the posterior rectal wall, 1.5 cm from the anal verge, with a 27-gauge needle. Two weeks after inoculation, the tumors grew to an average diameter of 20 mm, with a small amount of erosion on the top. All animals received humane care, in compliance with the guidelines in "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and the *Guide to the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, (U.S.) National Research Council, and published by the National Academy Press, revised in 1996.

Preparation of CDDP Suppositories

The CDDP powder (100 mg, Nippon Kayaku Co. Tokyo, Japan) was mixed with a base (a mixture of polyethyleneglycol 500 (15 g) and polyethyleneglycol 4000 (15 g)), and melted at 70°C. The melted mixture were poured into molds and cooled to 10°C to form rod suppositories 20 mm in length and 8 mm in diameter. The CDDP content in each suppository was 5 mg [10, 11].

Preparation of Mixed Micelles

For the enhancement of the intestinal absorption of CDDP, mixed micelles were administered with CDDP suppositories. Mixed micelles were composed of 1 M linolenic acid, 1 M sodium taurocholate (NaTC), and distilled water (1000 ml). These mixtures were subjected to ultrasonic waves for 4 minutes at 0°C.

Table 1. Changes in tumor diameters.

Group	14 day (mm)	22 day (mm)	Growth rate (%)
Control (n = 13)	19.4 ± 3.6	31.2 ± 8.3	61.4 ± 33.9
R (n = 9)	21.1 ± 3.2	28.9 ± 8.6	36.8 ± 36.1
CR (n = 10)	19.9 ± 3.1	22.1 ± 7.3	10.9 ± 30.3
CMR (n = 12)	18.9 ± 1.8	18.5 ± 4.9	−1.7 ± 25.4

Data are expressed as mean S.D.
*: $p = 0.002$.
†: $p = 0.001$.
‡: $p < 0.0001$.
R: irradiated group; CR: CDDP Suppositories plus irradiation group; CMR: CDDP Suppositorius plus micelles plus irradiation group.

Irradiation

An x-ray unit (Varian Nelac 1018, CA) was operated at 10 million V, delivering radiation to the tumor at a dose rate of 500 cGy/min. Anesthetized rabbits were placed in a supine position, and the entire pelvis was irradiated from one portal (12 cm × 10 cm). The total dose was 6 Gy (3 Gy/fraction, 2 times). No rabbit died after irradiation.

Experimental Design

The rabbits were divided into four groups as described below. In every group except the control group, rabbits were irradiated twice, 14 and 19 days after inoculation of VX2 tumor. No other treatment was provided in the control group (n = 13). Only irradiation (3 Gy/fraction × 2) was provided in the R group (n = 9). In the CR group (n = 10), CDDP suppositories were administered (1 rod/fraction), followed by irradiation 1 hour later. In the CMR group (n = 12), CDDP suppositories were administered just after enemas of 3 ml of mixed micelles each, followed by irradiation 1 hour later.

In every group, the anus was sutured after treatment, and opened 6 hours later. In each group, rabbits were killed by rapid intravenous administration of pentobarbital 22 days after inoculation, and the rectal tumor, regional lymph nodes, liver, lungs, and kidneys were immediately removed and fixed by 10% formalin. Lungs and livers were cut into 5-mm slices. Tissues were embedded in paraffin, sliced 3 μm thick, then stained with hematoxylin and eosin. They were then examined histologically. Body weight and tumor diameter were measured 14 and 22 days after inoculation. Because rectal tumors were palpable from the surface of the skin, tumor diameter was measured together with the skin with calipers. The thickness of the skin was assumed to be same in all rabbits. Platinum concentrations in tumors and regional lymph nodes were measured by atomic absorption spectrometry.

Platinum Plasma Concentration Curves

A CDDP suppository alone, a CDDP suppository and mixed micelles, and an intravenous CDDP (5 mg) solution were administered once each, and platinum plasma concentrations were measured in each group 15, 30, 60, and 90 minutes, and 2, 4, 6, and 24 hours after administration (n = 3, each group).

Statistical Analysis

Statistical analysis was carried out by means of Student's t-test, the Mann-Whitney U-test, and Fisher's exact probability test. All p values < 0.05 were considered significant. All group data were presented as mean standard deviation (S.D.).

Results

Body Weight Change

On day 14, the body weights of rabbits in each group were about 3 kg. Body weight changes from day 14 to day 22 were −0.10 kg in the control group, −0.06 kg in the R group, −0.14 kg in the CR group, and −0.14 kg in the CMR group. Body weight changes were not significantly different between groups.

Changes in Tumor Diameter

Table 1 shows tumor diameter changes among rabbits. On day 14, tumor diameters were about 20 mm in all groups. Tumor growth ratios were reduced significantly in the CR and CMR groups as compared with the ratio in the control group. When compared with the ratio in the R group, the ratio was not reduced significantly in the CR group, but the ratio was reduced significantly in the CMR group (Table 1, Fig. 1).

Microscopic Changes in Tumors

Microscopic changes in tumors were evaluated on maximum cut surfaces. Central necrosis of the tumor, which was observed in all tumors in the control group, was excluded. Histological effects were evaluated according to the Japanese classification of colorectal carcinoma in 5 grades, from grade 0 to grade 3 (Table 2) [12]. Effective response was defined when grade 1b, 2, or 3 changes were observed. Response rates were 0% in the control group, 33.3% in the R group, 70.0% in the CR group, and 91.7% in the CMR group (Table 3, Fig. 2).

Regional Lymph Node Metastasis

There was no significant difference between the groups as to the total number of regional lymph nodes recorded. The number of metastatic lymph nodes was not significantly different between the control group and the R group. The number of metastatic lymph nodes in the CR and CMR groups was significantly lower compared to the control group and the R group. The same result was observed with regard to the metastatic rate, a ratio of the number of metastatic lymph nodes to the number of total lymph nodes (Table 4).

Microscopic Changes in Metastatic Lymph Nodes

Microscopic changes in metastatic lymph nodes were evaluated in much the same way as the main tumor. The response rates

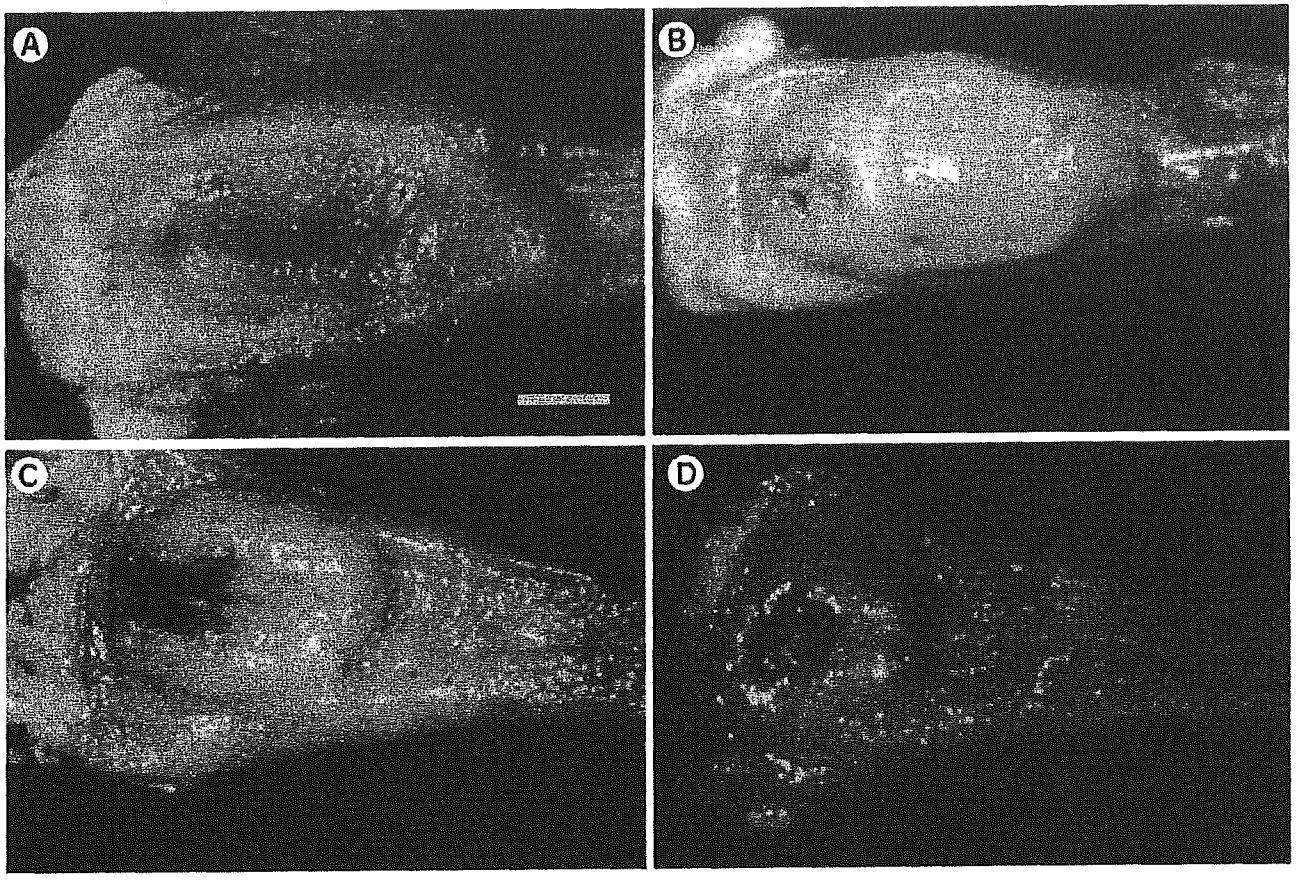

Fig. 1. Macroscopic findings of VX2 rectal tumors 22 days after inoculation. **A**. control group, **B**. R (irradiation) group, **C**. CR (CDDP + irradiation) group, **D**. CMR (CDDP + mixed micelles + irradiation) group. Bar: 1 cm; CDDP: cis-diamminedichloro platinum.

Table 2. Histological criteria.

Grade 0	No change Neither necrosis nor cellular or structural change can be seen throughout the lesion.
Grade 1	Mild change
1a	Necrosis or disappearance of the tumor is present in less than 1/3 of the lesion, or only cellular or structural changes are visible in variable amounts.
1b	Necrosis or disappearance of the tumor is present in less than 2/3 of the lesion.
Grade 2	Moderate change Necrosis or disappearance of the tumor is present in more than 2/3 of the lesion, but viable tumor cells still remain.
Grade 3	Severe change The whole lesion falls into necrosis and/or is replaced by fibrosis, with or without granulomatous changes. No viable tumor cells are observed.

were 0% in the control group, 11.1% in the R group, 40.0% in the CR group, and 41.7% in the CMR group. Response rates of metastatic lymph nodes in each group were lower than response rates in the main tumor. No metastatic lymph nodes were observed in three rabbits each in the CR and CMR groups (Table 5).

Lung and Liver Metastases

No lung metastases were observed in the control group or the CR group. Lung metastases were observed in three rabbits in the R group, and one rabbit in the CMR group. In every case, they were multiple metastases to the bilateral lung (Fig. 3). Lung metastatic rates were higher in the R group than in the control and CR groups, with marginal statistical significance. No liver metastases were found in any of the groups (Table 6).

Platinum Tissue Concentration

Platinum tissue concentrations increased significantly in both tumors and regional lymph nodes when mixed micelles were used (Fig. 4).

Platinum Plasma Concentration Curves

Platinum plasma concentrations in the mixed micelles group rose up to 10 times higher than in suppository group 30 minutes after administration, and maintained high levels. In the intravenous group, plasma concentrations rose rapidly to their maximum levels 15 minutes after administration of CDDP, and fell more rapidly thereafter than in the other two groups (Fig. 5).

Table 3. Microscopic changes in main tumor.

Group	Grade 0	Grade 1a	Grade 1b	Grade 2	Grade 3	Response rate[a] (%)
Control (n = 13)	13	0	0	0	0	0
R (n = 19)	0	6	3	0	0	33.3
CR (n = 10)	0	3	5	2	0	70.0
CMR (n = 12)	0	1	4	7	0	91.7

[a]Mann-Whitney U-test.
*$p < 0.001$.
†$p = 0.06$.
**$p = 0.08$.
‡$p = 0.002$.

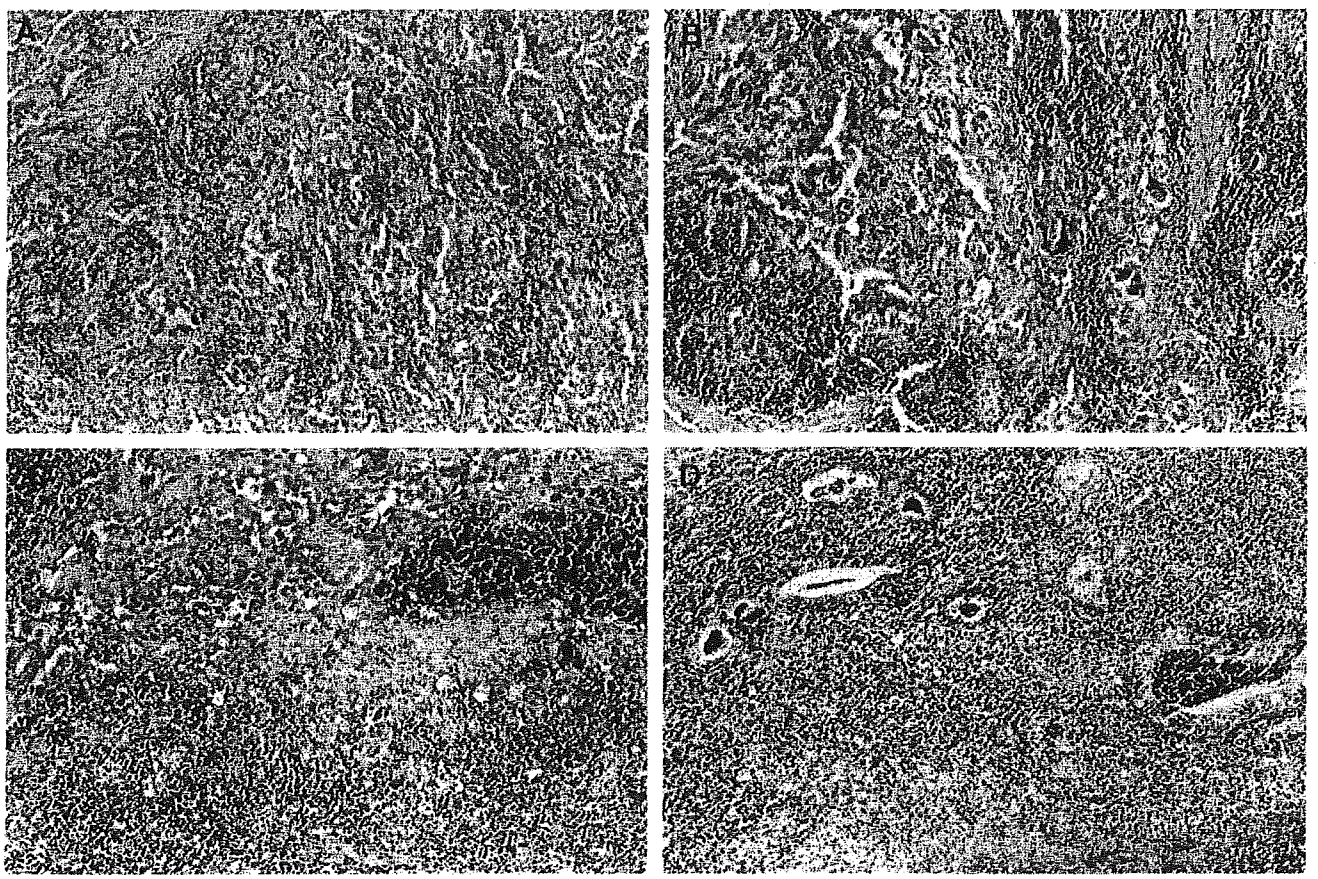

Fig. 2. Microscopic findings of VX2 tumors. **A.** control group (histological effect: grade 0), **B.** R group (grade 1a), **C.** CR group (grade 1b), **D.** CMR group (grade 2). Hematoxylin and eosin, × 100.

Discussion

Various preoperative chemoradiotherapies have been studied for the purpose of decreasing local recurrence and increasing disease-free survival in cases of advanced rectal carcinoma [1–9]. In this study, we investigated the efficacy of chemoradiotherapy, using cis-diamminedichloroplatinum II (CDDP) suppositories in rabbit VX2 rectal tumors. Two weeks after inoculation, VX2 tumors grew to a diameter of about 20 mm, with superficial ulceration. This model is thought to be useful for the study of regional cancer therapy [13].

Cisplatin or CDDP, is a potent cytotoxic agent for use against a wide range of tumors, and it has become one of the most

Table 4. Regional lymph node metastasis.

Group	Number of lymphnodes	Number of metastatic lymphnodes	Metastatic rate (%)
Control (n = 13)	5.8 ± 2.1	4.8 ± 2.1	81.5 ± 20.0
R (n = 9)	6.4 ± 1.5	4.7 ± 1.7	72.8 ± 24.4
CR (n = 10)	5.2 ± 2.0	1.9 ± 1.9	32.4 ± 32.9
CMR (n = 12)	5.6 ± 1.4	1.4 ± 1.3	23.2 ± 18.5

Data are expressed as mean S.D.
*$p < 0.001$.
†$p = 0.004$.
‡$p = 0.008$.
**$p = 0.91$.
††$p = 0.44$.

Table 5. Microscopic changes in lymph node metastasis.

Group	Grade 0	Grade 1a	Grade 1b	Grade 2	Grade 3	Meta (-)	Response rate[a] (%)
Control (n = 13)	13	0	0	0	0	0	0
R (n = 9)	1	7	0	1	0	0	11.1
CR (n = 10)	0	3	4	0	0	3	57.1
CMR (n = 12)	0	4	3	2	0	3	55.6

[a]Mann-Whitney U-test.
*$p = 0.06$.
†$p = 0.69$.
**$p = 0.09$.
‡$p = 0.07$.

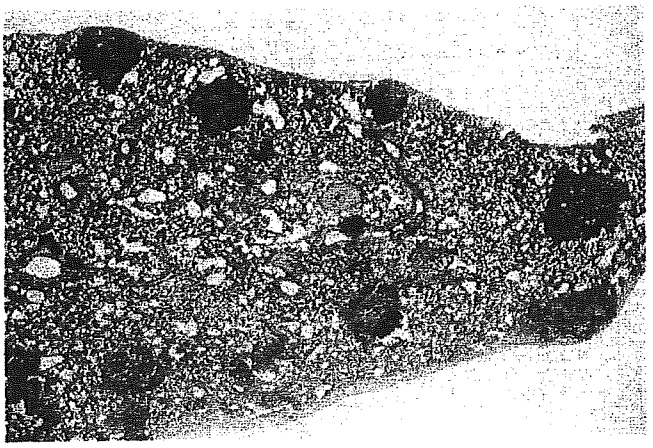

Fig. 3. Microscopic findings of multiple metastases in lung (R group). Hematoxylin and eosin, ×10.

Table 6. Metastasis to lung and liver.

Group	Lung[a]	Liver
Control (n = 13)	0	0
R (n = 9)	3	0
CR (n = 10)	0	0
CMR (n = 12)	1	0

[a] Fisher's exact probability test.
*$p = 0.06$.
†$p = 0.09$.

widely used cancer chemotherapeutic drugs. It was also found to have radiosensitizing properties [14–16]. There are several mechanisms that could contribute to the production of this kind of supra-additivity: (1) inhibition of repair of sublethal radiation damage, (2) radiosensitization of hypoxic cells, and (3) tumor reoxygenation subsequent to CDDP administration [17]. In addition, CDDP causes a greater-than-additive antitumor effect when administered a short time before a single dose of radiation [17].

Drug-delivery methods designed to increase the concentration of drug relative to the surrounding tissues may allow CDDP to function effectively as a radiosensitizing agent [18] . In this series,

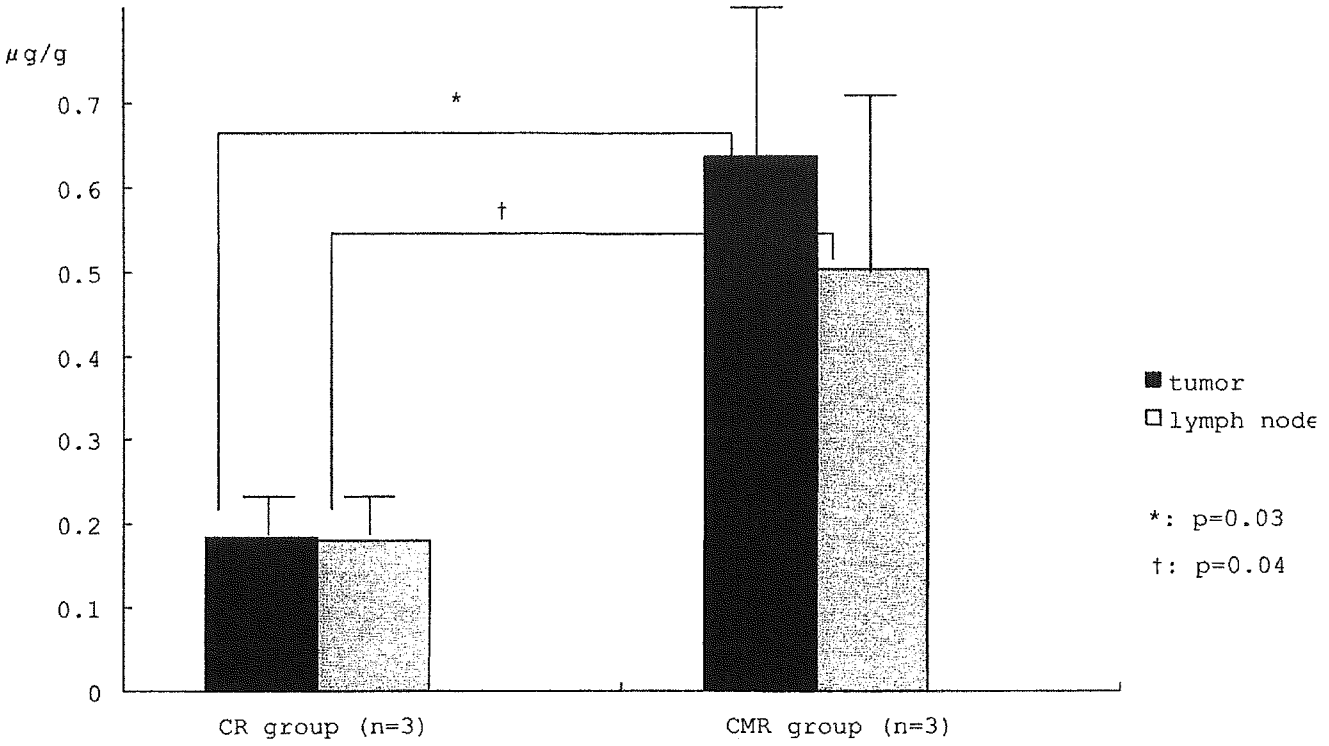

Fig. 4. Platinum tissue concentration.

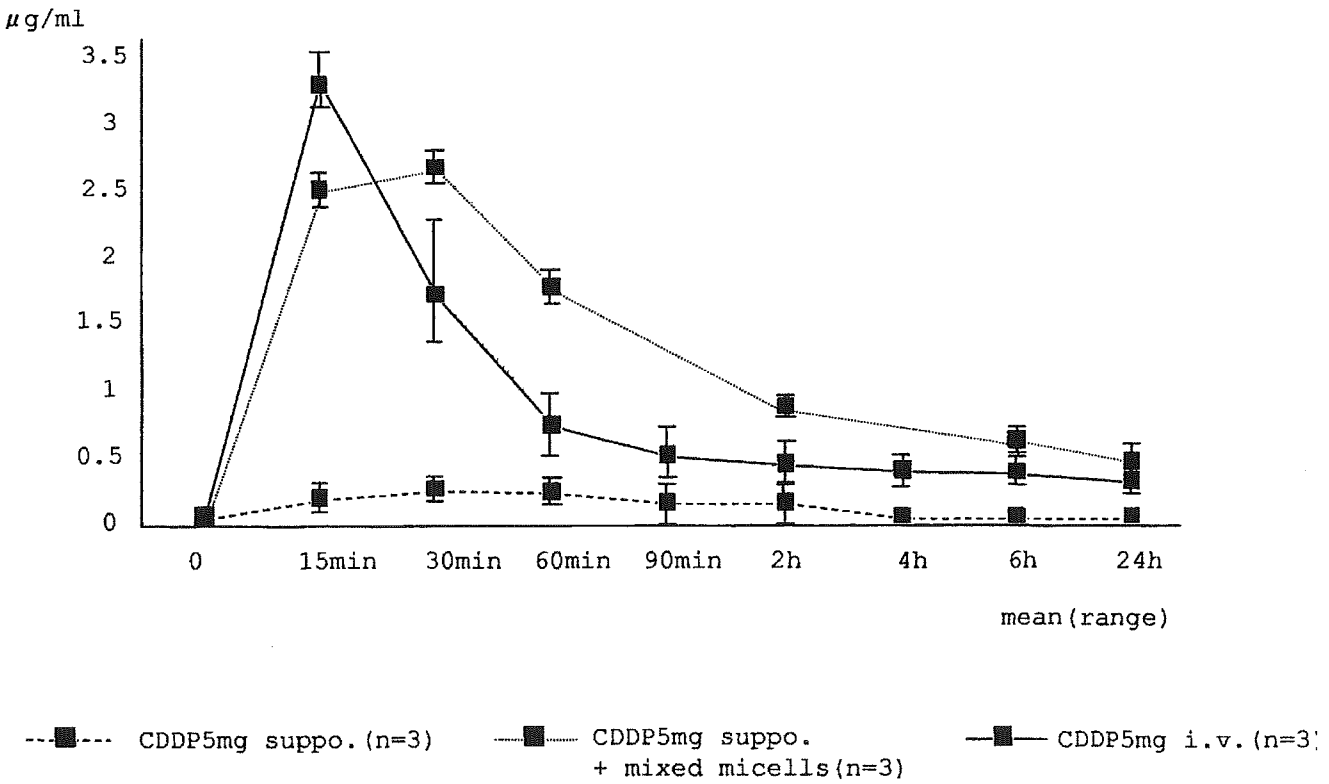

Fig. 5. Platinum plasma concentration curves.

we studied both the efficacy of chemoradiotherapy using CDDP suppositories, and the efficacy of adding mixed micelles as a means of increasing CDDP concentration in tumors. It may be speculated that the mechanism for inducing intestinal absorption of CDDP by mixed micelles is as follows [19–21]: The micellar state may facilitate incorporation of the lipid component of mixed micelles into the mucosal membrane. The incorporated lipid interacts with the polar region of the membrane phospholipids and enhances the fluidity and permeability of the mucous membrane. Consequently, CDDP can cross the mucosal membrane easily. In our study, CDDP concentrations in both tumors and regional lymph nodes were significantly higher in the CMR group than in the CR group. These results indicate that mixed micelles had increased CDDP concentrations in tumors and in lymph nodes.

The tumor growth rate was significantly lower in the CR and CMR groups than in the control group. As regards microscopic changes in the main tumors, response rates were higher in the CR and CMR groups than in the R group. Local control of the main tumor seemed to be better in the CR group than in the R group, and it was better in the CMR group to an even greater degree. It was not possible, however, to differentiate the simple additive effect from the synergistic effect.

As to the therapeutic effect on regional lymph nodes, the number of metastatic lymph nodes and the metastatic rates were significantly lower in the CR group than in the R group. Response rates of microscopic changes in metastatic lymph nodes were higher in the CR and CMR groups than in the R group. Although response rates of metastatic lymph nodes were lower than those of main tumors in all groups, chemoradiotherapy was also effective for regional lymph nodes.

Lung metastases were observed in three rabbits in the R group, and one rabbit in the CMR group. Luna-Pérez et al. reported that the presence of metastatic lymph nodes in the postirradiated specimen was an ominous prognostic factor for survival, and that such patients should be considered for adjuvant chemotherapy [22]. Qian et al. suggested that radiation might promote hepatocyte growth factor (HGF)-induced malignant biological behaviors of certain pancreatic cells through the upregulated HGF/c-Met signal pathway [23]. Koda et al. reported that some distant metastases were observed in the preoperative chemoradiotherapy group, and that this was why adjuvant chemoradiotherapy did not necessarily improve overall survival for rectal cancers, even when the local recurrence rate was reduced [24]. Moreover, Koda et al. insisted that neoadjuvant chemoradiotherapy impaired natural killer cell activity in selected patients [25]. These results indicate the necessity for concurrent chemotherapy and/or immunotherapy while performing preoperative radiotherapy.

In conclusion, chemoradiotherapy using CDDP suppository and mixed micelles is effective for local control in rabbit VX2 rectal tumor model.

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Diseases of the Colon & Rectum

Denervation of the Neorectum as a Potential Cause of Defecatory Disorder Following Low Anterior Resection for Rectal Cancer

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PURPOSE: The aim of this study was to determine whether denervation of the sigmoid colon during low anterior resection contributes to the postoperative motility characteristics of the neorectum and to the defecatory function of patients. METHODS: Sixty-seven patients who underwent either low or ultralow anterior resection for rectal cancer were evaluated. In accordance with the length of denervated neorectum, each patient was assigned to either the short-denervation or long-denervation group, determined by whether the inferior mesenteric artery was divided. Colonic propagated contraction was then measured by means of intraluminal pressure monitoring. Transit time was calculated with orally administered radiopaque markers. RESULTS: Propagated contraction down to the neorectum was significantly less common in the long-denervation group (14/36) than in the short group (12/15, $P < 0.05$), whereas spastic minor contraction at the neorectum was significantly more common in the long-denervation group (21/36) than the in short group (3/15, $P < 0.05$). Colonic transit time below the sigmoid colon was significantly longer in long group (6.4 hours) than in the short group (3.4 hours, $P < 0.01$). Although motility disorder of the neorectum was correlated with clinical defecatory malfunctions, including multiple evacuations, urgency, and soiling, no significant correlation was noted between the length of the denervated neorectum and the defecatory disorders. CONCLUSIONS: Motility of the neorectum following low anterior resection appears degraded by intraoperative maneuvers that cause denervation of the remnant sigmoid colon. Motility disorder of the neorectum, but not the length of the denervated neorectum causing the disorder, correlates well with several defecatory malfunctions. This finding suggests that postoperative defecatory disorder as a result of low anterior resection is caused by many factors in addition to denervation of the neorectum. [Key words: Rectal cancer; Low anterior resection; Defecatory function; Neorectum; Denervation; Motility disorder]

During standard low anterior resection (LAR) for rectal cancer, most of the rectum and some distal part of the sigmoid colon are removed along with accompanying vessels and nerve supplies during lymph node dissection. Because the healthy rectum and sigmoid colon are double innervated with ascending fibers from the pelvic plexus and descending fibers that run along the internal mesenteric artery (IMA),[1] the surgery-related maneuvers result in a denervated colonic segment of a length that varies from case to case (Fig. 1).

When the IMA is transected at its origin during removal of the specimen, most of the remaining sigmoid colon and the descending colon become a denervated colonic segment when the ascending fibers from the pelvic plexus and the descending fibers around the IMA are transected. In some cases, the

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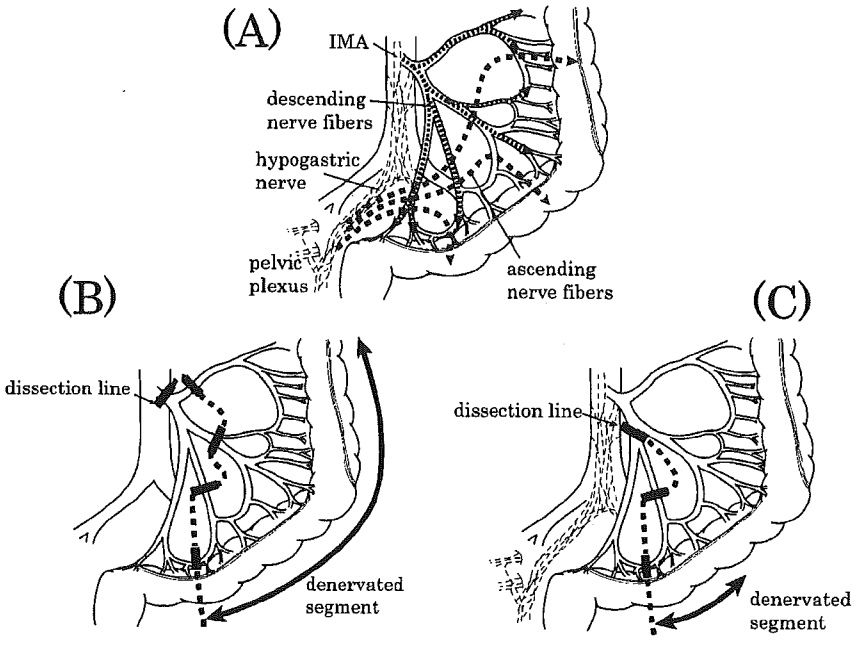

Figure 1. A. Innervation of healthy rectum and sigmoid colon. Both are double innervated with both ascending nerve fibers from the pelvic plexus and descending nerve fibers which run along the internal mesenteric artery (IMA). B. Long denervation of the neorectum when the IMA is transacted at its origin during removal of the specimen. C. Short denervation of the neorectum when the IMA and some sigmoid colon arteries are preserved together with the descending nerve fibers.

denervated colonic segment may be short if the IMA and some sigmoid colon arteries have been preserved along with the descending nerve fibers. However, the ascending nerve fibers originating from the pelvic plexus are always compromised (Figs. 1B, 1C). In most cases, the neorectum is reconstructed with the denervated sigmoid colon segment, thus the functionality of the neorectum may contribute to the patient's defecatory status following LAR for rectal cancer.

In the present study, we evaluated the correlation between motility characteristics and the length of the denervated neorectum to determine whether functional disorders of the neorectum contribute to postoperative defecatory status. Possible surgical maneuvers for LAR that preserve postoperative defecatory function are also discussed.

PATIENTS AND METHODS

Patients

Sixty-seven patients (35 men and 32 women; age range, 40–81 years; median age, 63 years) who underwent either LAR (n = 45) or ultralow anterior resection (n = 22) for middle or lower rectal cancer were enrolled in the present study. Informed consent was obtained from all patients. A colonic J-pouch of 6 to 7 cm in size was constructed with a side-to-side colocolic anastomosis by use of a linear stapler in 11 patients; 4 of these patients underwent pouch-anal anastomosis *via* the transanal handsewn method and the remaining 7 patients underwent a mechanical anastomosis with a circular stapler. A side-to-end anastomosis with a short stump was performed with a stapler in 18 cases and a straight end-to-end anastomosis by means of the double-stapling technique was performed in 38 cases. In this series, 11 patients received preoperative radiation therapy. The interval between initial surgery and postoperative physiologic study was one year in 31 cases (46 percent), two years in 10 cases (15 percent), and more than three years in 26 cases (39 percent) (Table 1).

Evaluation of Defecatory Dysfunction

Within one month before or after each examination, patients were interviewed with a self-administered questionnaire about their recent defecatory status. The patients were then categorized into groups according to the degree of defecatory dysfunction. The questionnaire consisted of 20 questions about bowel movement frequency, degree of urgency, multiple evacuations, and fecal soiling. Patients were first divided into three groups as follows: "severe urgency," in which a patient always or often could not defer defecation for ten minutes; "mild urgency," in which a patient only sometimes experienced such urgency; and "no urgency," in which a patient was always able to defer defecation for ten minutes. Next patients were divided into two groups

Table 1.
Patient Background and Length of Denervated Neorectum

Factor	Length of Denervated Neorectum		
	Short (n = 17)	Long (n = 50)	*P* Value[a]
Gender			<0.05
Male	6	33	
Female	11	17	
Period from operation			<0.01
1 year	15	16	
2 years	0	10	
>3 years	2	24	
Operation method			NS
Low anterior resection	9	36	
Ultralow anterior resection	8	14	
Reconstruction			NS
End-to-end	4	34	
Side-to-end	10	8	
J-pouch	3	8	
Preoperative radiation			NS
Yes	1	10	
No	16	40	

NS = not significant.
[a]Chi-squared test.

on the basis of evacuation frequency as follows: "multiple evacuations," in which a patient needed to use the toilet more than three times an hour for completion of evacuation, and "negative multiple evacuations," in which a patient was able to evacuate each time satisfactorily with one or two toilet visits. Fecal soiling was categorized on the basis of previously reported criteria, which take into account the degree and frequency of incontinence,[2] as follows: "soiling," in which a patient experienced fecal incontinence with liquid stool more than once a month, and "negative soiling," in which a patient rarely or never experienced incontinence. In addition, patients also categorized their own defecatory status as excellent, good, fair, poor, or totally unsatisfactory.

Monitoring of Contraction Waves

A 3-mm-wide pressure transducer that consisted of four sensors 20 cm apart was specially manufactured. At the time of postoperative follow-up colonoscopy performed on patients who underwent low or ultralow anterior resection, the tip of the catheter was introduced up to the cecum. Following completion of the routine examination, the position of the catheter was adjusted under x-ray so that the fourth sensor was located inside the neorectum, which was approximately 10 cm from the anal edge. In most cases, the first sensor was located at the transverse colon, the second around the descending colon close to the splenic flexure, and the third around the sigmoid colon–descending colon junction. The catheter was then connected to a pressure amplifier (Nihon Koden Inc., Tokyo, Japan) and contractions of the colon were recorded as variations in intraluminal pressure. Patients were then asked to lie quietly for 30 to 60 minutes to monitor colonic motility. Waves with high amplitude (≥20 mmHg) of more than ten seconds in duration were regarded as contraction waves. Contraction waves that originated at sensor 1 or 2 and propagated accordingly down to the neorectum (sensor 4) were called "propagated waves." When there was at least one propagated wave seen during the examination period, the patient was allocated to the positive propagated-wave group. The monitoring of contractions was successfully performed in 51 patients from whom consent was obtained.

Calculation of Transit Time

Colonic transit time was determined in 48 patients by means of Sitzmarks capsules (Konsyl Pharmaceuticals Inc., Fort Worth, TX), which consist of 20 radiopaque markers within a gelatin capsule. On three consecutive days, a Sitzmarks capsule was taken with water after dinner. On Day 4, two flat-plate abdominal x-rays were taken of the patient in the supine and upright positions. From one day before to the day of completion of the examination, patients were asked not to take laxatives or other medicines that might affect intestinal motility. Segmental colonic transit time was calculated with the computerized Sitzmarks Analysis Program (Kaigen Inc., Osaka, Japan), which is based on Arhan *et al.*'s theory.[3] In this theory, the number of markers that pass through one intestinal point exhibits a bell-shaped distribution curve. By measuring the total time for all radiopaque markers to pass each colonic segment, the mean passage time in the corresponding colonic segment was calculated. The transit times of the ascending colon, transverse colon, descending colon, and neosigmoid colon and neorectum were calculated.

Statistical Analysis

Continuous variables were analyzed with the Student's *t*-test. Categoric variables were analyzed with the chi-squared test. Ordered categories were ana-

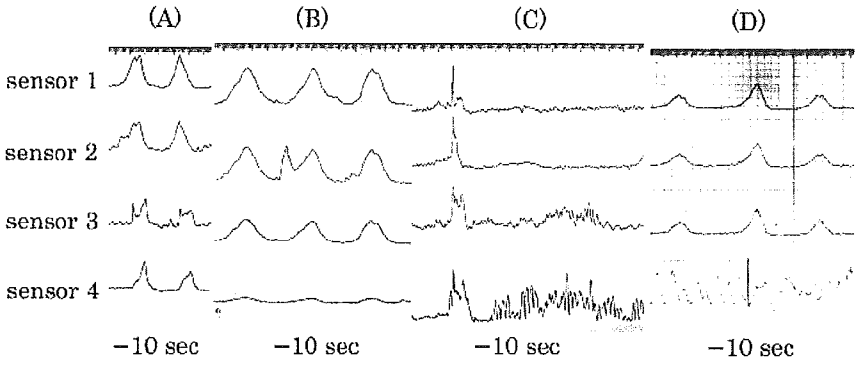

Figure 2. Pattern of contraction waves seen in patients who underwent low or ultralow anterior resection for rectal cancer. A. Typical propagated contraction waves down to the neorectum (sensor 4, n = 17). B. Vanished propagated waves at the neorectum (n = 9). C. Propagated waves followed by minor spastic waves (n = 9). D. Minor spastic waves without any propagated waves (n = 16).

lyzed with Wilcoxon's signed-rank test. All statistical calculations were performed by means of the SPSS® program (SPSS, Inc., Chicago, IL) under the direction of a statistician. A P value of <0.5 was considered to indicate statistical significance.

RESULTS

Length of Denervated Neorectum

Of the 67 patients, the IMA was divided at its origin in 35 patients with advanced-stage tumor and N2 (more than 3) lymph node metastases suspected either preoperatively or intraoperatively. In another 15 patients with N1 (1 to 3) lymph node metastases suggested, the IMA was preserved but the surrounding tissue was removed for lymph node dissection. In 50 patients, the descending nerve fibers that otherwise would have serviced the neorectum were compromised during lymph node dissection. Thus, the neorectums of these 50 patients were composed of long denervated segments of sigmoid colon (the long-denervation group; Fig. 1B). In contrast, in the remaining 17 patients without any lymph node metastases diagnosed preoperatively or intraoperatively, the superior rectal artery was divided at its origin, thereby preserving the descending nerve fibers that service the neorectum. The descending nerve fibers along with the IMA and several sigmoid colon arteries were preserved in these patients (the short-denervation group; Fig. 1C). Table 1 summarizes patients' characteristics based on the theoretical length of the denervated neorectum. Male patients who had received the operation more than three years ago tended to have a long denervated neorectum.

Pattern of Colonic Motility After Low Anterior Resection

Of the 67 patients, colonic motility was measured in 51 cases by a pressure transducer. Figure 2 summarizes the pattern of colonic contractions following LAR. In this examination, we focused on two major findings: existence of strong propagated contractions down to the neorectum (sensor 4), and existence of spastic minor waves in the neorectum (sensor 4; Fig. 2). Contraction waves were categorized and detected as follows: Type A, strong contraction waves that propagated down to the neorectum without any spastic waves, found in 17 patients; Type B, strong contractions that diminished at the neorectum and were not associated with spastic waves, found in 9 patients; Type C, strong contractions accompanied by spastic-waves at the neorectum, found in 9 patients; and Type D, spastic waves only irregularly seen at sensor 4 without any propagated waves, found in16 patients.

Segmental Colonic Transit Time and Colonic Motility

The mean segmental colonic transit time in relation to propagated contraction down to the neorectum is summarized in Table 2. The transit time at the neosigmoid colon or neorectum was longer (with marginal statistical significance) in patients who did not have propagated contraction waves down to the neorectum (Type B or Type D) than in patients who had propagated contractions (Type A or Type C). No other difference in transit time was observed at any other colonic segment among the various motility patterns; spastic waves did not correlate with segmental colonic transit time (data not shown).

Table 2.
Mean Segmental Colonic Transit Time in Relation to Propagated Contraction Waves

Colonic Segment	Mean Transit Time (hours)		
	Positive Propagated Contractions (n = 14)	No Propagated Contractions (n = 18)	*P* Value[a]
Ascending colon	12.9 ± 9.6	13.1 ± 5.4	NS
Transverse colon	8.7 ± 4.2	8.6 ± 5.9	NS
Descending colon	4.3 ± 3.5	5.9 ± 4.6	NS
Sigmoid colon/neorectum	3.7 ± 2.1	6.5 ± 5.0	0.06
Total	29.7 ± 8.8	34.5 ± 10.8	NS

NS = not significant.
[a]Student's *t*-test.

Length of Denervated Neorectum and Colonic Motility

Monitoring of colonic contraction with a pressure transducer revealed that 12 of 15 patients (80 percent) who belonged to the short-denervation group had propagated contraction waves (Types A and C; Fig. 2), which was a significantly higher percentage than that seen in the long-denervation group (38.9 percent; $P < 0.01$; Table 3).

In contrast, 21 of 36 patients (58.3 percent) in the long-denervation group showed spastic waves at the neorectum (Types C and D; Fig. 2) irrespective of the existence of propagated contraction waves, which was a significantly higher percentage than that seen in the short-denervation group (20 percent; $P < 0.05$; Table 3).

The mean transit time in relation to length of the denervated neorectum is summarized in Table 4. The transit time at the neosigmoid colon or neorectum was greatly prolonged in patients with a long denervated neorectum. In contrast, the transit time at the transverse colon was only slightly prolonged in patients with a short denervated neorectum.

Motility of Neorectum and Defecatory Function

Table 5 summarizes the correlation between colonic contraction pattern and defecatory disorders. The loss of propagated contraction correlated well with urgency and slightly with multiple evacuations. There was no correlation seen between propagated contraction and soiling. By contrast, spastic minor contractions in the neorectum correlated significantly with several major defecatory disorders, specifically urgency, multiple evacuations, and major soiling ($P < 0.01$). The loss of propagation and occurrence of spastic minor contractions in the neorectum also correlated with patients' self-assessment of bowel function. The majority of patients who did not have a spastic neorectum (24/26) and most patients with propagated waves (23/26) replied that theirfunction was either satisfactory or fair.

Length of Denervated Neorectum and Defecatory Functions

Table 6 summarizes the correlation between length of the denervated neorectum and clinical defecatory disorders seen in patients. Patients with a long denervated neorectum tended to have multiple evacuations; however, the difference did not reach statistical significance.

DISCUSSION

Functional disorders in defecation following LAR for rectal cancer have often been discussed as postoperative sphincter malfunctions. Manometric studies have revealed that anal resting pressure and maximum squeeze pressure are both degraded following surgery,[4] with reduced anal sensation[5] or reduced physiologic rectoanal inhibitory reflex.[6] These sphincter malfunctions may be caused by direct injury during surgery[7] or be a result of denervation to the internal sphincter,[8] which is controlled by the hypogastric nerve.[9] In addition to postoperative malfunction of the sphincter, compliance of the neorectum constructed at the LAR is also reported to contribute to postoperative defecatory function,[10] although low compliance may recover with time, unlike damage to the anal sphincter.[11] Similarly, the volume[12] and diameter[13] of the neorectum have been reported to correlate with postoperative bowel functioning.

More recently, efforts have been made to evaluate

Table 3.
Length of Denervated Neorectum and Colonic Contraction Pattern

	Contraction Pattern of Neorectum			
	Pattern A	Pattern B	Pattern C	Pattern D
Long denervation (n = 36)	8	7	6	15
Short denervation (n = 15)	9	2	3	1

Table 4.
Mean Colonic Transit Time in Relation to Length of Denervated Neorectum

	Mean Transit Time (hours)		
Colonic Segment	Short-Denervation Group (n = 11)	Long-Denervation Group (n = 37)	*P* Value
Ascending colon	12.4 ± 6.7	13.4 ± 7.1	NS
Transverse colon	11.7 ± 5.3	8.6 ± 5.9	<0.05
Descending colon	4.9 ± 3.4	6.6 ± 5.3	NS
Sigmoid colon/ neorectum	3.4 ± 1.6	6.4 ± 5.4	<0.01
Total	30.1 ± 7.4	34.4 ± 11.1	NS

NS = not significant.

Table 5.
Colonic Contraction Pattern and Defecatory Disorders

	Propagated Contractions			Spastic Neorectum		
	Yes (n = 26)	No (n = 25)	*P* Value	Yes (n = 25)	No (n = 26)	*P* Value
1) Urgency			<0.01[a]			<0.01[a]
None	16	8		7	17	
Mild	9	12		12	9	
Severe	1	5		6	0	
2) Multiple evacuations			<0.05[b]			0.01[b]
Yes	8	16		17	7	
No	18	9		8	19	
3) Major soiling			NS[b]			<0.01[b]
More than sometimes	4	6		9	1	
Almost none	22	19		16	25	
4) Patients' self-assessment of defecation			<0.01[a]			<0.01[a]
Excellent	7	5		2	10	
Good	11	11		11	11	
Fair	5	2		4	3	
Poor	3	6		7	2	
Unsatisfied	0	1		1	0	

NS = not significant.
[a]Wilcoxon's signed-rank test.
[b]Chi-squared test.

the motility of the neorectum. Oya *et al.* reported that the time-activity curve of evacuation seen in cases of a neorectum closely correlated with postoperative bowel functions following anterior resection.[14] Seike *et al.* showed that patients who complain of evacuation difficulty postoperatively tend to have a high volume of left-sided colon gas, which suggests a motility disorder at the neosigmoid or neorectum.[15] In healthy individuals who have not undergone any surgery, rectal function has been reported to play a major role in fecal continence[16] and chronic constipation.[17]

In the present study, we evaluated the dynamic motility characteristics of the neorectum in conjunction with motility of the oral colonic segments to determine if collaborative movements of the postoperative residual colon correlate with evacuative malfunction following LAR. We found that the propagated contraction waves that originated from the up-

Table 6.
Length of Denervated Neorectum and Defecatory Disorders

	Length of Denervated Neorectum		
	Long (n = 50)	Short (n = 17)	*P* Value
1) Urgency			NS[a]
None	20	10	
Mild	21	7	
Severe	9	0	
2) Multiple evacuations			0.07[b]
Yes	27	5	
No	23	12	
3) Major soiling			NS[b]
More than sometimes	12	2	
Almost none	38	15	
4) Patients' self-assessment of defecation			NS[a]
Excellent	8	5	
Good	19	9	
Fair	11	2	
Poor	11	1	
Unsatisfied	1	0	

NS = not significant.
[a]Wilcoxon's signed-rank test.
[b]Chi-squared test.

per part of the colon disappeared at the neorectum in 25 of 51 patients (Fig. 1). The loss of propagation at the neosigmoid colon or neorectum seemingly correlated with the tardiness of transit in that part of the bowel. Clinically, most patients who showed a loss of propagating waves also experienced multiple evacuations and urgency, which suggests that their evacuation each time was incomplete because of a motility disorder of the neorectum. We would like to emphasize that the patients who showed loss of propagating waves predominantly had a long denervated neorectum, in which the IMA was either divided or its surrounding tissue was removed for lymph node dissection at the time of surgery (Table 3).

The other important finding regarding contraction pattern was the existence of spastic minor contractions at the neorectum. Although having a spastic neorectum did not correlate with segmental transit time, a spastic neorectum was very closely associated with clinical symptoms such as multiple evacuations, urgency, and major soiling. Importantly, spastic contraction was more commonly seen in patients with a long denervated neorectum (Table 3). This finding suggests that denervation of the neorectum during surgery may be a causative factor of the spastic neorectum and of the loss of contraction waves to the neorectum.

The long denervated neorectum, which is the result of dissection of the IMA or its surrounding tissue, may cause motility disorders in the neorectum constructed during LAR for rectal cancer. No significant correlation was noted between the length of the neorectum and the manifestation of defecatory disorders, which suggests that many other factors also contribute to postoperative defecatory function. Denervation to the neorectum may be one factor that indirectly relates to and exerts influence on postoperative function. Therefore, IMA and the surrounding nerve fibers should be preserved whenever feasible. Further study is warranted to elucidate the influence of IMA dissection on functional outcome for defecation. In addition, a time-course study is needed to investigate whether these motility disorders will recover with time, given that patients' clinical symptoms sometimes gradually improve.

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A New Method for Isolating Colonocytes From Naturally Evacuated Feces and Its Clinical Application to Colorectal Cancer Diagnosis

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Background & Aims: The early detection of colorectal cancer is desired because this cancer can be cured surgically if diagnosed early. The purpose of the present study was to determine the feasibility of a new methodology for isolating colonocytes from naturally evacuated feces, followed by cytology or molecular biology of the colonocytes to detect colorectal cancer originating from any part of the colorectum. *Methods:* Several simulation studies were conducted to establish the optimal methods for retrieving colonocytes from any portion of feces. Colonocytes exfoliated into feces, which had been retrieved from 116 patients with colorectal cancer and 83 healthy volunteers, were analyzed. Part of the exfoliated colonocytes was examined cytologically, whereas the remainder was subjected to DNA analysis. The extracted DNA was examined for mutations of the APC, K-*ras*, and p53 genes using direct sequence analysis and was also subjected to microsatellite instability (MSI) analysis. *Results:* In the DNA analysis, the overall sensitivity and specificity were 71% (82 of 116) of patients with colorectal cancer and 88% (73 of 83) of healthy volunteers. The sensitivity for Dukes A and B was 72% (44 of 61). Furthermore, the sensitivity for cancers on the right side of the colon was 57% (20 of 35). The detection rate for genetic alterations using our methodology was 86% (80 of 93) when the analysis was limited to cases in which genetic alterations were present in the cancer tissue. *Conclusions:* We have developed a new methodology for isolating colonocytes from feces. The present study describes a promising procedure for future clinical evaluations and the early detection of colorectal cancers, including right-side colon cancer.

Colorectal cancer is one of the most common malignancies worldwide. In Japan, colorectal cancer is the third and second leading cause of death from cancer in men and women, respectively.[1] However, colorectal cancer is curable by surgical resection if diagnosed at a sufficiently early stage. This incentive has prompted investigators to develop new methods enabling the early diagnosis of colorectal cancer and has led to the introduction of cancer screening programs in many countries. For mass cancer screenings, a simple, economic, and noninvasive method of cancer detection is desired. The Hemoccult test is currently used in many countries for this purpose.[2–6] However, this test is nonspecific and is not sufficiently sensitive to detect early stage colorectal cancer, although a higher sensitivity has been reported for advanced-stage colorectal cancer.[7] Radioimmunoassays using tumor markers, such as carcinoembryonic antigen, also are not suitable for the detection of early cancer, although such tests can be used to monitor patients for an increasing tumor burden or tumor recurrence. Diagnosis by barium enema study and fiberoptic colonoscopy is accurate but time-consuming, expensive, and invasive. Therefore, an urgent need exists to establish a sensitive, reliable, and noninvasive method for the detection of colorectal cancer at an early stage.

To date, several screening methods for colorectal cancer based on the detection of mutated DNA in feces have been reported.[8–20] These methods, however, are time-consuming and are not sufficiently sensitive. The major reason for this inaccuracy is the fact that

Abbreviations used in this paper: APC, adenomatous polyposis coli; MSI, microsatellite instability; OMIM, Online Mendelian Inheritance in Man.

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nucleic acids in feces are derived from an enormous number and variety of bacteria and normal cells. Accordingly, the proportion of genes derived from cancer cells in feces is as low as 1%, at most.[9] This makes the application of gene-detecting methods difficult in clinical practice.

We previously reported that the expression of CD44 variants in exfoliated colonocytes isolated from feces according to the Percoll centrifugation method could serve as a noninvasive diagnostic marker for early colorectal cancer.[21] However, the repetition of the Percoll centrifugation method was found to distort the morphology of the exfoliated colonocytes. Accordingly, the sensitivity of this method also appeared to be unsatisfactory because of the low retrieval rate of the exfoliated colonocytes. Another study described a processing method that involved scraping or washing the stool's surface with a buffer to collect exfoliated colonocytes.[22] In the ascending colon, however, the feces remains unformed. Therefore, most cancer cells exfoliated from the walls of the ascending colon would be incorporated into the inner core of the feces during the course of its formation. Thus, recovering cancer cells that originated from the ascending colon might be difficult using methods that involve scraping or washing solid feces.

Under these circumstances, we succeeded in developing a new, very effective methodology that allows the simple isolation of exfoliated colonocytes from not only the surface but also the central portion of feces while maintaining the colonocytes' initial morphology. Currently, we are attempting to apply a molecular biologic tool to purified colonocytes exfoliated into feces to detect cells from early colorectal cancers, including right-side colon cancer.

Materials and Methods

Study Design

This was a prospective study conducted between December 2002 and August 2004. The study protocol was reviewed and approved by the Institutional Review Board of the National Cancer Center, Japan. Written informed consent was obtained from all patients and healthy volunteers. No modifications to the protocol procedures were made during the course of the study.

Study Population

A total of 116 patients with histologically confirmed colorectal cancer and 83 healthy volunteers were enrolled. The healthy volunteers consisted of 37 men and 46 women with no apparent abnormalities, such as adenoma or carcinoma (including hyperplastic polyps), found during a total colonoscopy performed at the National Cancer Center Research Center for Cancer Prevention and Screening. The median age of these volunteers was 58.4 years (range, 40–70 years). The characteristics of the patients and healthy volunteers are summarized in Table 1. All the patients with colorectal cancer had undergone surgical resection of their primary tumor at the National Cancer Center Hospital, Tsukiji, or at Hospital East, Kashiwa, Japan. The median age of the patients was 62.0 years (range, 32–82 years). There were 69 men and 47 women patients. The primary tumors were located in the following sites: rectum in 53 patients, sigmoid colon in 21 patients, descending colon in 7 patients, transverse colon in 6 patients, ascending colon in 23 patients, and cecum in 6 patients. The clinical stage of the patients according to Dukes' classification was as follows: Dukes' stage A in 30 patients, stage B in 31 patients, stage C in 53 patients, and stage D in 2 patients.

Table 1. Characteristics of Patients and Healthy Volunteers

Characteristic	Patient (N = 116)	Healthy volunteer (N = 83)
Age, *y*		
Mean	62.0	58.4
Range	32–82	40–70
Sex, no (%)		
Male	69 (59.5)	37 (44.6)
Female	47 (40.5)	46 (55.4)
DNA, ng/gram of stool		
Mean	570.8	175.3
Range	2.0–7462.8	0.2–1907.5
Tumor location, no (%)		
Cecum	6 (5.2)	
Ascending colon	23 (19.8)	
Transverse colon	6 (5.2)	
Descending colon	7 (6.0)	
Sigmoid colon	21 (18.1)	
Rectum	53 (45.7)	
Size, mm		
Mean	40.0	
Range	4.0–120.0	
Histology, no (%)		
W/D	55 (47.4)	
M/D	56 (48.3)	
P/D	2 (1.7)	
Mucinous carcinoma	2 (1.7)	
Carcinoid tumor	1 (0.9)	
Depth, no (%)		
T1	10 (8.6)	
T2	32 (27.6)	
T3	71 (61.2)	
T4	3 (2.6)	
Dukes' stage, no (%)		
A	30 (25.9)	
B	31 (26.7)	
C	53 (45.7)	
D	2 (1.7)	

W/D, Well-differentiated adenocarcinoma; M/D, moderately differentiated adenocarcinoma; P/D, poorly differentiated adenocarcinoma.

Stool Samples

Before surgical resection, stool samples were obtained from 116 patients with colorectal cancer. Stool sam-

ples were also obtained from 83 healthy volunteers a few weeks after they had undergone a total colonoscopy. Naturally evacuated feces from subjects who had not taken laxatives were used as stool samples. Each patient was instructed to evacuate into a polystyrene disposable tray (AS one, Osaka, Japan) measuring 5 × 10 cm in size at home and bring the sample to the reception counter at the outpatient clinic or the Cancer Prevention and Screening Center of the National Cancer Center. The samples were collected and transferred to a laboratory at which they were allowed to stand at room temperature. Preparation of the stool samples for examination was conducted within 1–6 hours after the evacuation.

Magnetic Beads

Dynabeads Epithelial Enrich are uniform, superparamagnetic, polystyrene beads (4.5-μm diameter) coated with a mouse IgG1 monoclonal antibody (mAb Ber-EP4) specific for the glycopolypeptide membrane antigen Ep-CAM, which is expressed on most normal and neoplastic human epithelial tissues (Dynal, Oslo, Norway). Ep-CAM is widely expressed in the highly proliferative cells of the intestinal epithelium, from the basal cells to cells throughout the crypts at the basolateral membranes, and only the apical membrane facing the lumen is negative. The development of adenomas has been reported to be associated with increased Ep-CAM expression, and Ep-CAM over expression (mAb GA733) has frequently been demonstrated in colorectal carcinomas.[23–25]

Simulation Studies

A series of simulation studies were conducted to establish the optimal conditions for retrieving HT-29 colorectal cancer cells from feces. Feces from healthy volunteers were divided into several portions, each of which was seeded with 100 μL HT-29 cells (1×10^6/approximately 5 g feces). The cells were retrieved under several different conditions as follows: use of a Hank's solution and 25 mmol/L Hepes buffer (pH 7.35); processed feces of 5, 10, or 30 g volume; filter with a pore size of 48, 96, 512, or 1000 μm; incubation of homogenized solution with magnetic beads at 4°C or room temperature; application of 20, 40, 80, 200, or 400 μL magnetic beads; incubation of homogenized solution with magnetic beads under gentle rolling at 15 rounds/minute in a mixer for 10, 20, 30, or 40 minutes; and the reaction time between the cell-magnetic bead complexes and a magnet on a shaking platform for 0, 2, 10, 20, 30, 40, 50, or 60 minutes. Finally, the cell retrieval rate calculated for the magnetic beads method under the conditions determined to be the most suitable for this simulation study was compared with that calculated for the Percoll centrifugation method. The retrieval rate was calculated by dividing the number of cells that bound to the retrieved beads by the number of cells initially added to the feces. The cells were counted using a NucleoCounter (ChemoMetec A/S, Allerød, Denmark).

Isolation of Exfoliated Cells From Feces

The procedure was conducted using the most suitable and optimal conditions determined by the simulation study (Figure 1). Approximately 5–10 g of naturally evacuated feces were used to isolate exfoliated cells. Feces were collected into Stomacher Lab Blender bags (Seward, Thetford, United Kingdom). The stool samples were homogenized with a buffer (200 mL) consisting of Hank's solution, 10% fetal bovine serum (FBS), and 25 mmol/L Hepes buffer (pH 7.35) at 200 rpm for 1 minute using a Stomacher (Seward). The homogenates were then filtered through a nylon filter (pore size, 512 μm), followed by division into 5 portions (40 mL each). Subsequently, 40 μL of magnetic beads were added to each homogenized solution portion, and the mixtures were incubated for 30 minutes under gentle rolling in a mixer at room temperature. The samples on the magnet were then incubated on a shaking platform for 15 minutes at room temperature. Colonocytes isolated from 5 tubes were smeared onto slides and then stained using the Papanicolaou method. The remainder of the samples was centrifuged, and the sediments were stored at −80°C until DNA extraction.

Extraction of DNA

Fresh tissue samples were obtained from the surgically resected specimens of 116 patients with colorectal cancer. The samples were snap frozen in liquid nitrogen within 20 minutes of their arrival at the pathologic specimen reception area and were stored in liquid nitrogen until analysis.

Genomic DNA was extracted from each tumor tissue specimen using a DNeasy kit (QIAGEN, Valencia, CA). Genomic DNA was also extracted from colonocytes isolated from feces using the SepaGene kit (Sanko-Junyaku, Tokyo, Japan).

Direct Sequence Analysis

Direct sequencing was conducted to identify mutations in the APC codon 1270–1594, in codons 12 and 13 of the K-*ras* gene, and in exons 5, 6, 7, and 8 of the p53 gene.

The PCR primers used in this study were as follows: APC (5′-AAACACCTCAAGTTCCAACCAC-3′, 5′-GGTAATTTTGAAGCAGTCTGGGC-3′); K-*ras* (5′-CTGGTGGAGTATTTGATAGTG-3′, 5′-CCCAAGGAAAGTAAAGTTC-3′); p53 exon 5 (5′-GCCGTCTTCCAGTTGCTTTAT-3′, 5′-CCAAATACTCCACACGCAAAT-3′); p53 exon 6 (5′-CATGAGCGCTGCTCAGATAG-3′, 5′-TGCACATCTCATGGGGTTATAG-3′); p53 exon 7 (5′-CTTGGGCCTGTGTTATCTCCTA-3′, 5′-AAGAAAACTGAGTGGGAGCAGT-3′); and p53 exon 8 (5′-ACCTCTTAACCTGTGGCTTC-3′, 5′-TACAACCAGGAGCCATTGTC-3′).

The sequence primers used in this study were as follows: APC (5′-CAAAAGGCTGCCACTTGCAAAG-3′, 5′-AAAATAAAGCACCTACTGCTG-3′, 5′-GAATCAGCCAGGCACAAAGC-3′); K-*ras* (5′-CTGGTGGAGTATTTGATAGTG-3′); p53 exon 5 (5′-CCAAATACTCCACACGCAAAT-3′); p53 exon 6 (5′-CATGAGCGCTGCTCAGATAG-3′); p53 exon 7 (5′-AAGAAAACTGAGTGGGAGCAGT-3′); and p53 exon 8 (5′-

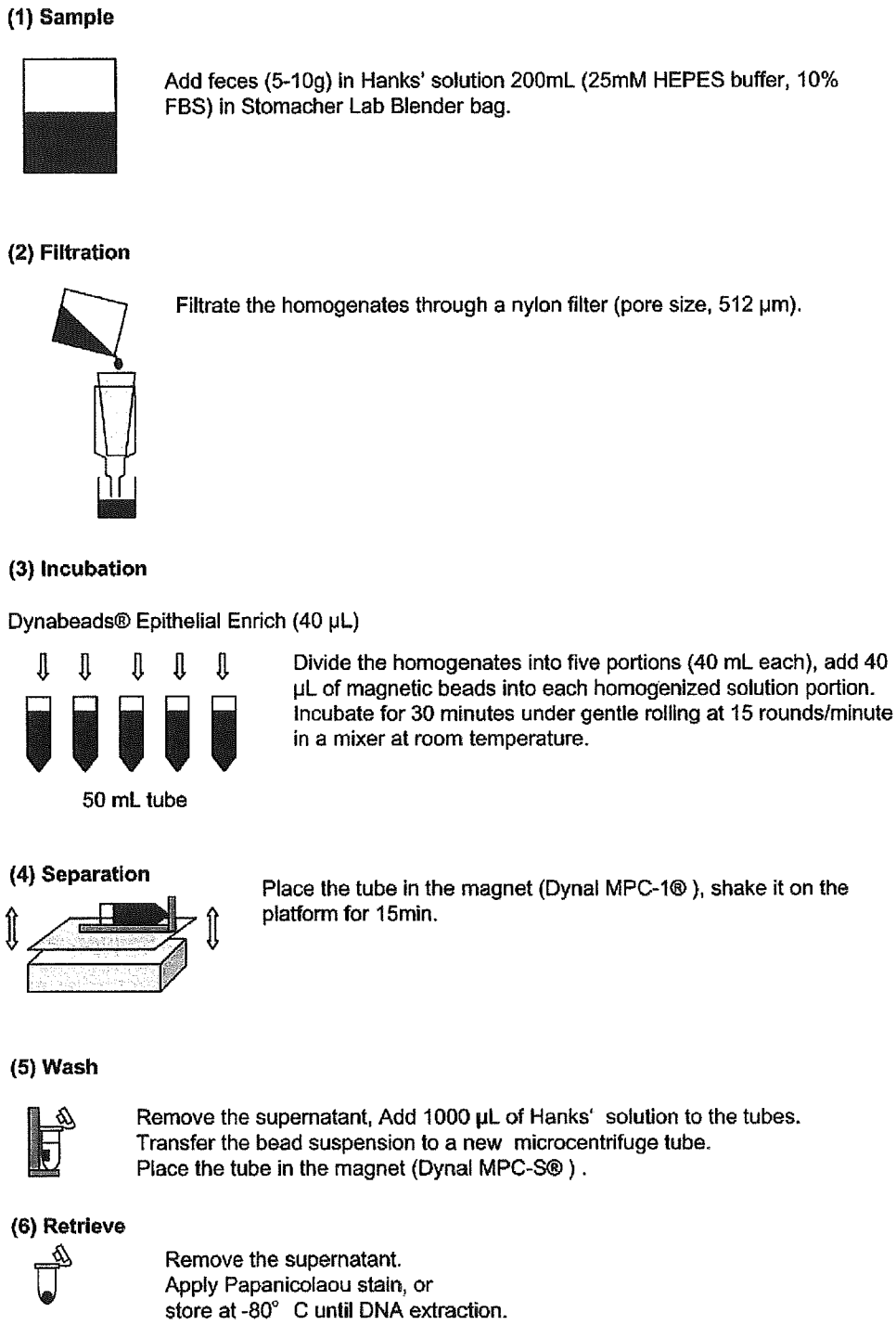

Figure 1. Schematic of procedure for isolating colonocytes from feces.

ACCTCTTAACCTGTGGCTTC-3′). Each fragment was sequenced by direct sequencing using the Big Dye Terminator v 3.1/1.1 cycle kit (Applied Biosystems, Forester City, CA).

All obtained sequences were aligned with previously published sequences (National Center for Biotechnology Information [NCBI] Genbank accession No. M74088 [APC], M54968 [K-*ras*], and X54156 [p53]) for each of the target genes and were analyzed using Phred/Phrp/DNASIS pro (Hitachi Software Engineering, Tokyo, Japan). The presence and nature of each mutation were confirmed by repeated PCR and sequencing.

BAT26

The BAT26 gene, an indicator of microsatellite instability (MSI), was amplified by PCR. Each fragment was elec-