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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′-

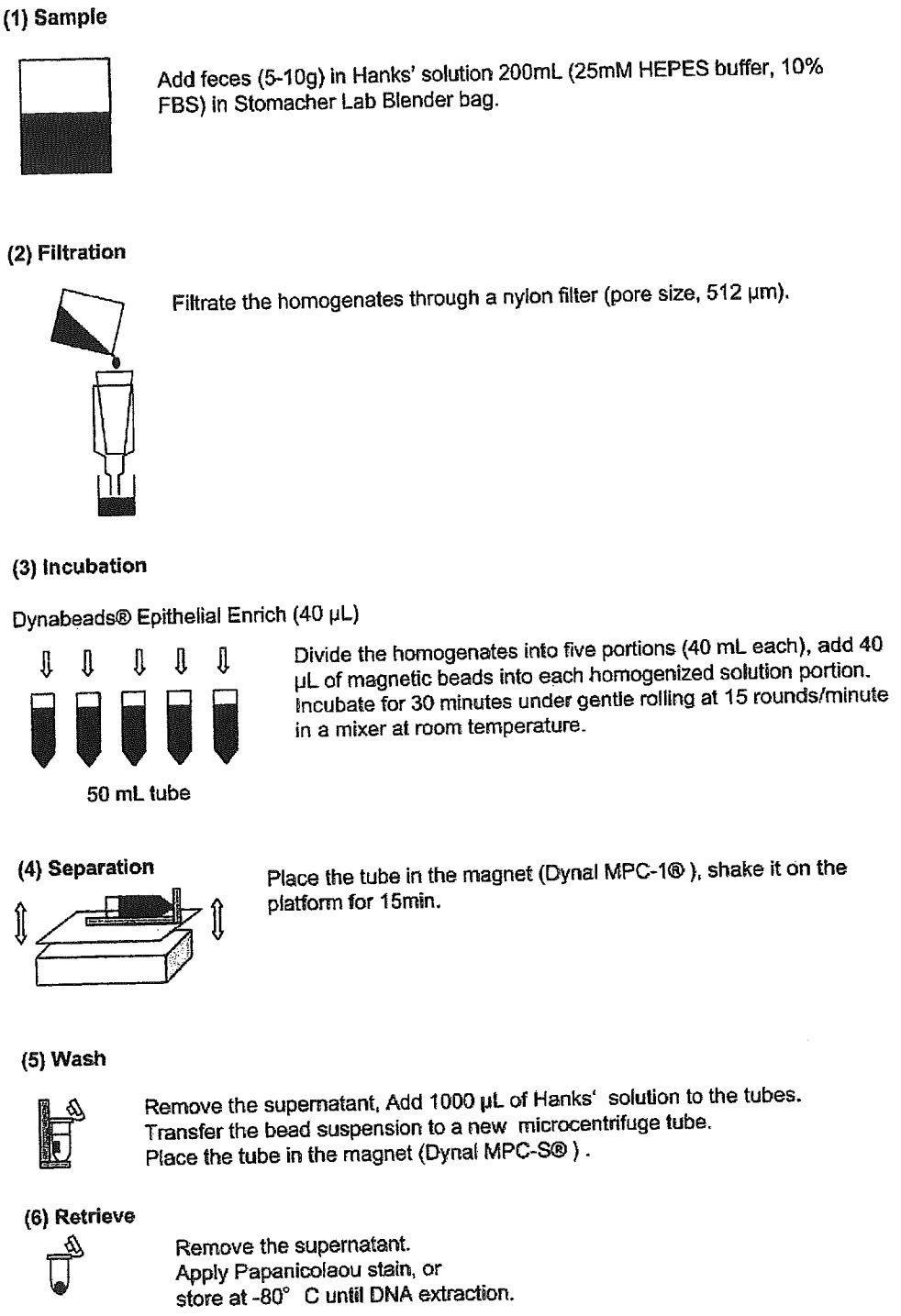

Figure 1. Schematic of procedure for isolating colonocytes from feces.

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-

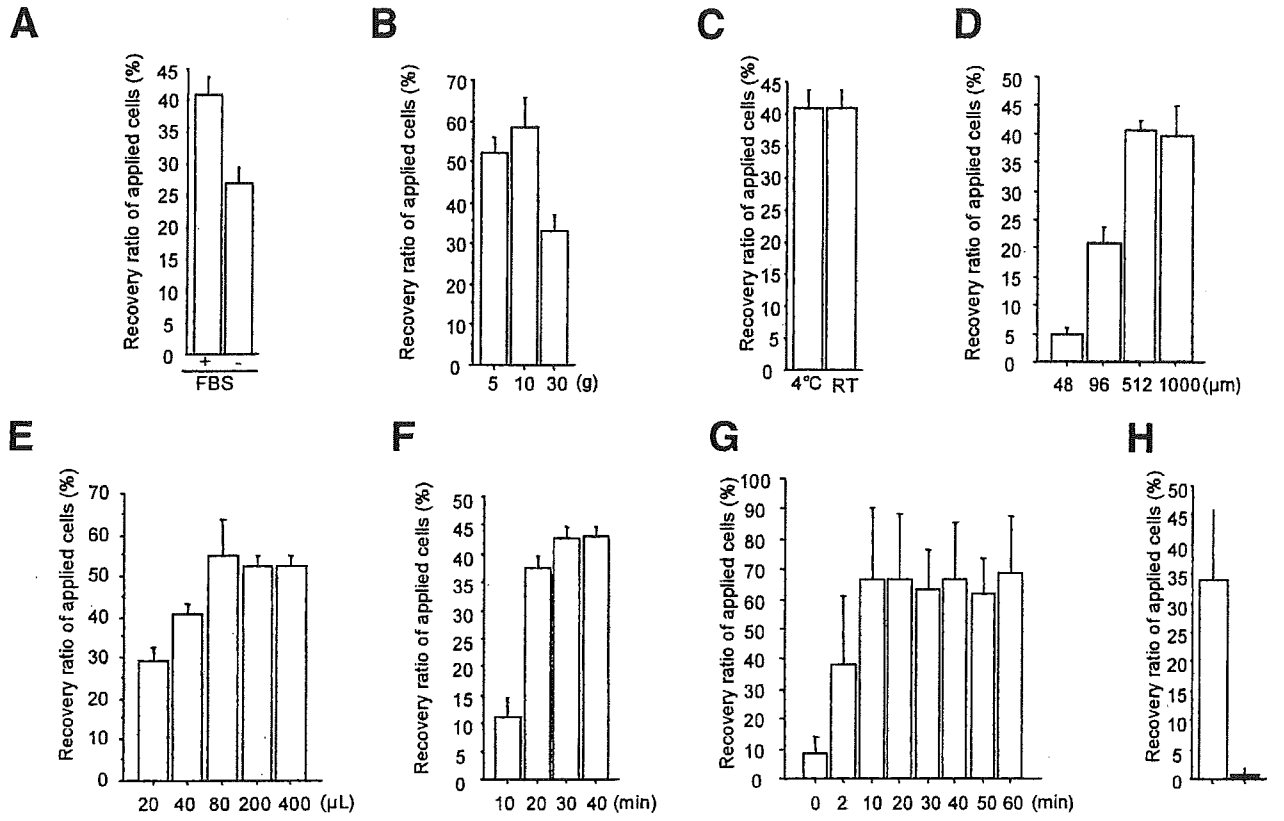

Figure 2. Simulation study to establish the optimal conditions for retrieving HT-29 colorectal cancer cells from feces and to compare the cell retrieval rates for the magnetic beads methods and the Percoll centrifugation method. Feces from healthy volunteers were divided into several portions, each of which was seeded with 100 μL HT-29 colorectal cancer cells (1×10^6/approximately 5 grams of feces). The procedure for retrieving the HT-29 cells was conducted under various conditions as follows: (*A*) homogenizing buffer with or without FBS; (*B*) stool weight (5, 10, or 30 g); (*C*) temperature during the cell-yielding procedure (4°C or room temperature); (*D*) filter pore size (48, 96, 512, or 1000 μm); (*E*) volume of applied magnetic beads (20, 40, 80, 200, or 400 μL); (*F*) incubation time of the homogenized solution with the magnetic beads under gentle rolling in a mixer (10, 20, 30, or 40 minutes); and (*G*) reaction time for the cells-magnetic bead complexes and the magnet on the shaking platform (0, 2, 10, 20, 30, 40, 50, or 60 minutes). The cell retrieval ratio (%) was calculated using the following formula: 100 × number of HT-29 cells retrieved/number of applied HT-29 cells. (*H*) Comparison of cell retrieval rates for the magnetic beads methods (*open column*) and the Percoll centrifugation method (*solid column*).

trophoresed using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) and then analyzed by GeneScan v 3.7 (Applied Biosystems). The PCR primers used in this study were 5′-TGACTACTTTTGACTTCAGCC-3′ and 5′-AACCATTCAACATTTTTAACCC-3′.

Cytology

Colonocytes isolated from feces were examined by 2 experienced cytotechnologists after Papanicolaou staining.

Study Blinding

We followed the guidelines of our medical institution for preparing blinded samples. Technicians processed the stool samples and prepared the slides for cytology and the cell pellets for DNA extraction. The samples were blinded to prevent the identification of individuals and the samples' origins. Two cytologists assessed the blinded samples, and the Life Science Group of Hitachi, Ltd, analyzed the DNA sequences.

Statistical Analysis

A Fisher exact test was used to compare all proportions. All reported P values are 2-sided. A value of $P < .05$ was considered statistically significant.

Results

Simulation Studies

The cell retrieval rate was found to decrease when Hank's solution without FBS was used, thus indicating the effectiveness of adding serum to the homogenizing buffer (Figure 2*A*). The cell retrieval rate was found to decrease when more than 30 g of feces were processed (Figure 2*B*). The cell retrieval rates were similar when incubation was conducted at room temperature and at 4°C (Figure 2*C*). Filtering of the stool suspension with the 48- or 96-μm filter resulted in significant clogging and thus hampered cell retrieval. However, a lot of fecal

residue remained after filtering with the 1000-μm filter, hindering the handling of the stool suspension thereafter. We therefore decided to use the 512-μm filter (Figure 2*D*). The dose of the magnetic beads applied was also examined. The cell retrieval rate increased in a dose-dependent manner up to 80 μL. In reality, a sufficient amount of genomic DNA derived from exfoliated colonocytes was obtained, even when 40 μL of magnetic beads were used (Figure 2*E*). Regarding the optimal incubation time of the magnetic beads for the complete binding of HT-29 cells to the beads, 30 minutes of incubation was found to be sufficient for the satisfactory binding of HT-29 cells to the beads (Figure 2*F*). For the retrieval of the cell-magnetic bead complexes on the magnet, a 10-minute reaction period was sufficient (Figure 2*G*).

The cell retrieval rates were 0.8% and 33.5% using the Percoll centrifugation method and the magnetic beads method, respectively, thus underscoring the advantage of the magnetic beads method (Figure 2*H*).

Cytology

Atypical cells were observed in colonocytes isolated from the feces of 32 of 116 patients with colorectal cancer, with a sensitivity rate of 28% (95% CI: 20–37; Table 2, Figure 3*A* and 3*B*). No atypical cells were observed in any of the 83 healthy volunteers, with a specificity rate of 100% (95% CI: 96–100). A significant difference ($P < .0001$) was found in the positivity rate between the patient group and the healthy volunteer group. The sensitivity rates for Dukes' A, B, and C or D colorectal cancers were 23% (7 of 30; 95% CI: 10–42), 32% (10 of 31; 95% CI: 17–51), and 27% (15 of 55; 95% CI: 16–41), respectively. No significant differences in the positivity rates were found among any of the stages. Furthermore, the sensitivity rates for cancers on the right side of the colon, including the cecum, ascending colon, and transverse colon, and for those on the left side of the colon, including the descending colon, sigmoid colon, and rectum, were 9% (3 of 35; 95% CI: 2–23) and 36% (29 of 81; 95% CI: 25–47), respectively. Therefore, the positivity rate was significantly higher for cancers on the left side of the colon ($P < .01$).

DNA Analysis

Overall analysis of stool samples. Sequence analysis showed distinct mutations in each of the analyzed genes in the tumor tissue and colonocytes isolated from feces (Figure 3*C–F*). Genetic alterations were observed in the colonocytes isolated from the feces of 82 of the 116 patients with colorectal cancer, yielding a sensitivity rate of 71% (95% CI: 62–79; Table 2). However, 10 of the 83 healthy volunteers were also positive for genetic alterations, producing a specificity value of 88% (95% CI: 79–94). A significant difference ($P < .0001$) was noted in the positivity rates of the patient group and the healthy volunteer group.

Genetic alterations were observed in 18 of the 30 patients with Dukes' A colorectal cancer, yielding a sensitivity rate of 60% (95% CI: 41–77). Furthermore, genetic alterations were observed among 26 of the 31 patients with Dukes' B colorectal cancer (84%; 95% CI: 66–95) and 38 of the 55 patients with Dukes' C or D colorectal cancer (69%; 95% CI: 55–81). No significant difference in sensitivity was found among any of the stages.

Genetic alterations were observed in colonocytes isolated from feces in 20 out of 35 patients with cancers originating on the right side of the colon (57%; 95% CI: 39–74) and in 62 out of 81 patients with cancers originating on the left side of the colon (77%; 95% CI: 66–85). No significant differences in the sensitivity rates were observed, although the sensitivity rate tended to be higher for cancers on the left side of the colon.

DNA analysis limited to colonocytes isolated from the feces of patients with colorectal cancer tissue involving genetic alterations. We assessed the performance of the present methodology for isolating cancer cells by examining the positivity rate of genetic alterations in colonocytes isolated from the feces of patients who showed alterations in their cancer tissues (Table 3). Among the 116 patients, a total of 93 (80%; 95% CI: 72–87) exhibited genetic alterations in the APC, K-*ras*, or p53 genes or BAT26 positivity in their cancer tissue: 51 patients exhibited APC mutations (44%, 95% CI: 35–53), 33 patients exhibited K-*ras* mutations (28%; 95% CI: 20–38), 62 patients exhibited p53 mutations (53%; 95% CI: 44–63), and 6 patients exhibited BAT26 positivity (5%; 95% CI: 2–11). Among the 93 patients with genetic alterations in their cancer tissues, the alterations were also successfully detected in colonocytes isolated from the feces of 80 patients (86%; 95% CI: 77–92). Among the 39 patients with Dukes' C or D advanced cancer who exhibited a genetic alteration in their cancer tissues, 36 patients exhibited genetic alterations in colonocytes isolated from their feces (92%; 95% CI: 79–98). Furthermore, genetic alterations were detected in colonocytes isolated from the feces of 18 of 24 patients with Dukes' A cancer (75%; 95% CI: 53–90) and 26 of 30 patients with Dukes' B cancer (87%; 95% CI: 69–96). No statistically significant difference was found among these 3 groups. In addition, genetic alterations could be detected in colonocytes isolated from the feces of 20 of 27 patients with cancers originating on the

Table 2. Incidences of Genetic Alterations of the APC, *K-ras*, p53, and MSI (BAT26) Genes as Well as Results From Cytology in all Patients and Healthy Volunteers

		Patient				Healthy volunteer	
		Tumor tissue		Isolated cell		Isolated cell	
	Marker	No.	Positivity (%) (95% CI)	No.	Sensitivity (%) (95% CI)	No.	Specificity (%) (95% CI)
Overall Patients (n = 116), healthy volunteers (n = 83)	Combined marker	93	80 (72–87)	82	71 (62–79)	10	88 (79–94)
	APC	51	44 (35–53)	47	41 (32–50)	1	99 (93–100)
	K-*ras*	33	28 (20–38)	33	28 (20–38)	1	99 (93–100)
	p53	62	53 (44–63)	45	39 (30–48)	6	93 (85–97)
	BAT26	6	5 (2–11)	4	3 (1–9)	3	96 (90–99)
	Cytology			32	28 (20–37)	0	100 (96–100)
Dukes' stage A (n = 30)	Combined marker	24	80 (61–92)	18	60 (41–77)		
	APC	14	47 (28–66)	11	37 (20–56)		
	K-*ras*	6	20 (77–39)	5	17 (6–35)		
	p53	6	20 (77–39)	9	30 (15–49)		
	BAT26	1	3 (1–17)	1	3 (1–17)		
	Cytology			7	23 (10–42)		
Dukes' stage B (n = 31)	Combined marker	30	97 (83–100)	26	84 (66–95)		
	APC	17	55 (36–73)	17	55 (36–73)		
	K-*ras*	10	32 (17–51)	9	29 (14–48)		
	p53	18	58 (39–75)	13	42 (25–61)		
	BAT26	2	6 (1–21)	1	3 (1–17)		
	Cytology			10	32 (17–51)		
Dukes' stages C and D (n = 55)	Combined marker	39	71 (57–82)	38	69 (55–81)		
	APC	20	36 (24–50)	19	35 (22–49)		
	K-*ras*	17	31 (19–45)	19	35 (22–49)		
	p53	27	49 (35–63)	23	42 (29–56)		
	BAT26	3	5 (1–15)	2	4 (0–13)		
	Cytology			15	27 (16–41)		
Right-sided colon cancer (n = 35)	Combined marker	27	77 (60–90)	20	57 (39–74)		
	APC	11	31 (17–49)	8	23 (10–40)		
	K-*ras*	16	46 (29–63)	12	34 (19–52)		
	p53	17	49 (31–66)	11	31 (17–49)		
	BAT26	2	6 (1–19)	1	3 (1–15)		
	Cytology			3	9 (2–23)		
Left-sided colon cancer (n = 81)	Combined marker	66	81 (71–89)	62	77 (66–85)		
	APC	40	49 (38–61)	39	48 (37–60)		
	K-*ras*	17	21 (13–31)	21	26 (17–37)		
	p53	45	56 (44–67)	34	42 (31–53)		
	BAT26	4	5 (1–12)	3	4 (1–10)		
	Cytology			29	36 (25–47)		

right side of their colon (74%; 95% CI: 54–89) and 60 of 66 patients with cancers originating on the left side of their colon (91%; 95% CI: 81–97). A statistically significant difference was found between the right- and left-side colon cancer patient groups ($P = .03$).

Discussion

We have devised a simple, highly reliable methodology for isolating colorectal cancer cells from nonlaxative-induced, naturally evacuated feces from most patients with colorectal cancer. To date, several methods of isolating colorectal cancer cells from feces have been reported.[21,22,26,27]

Our new funnel-shaped filter system extensively improved the filtration efficiency of the stool suspension by enlarging the filtration area and selecting the optimal pore size; the system was capable of filtrating the entire stool suspension without filter clogging. These properties permit the omission of centrifugation and simplify the overall process because all steps can be performed at room temperature. Furthermore, the use of serum successfully increased the cell retrieval rate. We presume that this increase may be attributed to the suppression of protease activity or the inhibition of nonspecific reactions of the antibodies on the bead surface. Consequently, our new methodology also allows the extraction of high-quality DNA or RNA from exfoliated colonocytes. Very recently, Imperiale et al compared a panel of fecal DNA markers and Hemoccult II as screening tests for colorectal cancer. It is worth noting that, in their study, colonoscopy as a reference standard was used

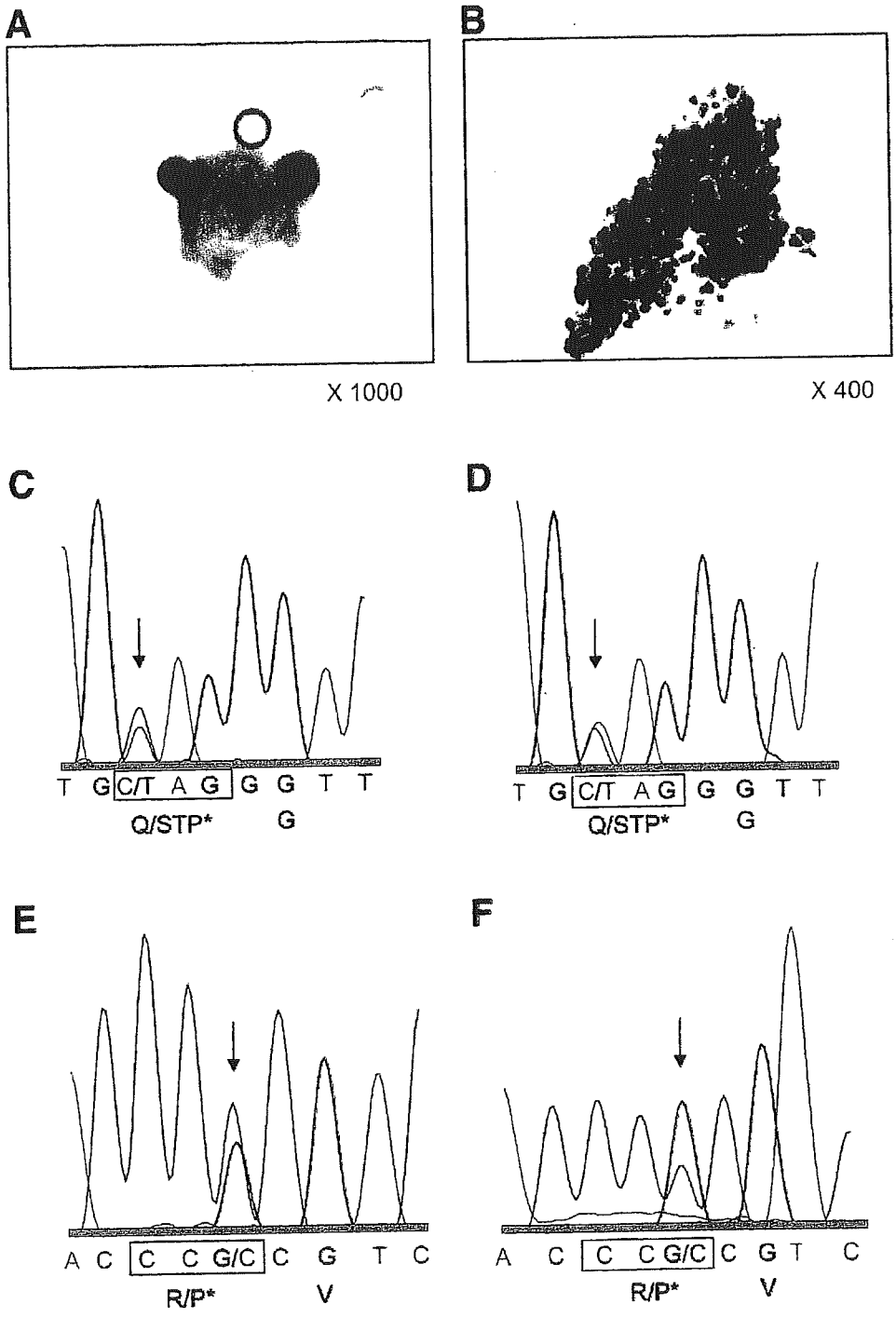

Figure 3. Cytology and DNA sequencing. Papanicolaou staining of colonocytes isolated from the feces of patients with colorectal cancer. (*A*) A patient with ascending colon cancer, Dukes' stage A. (*B*) A patient with rectal cancer, Dukes' stage C. Detection of mutations in tumor tissues and colonocytes isolated from the feces of patients with colorectal cancer. (*C*) A point mutation of the APC gene in a tumor tissue specimen obtained from a patient with rectal cancer, Dukes' stage B. (*D*) An identical mutation was detected in colonocytes isolated from the feces of the patient. (*E*) A point mutation of the p53 gene in a tumor tissue specimen obtained from a patient with ascending colon cancer, Dukes' stage A. (*F*) An identical mutation was detected in colonocytes isolated from the feces of the patient. *Wild/mutant.

in all subjects. They conducted those tests in a blinded fashion and showed that sensitivity of DNA analysis was 4-fold higher than that of Hemoccult test.[28] We believe that this report may prompt a study of fecal DNA test for colorectal cancer screening.

The idea to isolate cancer cells from feces originally derived from a study that described the abnormal expression of the CD44 gene in many tumors, including colon cancer and bladder cancer.[21,29,30] In the course of a series of studies, we predicted that normal mucous cells would die and be exfoliated during turnover and that the cancer cells would likely survive for a long time in the feces.

Although cytology is highly specific compared with direct sequence analysis, its sensitivity, especially for cancers on the right side of the colon is relatively low. From a technical aspect, our cytology method does not allow the

Table 3. Incidences of Genetic Alterations in Colonocytes Isolated From the Feces of Patients With Colorectal Cancer Tissue Involving Genetic Alterations of the APC, K-*ras*, p53, or MSI (BAT26) Gene

	Combined marker		APC		K-ras		p53		BAT 26	
	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)
Overall	80/93	86% (77–92)	46/51	90% (79–97)	29/33	88% (72–97)	42/62	68% (55–79)	4/6	67% (22–96)
Dukes' stage A	18/24	75% (53–90)	11/14	79% (49–95)	5/6	83% (36–100)	5/6	83% (36–100)	1/1	100% (3–100)
Dukes' stage B	26/30	87% (69–96)	16/17	94% (71–100)	9/10	90% (56–100)	12/18	67% (41–87)	1/2	50% (1–99)
Dukes' stages C and D	36/39	92% (79–98)	19/20	95% (75–100)	15/17	88% (64–99)	21/27	78% (58–91)	2/3	67% (9–99)
Right-sided	20/27	74% (54–89)	8/11	73% (39–94)	12/16	75% (48–93)	11/17	65% (38–86)	1/2	50% (1–99)
Left-sided	60/66	91% (81–97)	38/40	95% (83–99)	17/17	00% (81–100)	31/45	69% (53–82)	3/4	75% (19–99)

NOTE. Number of positive cases in tumor tissue and colonocytes isolated from feces/number of positive cases in tumor tissue, with 95% confidence interval.

observation of cells unless there are 5×10^4 cells per slide. Technical improvements might increase the benefits of feces cytology. However, we believe that cytology is not suitable as a method for identifying cancer because of its low sensitivity, at least at present. From a practical point of view, we have conducted a study to determine the effect of the time and temperature after evacuation on the recovery rates of fecal colonocytes, and we have found that we can obtain almost the same number of colonocytes from stool materials 3 days after evacuation in comparison with 6 hours after evacuation if fecal material is kept at 4°C (data not shown). This observation may be important for the potential clinical application of this method.

Direct sequence analysis of colonocytes isolated from the feces of 83 healthy volunteers revealed mutations in 8 subjects (9%; 95% CI: 4–18), the breakdown of which was as follows: 1 APC1 mutation, 1 K-*ras* mutation, and 6 p53 mutations. Points of mutations identified of the p53, APC, and K-*ras* genes observed in the 83 healthy volunteers in this study were identical to that reported previously in tumors. These mutations of p53, APC, and K-*ras* in tumors are recorded in the database of OMIM. PCR errors were unlikely because multiple PCR reactions and sequence reactions were separately conducted. However, genetic alterations in precancerous lesions may have been present, although endoscopy findings macroscopically verified the absence of adenoma and carcinoma. The individuals in whom the present methodology revealed genetic alterations should be monitored to assess whether these findings were false-positive results or a predictor of tumorigenesis.

Oncogenes in feces are presumably derived from cancer cells exfoliated from the cancer tissue, and genetic alterations would not be detected in colonocytes isolated from feces if the original cancer tissue did not contain genetic alterations. In fact, among the 93 patients who exhibited genetic alterations in their cancer tissues, alterations were detected in colonocytes from the stools of 80 patients, producing a true sensitivity rate of 86% (80 of 93), although the present overall sensitivity was 71%. Furthermore, our methodology allows the isolation and retrieval of colorectal cancer cells from both early stage cancer and right-side colon cancer. Because the methodology allows processing at room temperature, we are currently constructing an automated, mechanized processing system on a commercial basis. A problem of our test was its relatively low specificity for a screening test as described previously. We consider that mutations observed in the healthy subjects might be attributable to the fact that they belonged to a high-risk group for colorectal cancer because these 83 volunteers were selected from among colonoscopy examinees recruited by the newly established National Cancer Center Research Center for Cancer Prevention and Screening, and the detection rate of cancers appeared to be considerably higher in the all examinees at the center than in the general population in Japan (unpublished observation). Therefore, we speculate that precancerous lesions with mutations of the genes tested might have been present in the colorectal epithelium of some of these healthy volunteers. We think that a prospective randomized study would be needed to determine the actual specificity of our method in a real screening population and to verify its clinical usefulness.

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Thymidine phosphorylase expression and efficacy of adjuvant doxifluridine in advanced colorectal cancer patients

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Abstract. To clarify the correlation between the expression level of thymidine phosphorylase (TP) and efficacy of doxifluridine (5'-DFUR) and 5-fluorouracil (5-FU), samples from 177 colorectal cancer patients who underwent curative resection were evaluated by immunohistochemical staining using a newly developed monoclonal antibody 1C6-203. Patients were randomly given either oral 5'-DFUR or 5-FU as postoperative adjuvant chemotherapy. In Dukes' C staged colon cancer patients treated with 5'-DFUR, better survival was observed in the high TP patients than the low TP patients (P=0.025 by the log-rank test). The observed 5-year survival rates were 91.2 and 74.8%, respectively. No correlation between TP expression and patient prognosis was detected in the 5-FU group. In Dukes' C staged colon patients with high TP expression, the 5'-DFUR group had slightly better survival than the 5-FU group. These findings suggest that TP may be a chemosensitive marker for 5'-DFUR as postoperative adjuvant chemotherapy for advanced colon cancer patients.

Introduction

Although intravenous administration of 5-fluorouracil (5-FU) + leucovorin (LV) was introduced worldwide in the mid-1990s as the standard postoperative adjuvant therapy for colorectal cancer (1), oral cancer drugs such as capecitabine have recently been re-evaluated due to a favorable benefit:risk ratio compared with 5-FU+LV (2,3), while in Japan, oral fluoropyrimidines have been widely used as postoperative adjuvant chemotherapy since the early 1990s, due to better compliance and minimal toxicity. However, a major retardation of cancer chemotherapy is the lack of predictive markers for responsiveness, and selecting effective chemotherapy might not only offer a survival benefit but also avoid unnecessary adverse effects caused by unsuitable drugs.

Thymidine phosphorylase (TP), which is predominantly observed in tumor tissue (4-7), is a key enzyme in the metabolic activation of fluoropyrimidine by conversion of doxifluridine (5'-DFUR), which is an intermediate metabolite of capecitabine, to 5-FU (8). Thus, administration of 5'-DFUR against tumors with high TP expression is expected to yield high concentrations of 5-FU in tumor tissue, and thereby a good chemotherapeutic response. In fact, the clinical efficacy of 5'-DFUR has been demonstrated in colorectal cancer patients with high-TP-expression tumors compared to patients with low-TP tumors (9,10). However, the efficacy of 5'-DFUR has not previously been compared to that of other therapies.

Immunohistochemical staining of TP has been widely adopted to evaluate its relationships with clinicopathologic features and prognosis. However, these studies have produced conflicting results, and may not be definitive, as monoclonal antibody (MAb) 654-1, which stains stromal cells stronger than cancer cells, was used (11-14). A newly developed MAb, 1C6-203, is reportedly more sensitive to colorectal cancer cells than MAb 654-1 in 10% formalin-fixed specimens (15). Thus, MAb 1C6-203 appears to be more suitable than MAb 654-1 to assess TP expression.

We previously conducted a randomized controlled trial (RCT) to compare the usefulness of 5'-DFUR to oral 5-FU as postoperative adjuvant chemotherapy for 558 patients with colorectal cancer, and reported a survival benefit in 5'-DFUR therapy in Dukes' B or C staged patients, particularly in colon cancer patients (16). In the present study, we assessed colorectal cancer patients from the previous RCT who were strictly followed for more than 5 years, and examined the predictive value of TP expression for patients' prognoses by immunohistochemistry using the new sensitive antibody.

Materials and methods

Patients. A total of 177 colorectal cancer patients (103 men and 74 women, median age at surgery; 62.0 years (range; 42-78 years), who had enrolled in the previously mentioned RCT and whose paraffin-embedded specimens were available for immunohistochemical staining, were assessed. All patients were diagnosed as having TNM stage II or III primary colorectal cancer, and underwent curative resection at institutes

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affiliated with the Department of General Surgery, Graduate School of Medicine, Chiba University, Japan, from April 1993 to September 1996. No patient had any history of treatment for other colorectal cancers, other active cancers, or serious concurrent disease. Patients were randomly assigned to two groups; either the 5'-DFUR group (84 patients) or 5-FU group (93 patients) at each institute. No differences in patient characteristics were observed between the two groups (Table I). The present study was approved by the institutional review boards at participating centers. All patients provided fully informed consent.

Treatment schedule. Both groups were given 6.0 mg/m^2 of mitomycin C intravenously on the day of surgery and on the following day. Oral chemotherapy was started from 2 weeks after surgery; the 5'-DFUR group was given 460 mg/m^2/day and the 5-FU group was given 115 mg/m^2/day, daily for 1 year. Both groups were concomitantly given 3 g/day of polysaccharide kureha for 1 year. All patients were followed for more than 5 years after surgery, and the median follow-up period after surgery was 5.5 years. No patient had additional treatment unless the cancer recurred or another cancer developed.

Immunohistochemical staining. After reviewing hematoxylin-eosin-stained slides of surgical sections, we selected a paraffin block including the deepest invasion site. Immunohistochemical staining was performed using avidin-biotin-peroxidase complex (Peroxidase Vectastain ABC Kit®, Vector Laboratories Inc., Burlingame, CA, USA). The sections were deparaffinized with xylene and rehydrated with ethanol. The specimens were washed with phosphate buffered saline (PBS) for 5 min, and endogenous peroxidase was then blocked by incubating the preparations with 0.3% hydrogen peroxide in methanol for 30 min. After washing the preparations 3 times with PBS for 5 min, they were incubated for 15 min with biotin blocking solution, rinsed again with PBS for 5 min, and re-incubated with PBS containing 3% skim milk for 30 min. These preparations were then incubated with anti-TP mouse antibody, 1C6-203 (Nippon Roche Research Center, Kamakura, Japan), which were diluted 1:1000 with 0.5% normal horse serum/PBS at 4°C overnight in a moist chamber. The sections were then washed 3 times with PBS and incubated with peroxidase-labeled horse anti-mouse IgG monoclonal antibody for 30 min at room temperature. These were rinsed again and then incubated with avidin-biotin-peroxidase complex for 30 min at room temperature. After washing 3 more times, the preparations were incubated with diaminobenzidine substrate for 1-7 min. The specimens were rinsed again with distilled water, counterstained with Mayer's hematoxylin and mounted.

Evaluation of stained sections. TP expression was simultaneously evaluated on x100 and x50 fields using a two-headed light microscope by 2 investigators (Drs S. Hasegawa and N. Takiguchi) with no prior knowledge of the patients' clinico-pathologic characteristics and prognosis. Specimens with ≥5% cancer cells stained in the cytoplasm or nucleus were regarded as having high TP expression, while the rest were regarded as having low TP expression.

Table I. Patient characteristics.

	5'-DFUR (n=84)	5-FU (n=93)	P-value
Age (y)	61.2	62.7	0.252
Gender (M/F)	53/31	50/43	0.269
Location of tumor			
Colon	56	61	0.427
Rectum	28	32	
Histologic differentiation			
Well	47	50	0.727
Moderate	33	40	
Poor	3	3	
Mucinous	1	0	
Depth of tumor			
pT1	1	1	0.954
pT2	9	9	
pT3	47	56	
pT4	27	27	
Dukes' stage			
A	8	8	0.860
B	39	47	
C	37	38	

Statistical analysis. Statistically significant differences in continuous variables between groups were assessed by the t-test, and with categorical variables either by the χ^2 test or Mann-Whitney U-test. P-values <0.05 were considered significant. The survival curve was calculated by the Kaplan-Meier method, and differences between two groups were evaluated using the log-rank and Wilcoxon tests.

Results

Relationship between TP expression and clinicopathological characteristics. TP staining was observed in the nucleus and/or cytoplasm of cancer cells (Fig. 1). Most normal colorectal mucosal cells were not stained with anti-TP antibody. TP was highly expressed in 92 (52.0%) colorectal cancer patients.

Table II summarizes the relationships between TP expression and some clinicopathological characteristics. In the 5-FU group, patients with low TP had more advanced tumor depth than those with high TP, but no difference in Dukes' stage was observed between them. No other correlation between TP expression and clinicopathological characteristics was observed.

Correlation between TP expression and outcome of adjuvant chemotherapy. A comparison of overall survival time of the colorectal cancer patients and tumor TP expression revealed no correlation in either the 5'-DFUR or 5-FU group (Fig. 2).

Figure 1. Immunohistochemical staining with 1C6-203 for TP in colon cancer tissue. TP staining was seen in the cytoplasm and/or nucleus of cancer cells (original magnification x50).

Table II. Relationships between TP expression and clinicopathologic features.

	High TP (n=92)		Low TP (n=85)	
	5'-DFUR (n=42)	5-FU (n=50)	5'-DFUR (n=42)	5-FU (n=43)
Age (y)	60.7	63.3	61.8	61.9
Gender (M/F)	25/17	22/28	28/14	28/15
Location of tumor				
Colon	26	34	30	27
Rectum	16	16	12	16
Histologic differentiation				
Well	20	27	27	23
Moderate	21	21	12	19
Poor	1	2	2	1
Mucinous	0	0	1	0
Depth of tumor				
pT1	0	1	1	0
pT2	6	6	3	3
pT3	25	36	22	20
pT4	11	7	16	20
		[a]		
Dukes' stage				
A	4	6	4	2
B	18	27	21	20
C	20	17	17	21

[a]P=0.0062.

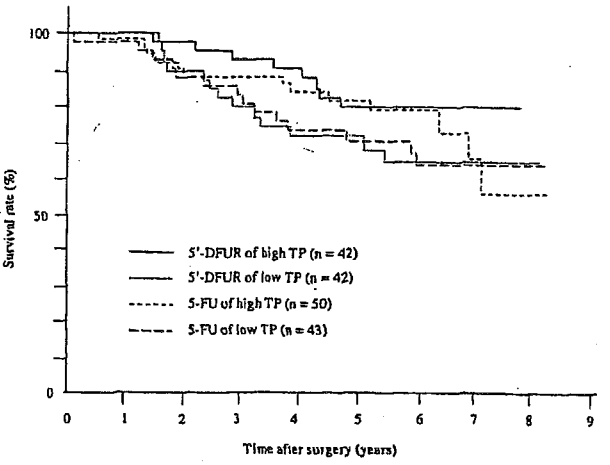

Figure 2. Overall survival curve between patients with high and low TP expression of the colorectal cancer patients in the two chemotherapy groups. Estimated overall 5-year survival rates in patients with high and low TP expression were 79.5 and 71.4% in the 5'-DFUR group, respectively, and 81.6 and 70.5% in the 5-FU group, respectively. No significant difference was detected among each group.

However, in the colorectal cancer patients with Dukes' C stage, slightly better survival was observed in the 5'-DFUR group with high TP expression than in the 5-DFUR group with low TP expression, while no significant difference was detected in the 5-FU group (Fig. 3). Evaluation of Dukes' C patients with only colon cancer revealed significantly better survival in the 5'-DFUR group with high TP expression than low TP expression. In addition, Dukes' C staged colon cancer patients with high TP expression had slightly better survival in the 5'-DFUR group than the 5-FU group, while in the low

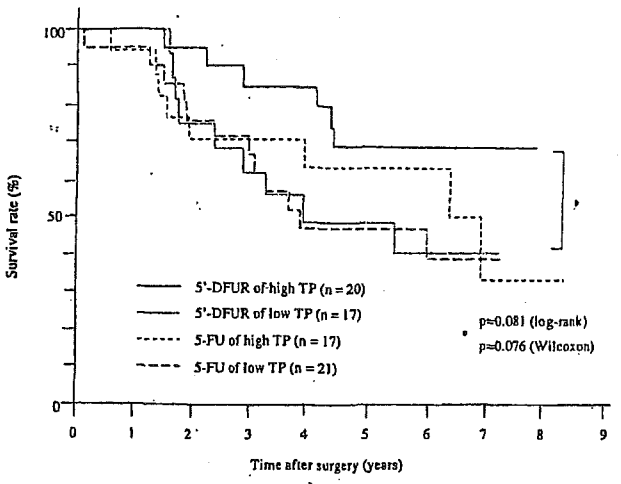

Figure 3. Overall survival curve between patients with high and low TP expression in Dukes' C staged colorectal cancer patients in the two chemotherapy groups. Estimated overall 5-year survival rates in patients with high and low TP expression were 69.1 and 49.2% in the 5'-DFUR group, respectively, and 63.5 and 47.6% in the 5-FU group, respectively. In the 5'-DFUR group, patients with high TP expression had slightly better survival than those with low TP expression.

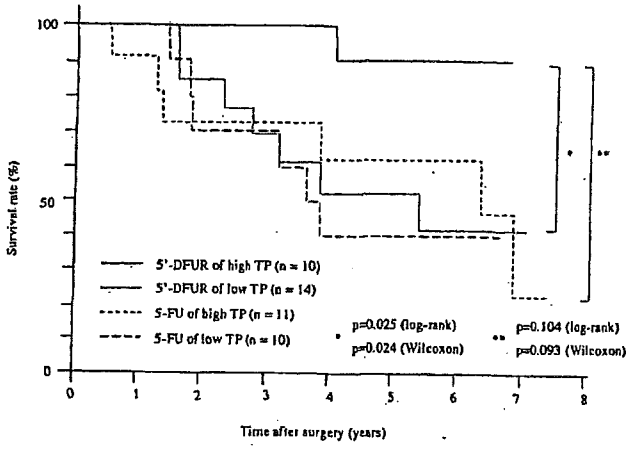

Figure 4. Overall survival curve between patients with high and low TP expression in Dukes' C staged colon cancer patients in the two chemotherapy groups. Estimated overall 5-year survival rates in patients with high and low TP expression were 91.2 and 74.8% in the 5'-DFUR group, respectively, and 82.2 and 71.1% in the 5-FU group, respectively. In the 5'-DFUR group, patients with high TP expression had significantly better survival than those with low TP expression. Of the patients with high TP expression, the 5'-DFUR group had slightly better survival than the 5-FU group.

TP patients, no difference in survival was observed between the 5'-DFUR and 5-FU groups (Fig. 4).

Discussion

TP activates 5'-DFUR to the active drug 5-FU by cleaving the 5-deoxyribose moiety, while by addition of 2-deoxyribose-1-phosphate TP can activate 5-FU to 5-fluoro-2'-deoxyuridine, a precursor of FdUMP, which inhibits thymidylate synthase, responsible for *de novo* thymidylate synthesis. Therefore, high levels of TP overexpression affect 5-FU sensitivity. However, several reports have demonstrated that high TP expression correlates with low sensitivity to 5-FU (17,18). Similarly, the present study was unable to demonstrate 5-FU efficacy in patients with high TP expression. We previously demonstrated that TP expression has no significant relationship with prognosis in gastric cancer patients treated with 5-FU, but that 5'-DFUR treated staged III gastric cancer patients with high tumor TP expression receive a significant survival benefit (19,20). The present study produced similar results, in that administration of 5'-DFUR contributed a higher survival benefit in Dukes' C staged colorectal cancer patients with high TP expression than those with low TP expression. In addition, slightly better survival was observed in the 5'-DFUR treated Dukes' C colon cancer patients than the 5-FU treated ones.

In the present study, a newly developed MAb, 1C6-203, was used for immunohistochemical analysis. In previous studies, MAb 654-1, which stained stromal cells stronger than cancer cells, was used, and thus TP expression was often evaluated by staining of cancer stromal cells not cancer cells (11-14,21,22). MAb 1C6-203 was raised against recombinant human TP, while MAb 654-1 was directed against human TP refined from a human colon cancer HCT 116 xenograft. The sensitivity of the new developed MAb 1C6-203 to colorectal cancer cells is 60%, whereas that of MAb 654-1 is 20% in 10% formalin-fixed specimens. In addition, this new antibody produced a 67% expression rate in stromal immune cells, while MAb 654-1 had a lower frequency of 47% in 10% formalin-fixed specimens (15). We also assessed TP expression in stromal cells (data not shown), and found that the relationships between TP expression and patient survival were similar to the results in cancer cells. Thus, MAb 1C6-203 appears more suitable than MAb 654-1 to assess TP expression in cancer cells, and contributes to the reliability of the results of the present study.

Our study demonstrated the clinical efficacy of 5'-DFUR in Dukes' C staged patients with high TP expression, particularly in colon cancer. In contrast to colon cancer, rectal cancer reportedly has different behavior in relapse, such as intrapelvic recurrence associated with or without anastomosis, even with the same histology and staging (23,24), and thus the operative outcome might significantly influence patient prognosis even if a curative resection was macroscopically performed. Thus, interpreting the results of postoperative adjuvant chemotherapy in rectal cancer patients requires caution, and it might be preferable to analyze only colon cancer patients if the chemotherapeutic responses are being strictly evaluated.

In general, the benefit from adjuvant chemotherapy has been clearly established in Dukes' C staged colon cancer patients. Mamounas *et al* (25) demonstrated the clinical efficacy of adjuvant chemotherapy in patients with Dukes' B staged colon cancer, as lymph node micro-metastasis, present in more than half the patients with Dukes' B staged colon cancer, significantly correlated with patient prognosis. Our study showed no survival difference in patients with Dukes' B staged colon cancer, but indicated the efficacy of 5'-DFUR in Dukes' C staged colon cancer patients with high TP expression, which supports the chemotherapeutic potential of 5'-DFUR as postoperative adjuvant treatment for advanced colon cancer.

TP is also an enzyme known as platelet-derived endothelial cell growth factor (26) or tumor-associated angiogenic factor (27), and correlates with tumor growth and metastasis not only in colorectal cancer but also in stomach and ovarian cancer (5,6,28,29). However, the relationship between TP expression and tumor malignant potential remains controversial. Studies have shown that TP expression in liver metastasis is higher than in primary colorectal cancer (30,31) and high TP expression is a risk factor for hematogenous metastasis in patients with Astler Coller B1/B2 colorectal cancer (17). In contrast to these studies, better survival in patients with high TP expression has been demonstrated in several studies (11,21). In the present study, no correlation was detected between TP expression and clinicopathologic features. This discrepancy was probably due to the various chemotherapy regimens or different analysis methods.

Based on these findings, immunohistochemical evaluation of TP expression might help predict patient response to oral 5'-DFUR. There is an increasing need for defining new factors that might be used to predict prognosis in colorectal cancer and its response to therapy. Several enzymes, such as thymidylate synthase, dihydropyrimidine dehydrogenase and orotate phosphoribosyl dehydrogenase, have been reported to be useful in predicting the sensitivity of colorectal cancer to 5-FU based chemotherapy (32,33). Hotta *et al* reported that TP level could be evaluated from preoperative endoscopic biopsy specimens (34). Establishing a predicting marker for chemo-sensitivity should contribute to effective neo-adjuvant chemotherapy in patients with high-risk of recurrence.

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特集 転移性肝癌のすべて—診断と治療方法の選択—

転移性肝癌に対する治療方針
大　腸　癌

Treatment for liver metastases from colorectal cancer

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大腸癌肝転移に対する治療法は，5-FUを中心とした全身化学療法と肝切除術，肝動注化学療法，熱凝固療法(マイクロ波凝固療法，ラジオ波焼灼療法)などの肝局所療法である．現在のところ外科的切除が唯一の根治的治療法であるが，切除適応となる症例は25～52%である．しかし，従来は切除不能とされた肝転移例に対し，肝動注化学療法や熱凝固療法を併用することにより，予後の改善や治療成績の向上が期待できる．

はじめに

大腸癌肝転移に対する治療法は，1970年代から開始された転移巣に対する外科的切除が唯一の根治的治療法である．つまり切除可能であれば外科的切除が治療の第一選択であり，予後も良好であることが示されている．しかし，切除不能例も多く存在し，これらの症例に対する治療法として，肝動注化学療法，マイクロ波凝固療法(MCT)・ラジオ波焼灼療法(RFA)などの肝局所療法がある(図1)．

本稿では，現在一般的に受け入れられている大腸癌肝転移に対する治療法に対して，最近の知見を加えて述べる．

I. 手術療法

現時点において，大腸癌肝転移に対する外科的切除は長期予後の改善が期待できる唯一の治療法である．近年，周術期管理の向上，術中超音波検査の普及[1]，術前門脈塞栓療法の併用[2)3)]，肝臓外科のテクニックの向上，手術器具などのさまざまな進歩[4]により，安全で確実な肝切除が可能になってきている[5]．

1．手術適応

大腸癌肝転移に対する外科的治療の適応は，

① 肝切除の安全性：肝切除の難易度
腫瘍の肝内占拠部位・範囲で決定される．

② 原発病巣がコントロールされている．

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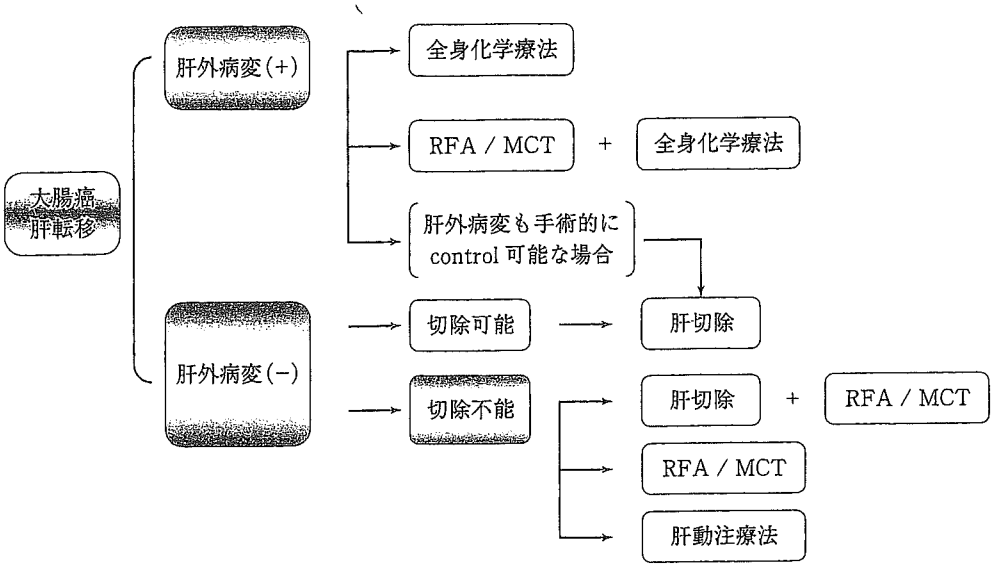

図1　大腸癌肝転移治療のフローチャート

③　適度な残肝量(正常肝の場合25～40%)を残して肝転移巣が完全に切除できる.

④　他臓器に転移病変を認めない.

肝切除が適応とならない条件

①　肝転移巣の完全切除が不可能である.

②　肝所属リンパ節転移が陽性である.

③　肝以外の遠隔転移巣を有する.

と，されてきたが肝切除術が安全に施行されるようになった現在では，たとえば肺転移があってもその転移巣が完全切除可能ならば肝切除が適応とされるようになってきている[6)7)]．また，完全切除が不可能でも熱凝固療法との組み合わせで治療効果の向上が期待されている.

2．肝切除の時期

同時性肝転移に対して，①原発巣のみを切除して，一定期間(約3ヵ月)後に切除可能であれば切除する方針と，②原発巣と同時に切除する方針がある.

異時切除の利点は，手術の時期を待つことにより，

①　初回診断時，画像上明らかでなかった微小転移巣が顕在化し，この転移巣を含めて切除できる点.

②　画像上，顕在化していなかった肝外病変が明らかとなり，手術非適応例の手術が避けることができる点.

不利な点は，

①　遅らせることで肝転移巣からの二次性の転移の危険性が高くなる点.

②　肝転移巣を持ったままのため，患者の病気に対する不安が持続する点.

である.

3．手術術式

解剖学的系統切除か非解剖学的部分切除のどちらの術式を基本術式とするかは，統一された見解は得られてはいない．しかし，腫瘍条件に関わらず多くの症例を手術適応にするには，肝部分切除術が良いとする意見がある[8)]．また，術中超音波検査の導入により，できるだけ非癌肝実質を温存し，かつ腫瘍を離断面に露出させずに切除することが可能となった．安全な外科的切除断端を確保することが重要である．症例によっては，転移巣が片葉やある区域にのみ偏在多発している場合は，片葉切除や区域切除術を選択する.

4．門脈塞栓術

技術的には肝転移巣を切除できるが，切除後の残肝量が少なくなり，手術適応からはずれる症例の場合，肝切除側の門脈を塞栓(通常右葉の塞栓)し，残肝量を術前に増やして肝切除術を施行する

方法である[2)3)9)]。残肝に腫瘍がないことが絶対的な条件となる。

5．肝所属リンパ節の郭清

肝門部リンパ節転移は肝外への転移病変の存在を示唆し，Rodgers と McCall による15研究を統合した review において，145名の肝所属リンパ節転移陽性大腸癌肝転移患者のうち，リンパ節転移陽性の5年生存者は5名であり，予後はきわめて不良であると報告した[10)]。大腸癌肝転移症例における病理組織学的に肝所属リンパ節転移を認める症例は11～28%である[11)-15)]。予防的肝所属リンパ節郭清については，予後に対する寄与を認めず，また治療効果に関しても不明である[13)]。また肝門部リンパ節郭清を標準手術術式にすることは，残肝再発の多い大腸癌肝転移では，再手術時の切除が難しくなるため，障害となることがある。

6．外科切除断端

肝離断面に腫瘍が露出した場合の予後が不良であることは明らかであるが[16)17)]，切除断端までの距離に関しては一定の見解が得られてはいない。つまり，10 mm 以上必要だとする施設と，腫瘍が露出していなければ良いとする施設の意見がある。腫瘍が肝離断面に露出するということは，腫瘍の遺残の可能性が高く絶対に避けなくてはならないが，切除断端までの距離を厳密に規定し切除の必要条件とすると，多発例の多い大腸癌肝転移の場合，手術適応例が制限されてしまう。そのため，切除断端距離は肝転移巣の数や部位，また切除技術のバランスにより決まるものである。

7．成　　績

肝切除方針・適応と肝切除手術技術に密接に関連するが，肝切除施行率は25～52%である[18)-20)]。

手術死亡率は，最近の手術技術の進歩や術中・術後管理の向上により0～3%である。本邦における厚生省がん研究・加藤班研究15施設で集積した410例の大腸癌肝転移肝切除例の累積5年生存率は50.1%であった[21)]。最近，欧米の報告でも58%という50%を超える報告がみられるようになり，症例選択基準の確立，肝切除技術の向上，再肝切除の施行が貢献していると考えられる[22)]。

8．予後因子(Prognostic factors)

一般的には，

① 原発巣組織型
② 転移腫瘍数
③ 外科切除断端陽性の有無
④ 肝所属リンパ節転移
⑤ 肝外病巣
⑥ 肝内微小転移(衛星病変)

切除病理標本から，

① 腫瘍偽被膜
② 肉眼的胆管侵襲
③ 肝内血管侵襲
④ リンパ管侵襲
⑤ 腫瘍内に巻き込まれた島状の肝細胞集団

一方，

① 肝転移の診断時期(同時・異時)
② 原発巣病期の進行度
③ 転移巣の肝内分布(片葉・両葉)
④ 切除術式(部分切除・解剖学的切除)
⑤ 切除断端の距離(10 mm 以上・未満)

などに関しては，各施設の報告により異なる。各研究における切除対象症例の基準の違いや，転移性肝腫瘍の病態の複雑さもあり，その予後には多くの因子が関与するため，一定の見解が得られにくいと思われる。

9．再肝切除

初回肝切除時に完全に切除された症例でも45～80%に再発を認め，そのうち約30%は肝のみの再発である[23)-25)]。

肝転移巣切除後の残肝再発に対して根治を得る可能性がある場合，積極的に再肝切除が行われる[24)26)]。Yamamoto ら[24)]は肝切除後の残肝再発75例に再肝切除を行い，3年生存率48%，5年生存率31%であったと報告している。他の報告でも5年生存率は30%前後であり，手術死亡も少なく安

全な治療となった．このことから，肝切除後残肝再発に対しては積極的な再肝切除にて治癒に持ち込むことができるようになったが，再肝切除術後再発率は70%と高い．

再肝切除の予後因子は初回切除時と同様で，

① 転移巣4個以上
② 外科切除断端陽性
③ 肝所属リンパ節陽性
④ 肝外病巣

が有意の予後不良因子である．

再肝切除の際の安全面から注意すべき点は，

① 初回肝切除後，補助肝動注療法施行症例では，残肝機能が低下している．

② 前回手術の影響による肝周囲の線維性癒着が術後の肝再生を阻害する場合がある．

以上のことに留意し，初回肝切除よりは残肝予備能に余裕をもたせることが必要である．

10. 肝切除後補助療法

治癒的肝切除後の残肝再発は前述したように45～80%であり，残肝再発予防として肝動注療法が試みられるが，結果は controversial である[27)28)]．その理由として，高い肺再発率がある．肝動注による残肝再発予防だけでは，予後の改善には必ずしも大きな比重を占めていない．一方 Kemeny ら[29)] は肝切除後の補助療法として，combined therapy (肝動注+全身化学療法) と monotherapy (全身化学療法) との比較試験を行い，2年生存率の比較において combined therapy 群の有意を明らかにしている．

II. 熱凝固療法

マイクロ波凝固療法 (MCT：microwave coagulation therapy)，ラジオ波焼灼療法 (RFA：radiofrequency ablation) は，肝腫瘍内にマイクロ波またはラジオ波を発生する電極を刺入し，一定範囲の熱凝固により腫瘍壊死を誘導する治療法である．

マイクロ波は電磁波であり，周波数2,450 MHz，原理は電子レンジと同じで，水分子の運動によって熱が生じる．体内にある水分子がマイクロ波により高速運動し，その際に発生した熱を利用して腫瘍組織を焼灼する治療法である[30)]．ラジオ波の周波数は，450～480 MHz で，RFA は病変に挿入した電極から周囲組織にラジオ波の交流が流れることによるイオンの変動が摩擦熱を発生し，熱凝固壊死を起こす局所治療法である[31)]．

熱凝固療法は長期に局所制御が可能でかつ根治も可能な治療法であり，肝切除が困難な症例でも施行可能である．大腸癌肝転移の治療における熱凝固療法の位置づけとしては，肝切除に次ぐ局所治療方法と考えられる．しかし，あくまでも肝切除に次ぐ局所療法であり，完全焼灼が不確実と考える場合は根治的な肝切除を追加することを常に念頭にいれて経過観察しなければならない．

1. 適　　応

MCT と RFA の適応条件は基本的には同じである．

① 原則的には，肝切除不能あるいは肝切除を行うにはリスクの高い症例．

② 超音波検査，CT 検査などで腫瘍の同定が可能．

③ 腫瘍が肝門部脈管や肝静脈根部に近接していない．

④ 腫瘍塞栓を認めない．

⑤ 高度な肝機能障害，高度な出血傾向を認める例は除外．

2. アプローチ法

経皮的，内視鏡下(腹腔鏡下・胸腔鏡下)，開腹，開胸下のアプローチがある[32)]．

経皮的アプローチの際，超音波検査にて同定が困難な症例に対して，CT や MRI 検査ガイド下で施行も可能である．一般的に経皮的アプローチは，大きさ2 cm 以下で深部に存在し，超音波検査，CT で同定が可能な病変を対象とする．内視鏡下アプローチは3 cm 以下で肝表在性の病変を対象とする．経皮的，内視鏡下のアプローチが困