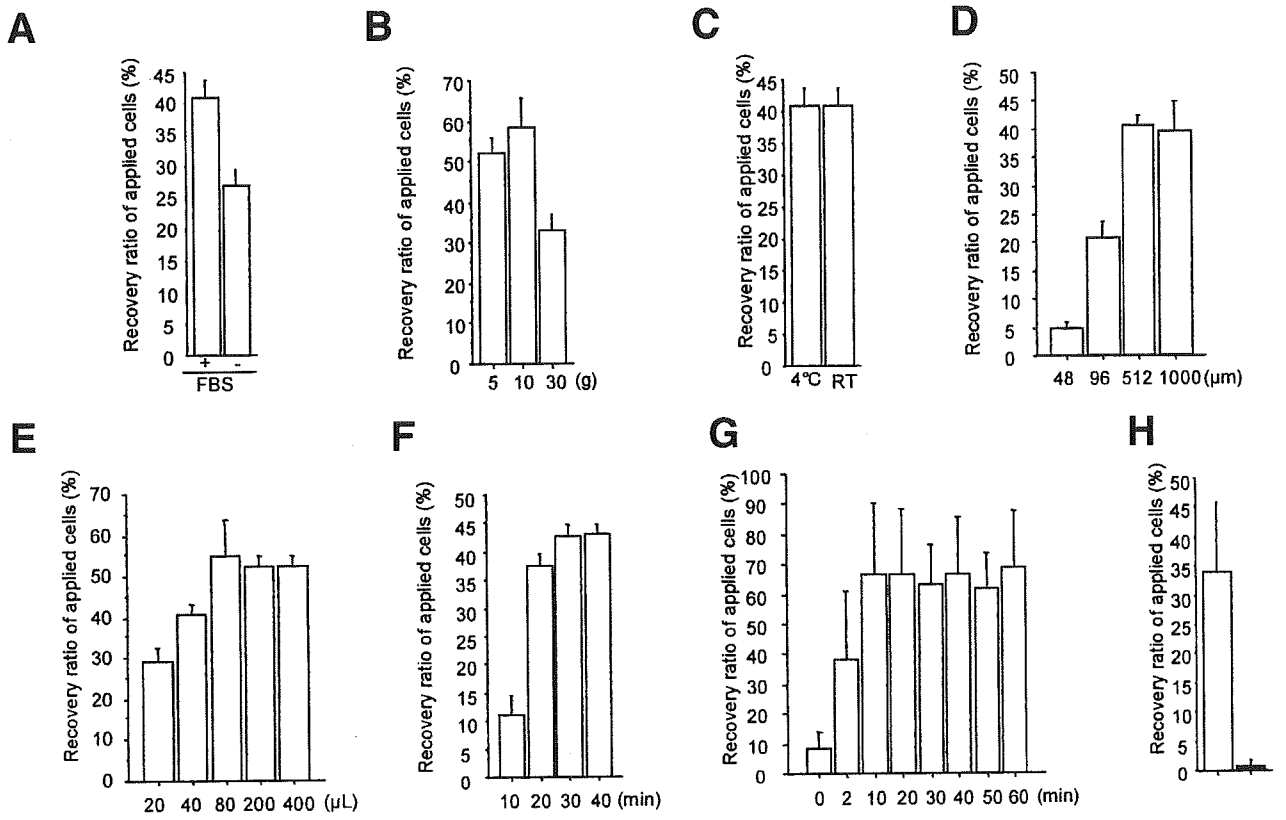

Figure 2. Simulation study to establish the optimal conditions for retrieving HT-29 colorectal cancer cells from feces and to compare the cell retrieval rates for the magnetic beads methods and the Percoll centrifugation method. Feces from healthy volunteers were divided into several portions, each of 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

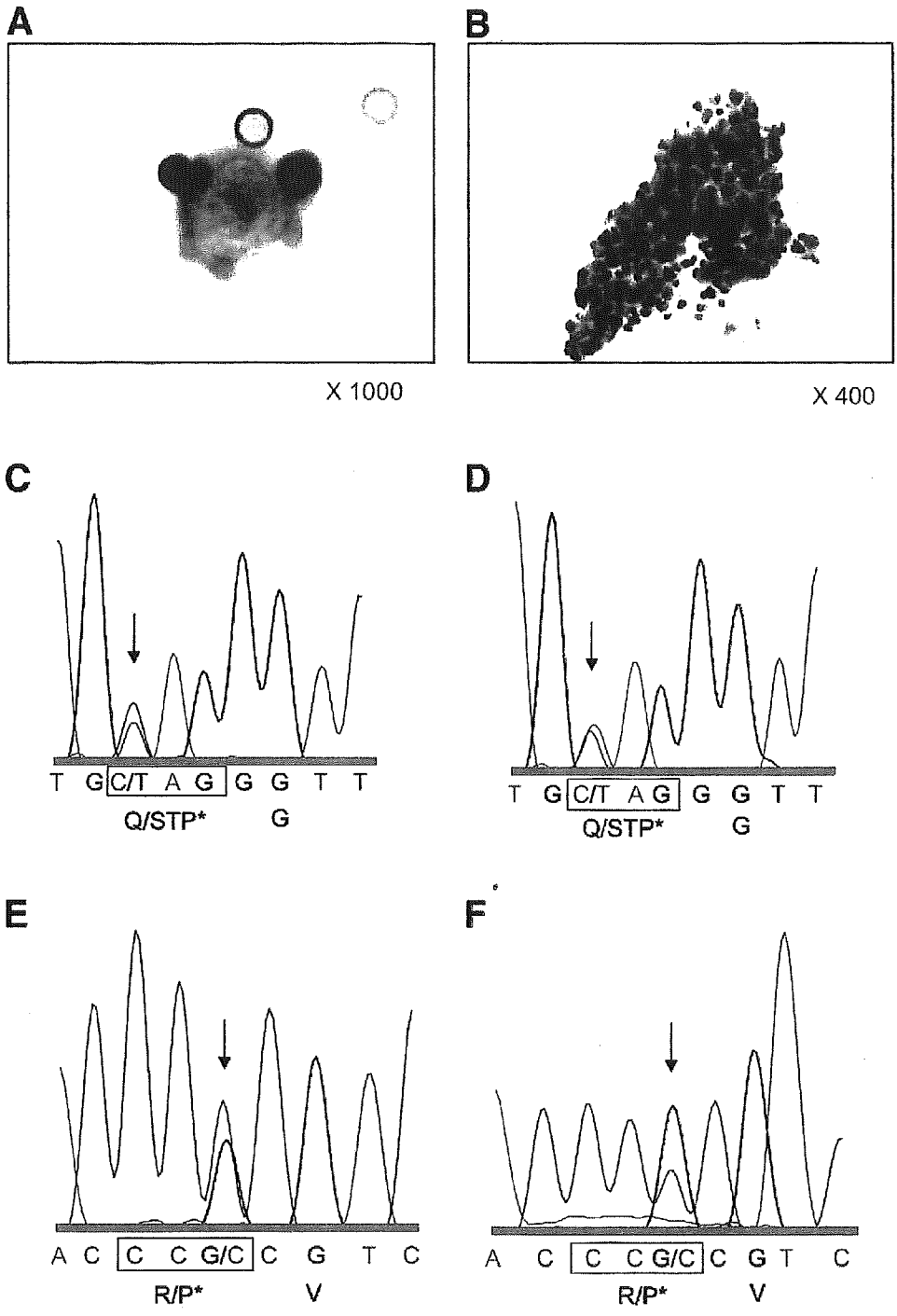

Figure 3. Cytology and DNA sequencing. Papanicolaou staining of colonocytes isolated from the feces of patients with colorectal cancer. (*A*) A patient with ascending colon cancer, Dukes' stage A. (*B*) A patient with rectal cancer, Dukes' stage C. Detection of mutations in tumor tissues and colonocytes 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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Original article

Feasibility of autonomic nerve-preserving surgery for advanced rectal cancer based on analysis of micrometastases

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Background: Autonomic nerve preservation has been advocated as a means of preserving urinary and sexual function after surgery for rectal cancer, but may compromise tumour clearance. The aim of this study was to determine the incidence of micrometastasis in the connective tissues surrounding the pelvic plexus.

Methods: The study included 20 consecutive patients who underwent rectal surgery with bilateral lymph node dissection for advanced cancer. A total of 78 connective tissues medial and lateral to the pelvic plexus and 387 lymph nodes were sampled during surgery. All connective tissue samples and 260 lymph nodes were examined for micrometastases by reverse transcriptase–polymerase chain reaction (RT–PCR) after operation. All patients were followed prospectively for a median of 36·0 months.

Results: Of 245 histologically negative lymph nodes, 38 (15·5 per cent) were shown by RT–PCR to harbour micrometastases. However, micrometastases to tissues surrounding the pelvic plexus were detected in only two (3 per cent) of 78 tissues, that is in two of 20 patients. Clinical follow-up showed that the two patients had a poor prognosis owing to distant metastases.

Conclusion: Autonomic nerve-preserving surgery may be feasible for advanced rectal cancer, but study of more patients positive for micrometastases is required.

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Introduction

The complex flow patterns of lymphatic fluid around the rectum were investigated in 1951 by Sauer and Bacon[1]. They regarded the lateral ligament as the tissue in which the inflow of lymphatic fluid from the rectum first occurs *en route* to the internal iliac artery. However, the high incidence of urinary and sexual dysfunction after rectal cancer surgery gradually led surgeons to realize that the lateral ligament plays an important role in postoperative urinary and sexual function[2]. The pelvic autonomic nerve system is comprised of the superior hypogastric plexus, hypogastric nerves, pelvic plexus (inferior hypogastric plexus) and pelvic splanchnic nerves[3]. The lateral ligament of the rectum consists of a neurovascular bundle derived from the combined redistributing sympathetic and parasympathetic nerves of the system, and dissection injury to these nerves may cause problems.

Inclusion of total mesorectal excision (TME) in rectal cancer surgery can improve the rate of cure[4]. Dissection of the rectum at the layer of TME allows preservation of the pelvic autonomic nerves, reducing postoperative urinary and sexual morbidity[5–7]. Extensive pelvic lymphadenectomy involving resection of both pelvic autonomic nerves and lateral lymph nodes was employed in Japan from the 1970s to the early 1980s with the aim of reducing local recurrence of rectal cancer[8]. However, postoperative urinary and sexual dysfunction invariably ensued, leading to marked reduction in quality of life. More recently, efforts have been made to preserve urinary and sexual function and to achieve local control of cancer, by a combination of autonomic nerve-preserving surgery and lateral lymph node dissection[9–12].

Recent pathological studies have warned against autonomic nerve preservation because histologically proven cancer foci might exist in the autonomic nerves

and surrounding connective tissues, and lead to local recurrence[13,14]. To aim of the present study was to examine the incidence of micrometastases in the region of the pelvic plexus by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis, which is more sensitive than conventional histological diagnosis[15,16].

Patients and methods

The study included 20 consecutive patients who underwent rectal cancer surgery with bilateral lymph node dissection at the Graduate School of Medicine, Osaka University between October 1999 and May 2001. Demographic data are shown in *Table 1*. Tumours were located 0–10 (median 6·5) cm above the anal verge and diagnosed before surgery as advanced cancer (T2,T3 and T4)[17]. The operative procedures are summarized in *Table 1*. Curative resection was performed in all patients. Tumour stages determined by postoperative histological examination are shown in *Table 1*[17]. Two patients with lateral node metastasis without distant organ metastasis were included in stage III (*Table 2*). Nine patients with stage III or IV tumours were treated with 5-fluorouracil-based chemotherapy but none had radiation therapy. The patients were reviewed at least every 3 months after operation with blood tests such as measurement of carcinoembryonic antigen (CEA), and underwent computed tomography (CT) or magnetic resonance imaging (MRI) every 6 months. The study protocol was approved by the Human Ethics Review Committee of Osaka University Graduate School of Medicine and a signed consent form for the study was obtained from each patient.

Table 1 Clinical characteristics of patients

Median (range) age (years)	63·5 (35–92)
Sex ratio (M : F)	15 : 5
Surgical procedure	
Low anterior resection	9
Abdominoperineal resection	7
Hartmann resection	3
Total pelvic exenteration	1
Autonomic nerve preservation	
Bilateral	18
Unilateral	2
TNM stage	
I	4
II	7
III	6
IV	3
Histological type	
Well differentiated	1
Moderately differentiated	16
Poorly differentiated	2
Mucinous	1
Tumour location (cm)*	
< 6	9
≥ 6	11

*Distance from anal verge. TNM, tumour node metastasis.

All patients underwent radical resection with autonomic nerve preservation and lymph node dissection[11]. The following is a brief description of the surgical procedures, with schematic presentation in *Fig. 1*, focusing on the collection of specimens. After isolating the sigmoid colon, the mesorectum was dissected from the parietal fascia of the sacrum at the layer of TME to preserve the hypogastric nerves, and the medial side of the pelvic plexus was exposed. At this stage, the connective tissues of the medial and lateral sides of the pelvic plexus were meticulously sampled taking care not to injure the plexus, with four specimens being obtained from each patient. However, if the tumour had macroscopically infiltrated the pelvic plexus, only tissue from the lateral side was collected because the infiltrated pelvic plexus was dissected *en bloc* with the tumour. The specimens were promptly frozen in liquid nitrogen and stored at −70°C pending RNA extraction. A total of 78 connective tissues were obtained from 20 patients (*Table 2*).

After removing the tumour and performing lateral node dissection, the lymph nodes were sampled from the mesorectum and the lateral area along the internal iliac artery and the obturator nerve outside the boundaries of TME (*Fig. 1*). In 12 patients, each of the 260 lymph

Table 2 Metastasis to lymph nodes and tissues surrounding pelvic plexus

		Site of metastasis			Survival at 1 year	
TNM stage	No. of patients	Upper lymph nodes (HE)	Lateral lymph nodes (HE)	Tissues surrounding pelvic plexus (micrometastases)	Relapse free	Overall
I	4	0	0	0 (16)	4	4
II	7	0	0	0 (28)	6	7
III	6	6	2	0 (24)	5	6
IV	3	3	3	2 (10)	0	0

Values in parentheses are number of tissues; HE metastasis detected by histological examination with haematoxylin and eosin staining. TNM, tumour node metastasis.

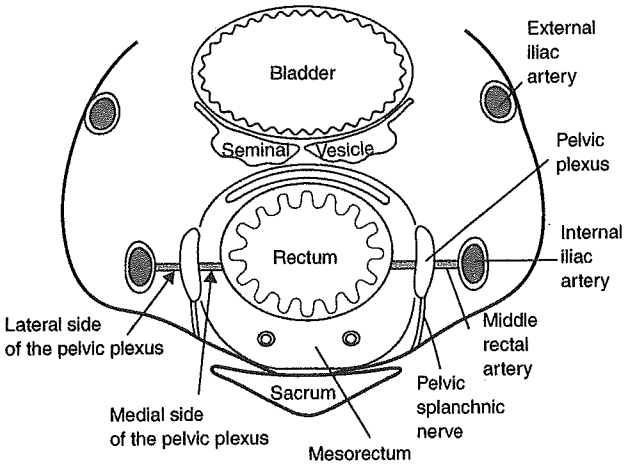

Fig. 1 Schematic presentation of the pelvic autonomic nerve system and connective tissues surrounding the pelvic plexus. The connective tissues comprise fat and lymphatic vessels including the middle rectal artery. The medial tissue is equivalent to the lateral ligament and the lateral tissue is located outside the boundaries of total mesorectal excision, between the plexus and internal iliac artery

node samples was halved, with one part being subjected to conventional histological diagnosis and the other being frozen in liquid nitrogen and stored at −70°C until extraction of RNA. In the other eight patients, 127 samples were collected and subjected to histological diagnosis only.

Total RNA was extracted by a single-step method as described previously[18]. Complementary DNA was generated with avian myeloblastosis virus RT using the procedure outlined by the supplier (Promega, Madison, Wisconsin, USA). *CEA* and *cytokeratin 20* (*CK-20*) transcripts were used as sensitive markers for micrometastases, and *phorphobilinogen deaminase* (*PBGD*) transcript was used to check for the presence of mRNA in samples[15]. PCR products were analysed by electrophoresis on 2 per cent agarose gels stained with ethidium bromide. The reproducibility of cDNA products was checked by repeated RT–PCR and gel electrophoresis. The sensitivity of PCR was determined by detecting *CEA* and *CK-20* transcripts in serial dilutions of a human colonic cancer cell line (HT29) mixed with human lymphocytes; the detection sensitivity was 100 HT29 cells among 10^6 lymphocytes.

Results

The presence of *PBGD* products was confirmed in all 78 connective tissue specimens and 260 lymph nodes examined for micrometastases by RT–PCR. The diagnosis was positive if bands specific for *CEA* or *CK-20* were present[15].

The 260 lymph nodes were collected from 12 patients (median 22 per patient). The appearance of both *CEA* and *CK-20* mRNAs was verified in all 15 histologically positive lymph nodes. Of the 245 histologically negative lymph nodes, 38 (15·5 per cent) harboured micrometastases (*Table 3*).

Metastases were identified histologically in the upper lymph nodes within the mesorectum in nine of 20 patients, and in the lateral lymph nodes along the internal iliac artery and the obturator nerve in five of 20, suggesting that many patients had very advanced cancer. Direct tumour invasion into the right or left pelvic plexus was observed macroscopically in two patients with stage IV disease. The pelvic plexus on the affected side was therefore resected *en bloc* with the tumour, whereas that on the other side was preserved.

Eighteen of 20 patients had neither *CEA* nor *CK20* mRNA in the four connective tissues surrounding the bilateral pelvic plexus. Two of three patients with stage IV disease were positive for both *CEA* and *CK20* mRNA in the lateral tissue from the resected pelvic plexus, but all other tissues surrounding the preserved pelvic plexus were negative. Micrometastases were identified in two (3 per cent) of 78 samples of connective tissue surrounding the pelvic plexus, that is in two of 20 patients (*Table 2*). All five patients with lateral node metastasis had metastasis to the upper lymph nodes, but only two had micrometastases to the connective tissues surrounding the pelvic plexus.

Median follow-up after surgery was 36·0 months. By 1 year after surgery, five of 20 patients had developed local recurrence or distant metastases, despite undergoing curative surgery and postoperative chemotherapy, and three had died from cancer (*Table 2*). Of three patients with pelvic recurrence, one patient showed relapse in the lateral area of the pelvis despite lateral node dissection, and two patients developed recurrent tumour in the rectum after a Hartmann's procedure. The autonomic

Table 3 Lymph node metastases detected by reverse transcriptase–polymerase chain reaction and histological examination with haematoxylin and eosin staining

	RT–PCR		
	Positive	Negative	Total
Haematoxylin and eosin			
Positive	15	0	15
Negative	38	207	245

RT–PCR, reverse transcriptase–polymerase chain reaction.

nerve system was preserved completely in 18 patients and unilaterally in two; no recurrence was found in this region by repeated follow-up CT and MRI. The overall survival rate, estimated by the Kaplan–Meier method, of the 18 patients without micrometastases surrounding the pelvic plexus was 94 per cent at 1 year and 88 per cent at 3 years. Neither of the patients with micrometastases was alive at 1 year after surgery.

Discussion

The lateral ligament is still regarded as a pathway of lymphatic vessels (middle lymphatic flow) from the lower rectum towards the lateral lymph nodes[1,19]. However, in the present study micrometastases to the connective tissues, including the lateral ligament, were identified by highly sensitive RT–PCR analysis in only two patients with distant metastases. Three of five patients with both upper and lateral lymph node metastases had no micrometastases in the connective tissues. A partial explanation for the discrepancy between the presence of lymph node metastases and the very low incidence of micrometastases to the connective tissues might be that lateral lymph node metastases developed via lower lymphatic flow rather than via middle lymphatic flow through the lateral ligament[19].

The autonomic nerve system was completely preserved in all but two patients in the present study, However, no local recurrence in the region of the preserved nerve system was observed by CT and MRI during follow-up. Contrary to expectation, micrometastases to the connective tissue surrounding the pelvic plexus were rare, verifying the feasibility of nerve preservation without oncological compromise in most patients.

Neither patient with micrometastases in the tissues surrounding the pelvic plexus survived for 1 year after surgery. Ueno *et al.*[14] performed complete dissection of the autonomic nerve system and pelvic lymph nodes with the aim of achieving local control in 61 patients with rectal cancer. They reported spread of cancer cells to the autonomic nerves in nine patients (15 per cent), six with Dukes' C and three with Dukes' 'D' lesions. The patients with Dukes' C tumours underwent curative radical resection, but all developed recurrence within 1 year and none survived for 4 years. The circumferential resection margin for TME is located inside the pelvic plexus whereas the pelvic nerve plexus and the lateral tissue are situated outside the margin. It has also been documented that TME in patients with tumour involvement of circumferential resection margin is associated with a poor prognosis[20].

The present results indicate that any patients with micrometastases in the preserved pelvic plexus already have advanced cancer, so their prognosis is unlikely to be affected by local recurrence that might develop if the autonomic nerves are preserved. Management of such patients should focus on maximizing the quality of remaining life.

The follow-up period in the present study was relatively short (median 36·0 months). However, some 50–80 per cent of local recurrences occur within 2 years after rectal cancer surgery, with a peak at 6–12 months[21]. The follow-up period should therefore have been sufficient for the analysis.

Based on examination of micrometastases, these results suggest that the autonomic nerve system should be preserved wherever possible, even in surgery for advanced rectal cancer. However, study of more patients positive for micrometastases is needed.

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Methylation and expression of p16^{INK4} tumor suppressor gene in primary colorectal cancer tissues

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Abstract. It is known that p16^{INK4} tumor suppressor gene expression in colon cancer cells is repressed by methylation at the CpG island of promoter, but *in vivo* silencing of p16 gene is not fully understood. Some studies showed that primary colorectal cancer (CRC) tissues often overexpress the p16 protein, while others showed the high incidence of p16 methylation. The aim of this study was to clarify p16 gene regulation *in vivo*. We used real-time methylation-specific PCR (MSP) to examine density of p16 methylation, and immunohistochemistry, Western blot analysis to determine p16 protein expression. Methylation was detected in 5 CRC cell lines tested and 9 of 21 (42.9%) CRCs. Four of 5 CRC cell lines did not express p16 mRNA, but 6 of 9 CRCs did express p16 mRNA even with methylation. Real-time MSP showed that CRC tissues had a wide variety in methylation density (methylation index: 0.28-0.91) and that highly methylated CRC tissues displayed significantly lower p16 mRNA expression than those with no-methylation or low-methylation. Immunohistochemistry showed that the majority of CRCs (53 of 55: 96.4%) overexpressed the p16 protein. Low p16 expression was associated with lymph node metastasis (p=0.003) and large tumor size (p=0.048). Western blot in a subset of non-tumor and tumor samples showed a consistent overexpression of the p16 protein. These results showed that CRC tissues displayed variable methylation density, which may be characteristics of p16 gene methylation *in vivo*. Our data suggest that a low p16 expression due to methylation may contribute to tumor enlargement and expansion of CRC.

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Key words: p16^{INK4}, colorectal cancer, methylation density, methylation-specific PCR

Introduction

The progression of the cell cycle is controlled by the cyclin-dependent kinase (CDK)/cyclin complex countered by CDK inhibitors (CKIs) (1,2). Cyclin D1/CDK4, CDK6 and cyclin E/CDK2 control the progression from the G1 to S phase of the cell cycle. Based on their structural and functional characteristics, CKIs are classified into two groups, the p21^{Waf1} family, which includes p27^{Kip1} and p57^{Kip2}, and another group, which consists of p16^{INK4a} (hereafter designated p16), p15^{INK4b}, p18^{INK4c} and p19^{INK4d}. The p16 gene is localized on chromosome 9p21 and the p16 family can form complexes with CDK4, CDK6 and D-type cyclins (3-5). Overexpression of INK4 proteins can arrest cells in the G1 phase through inhibition of cyclin D/CDK activity (6). On the other hand, p16-deficient mice develop spontaneous tumors at an early stage and are highly sensitive to carcinogens, suggesting that p16 is a tumor suppressor gene (7). Indeed, deletions and mutations of the p16 gene are frequently present in primary cancers of the brain, biliary tract, lung, pancreas and esophagus (8-12), although a panel of cancer cell lines tends to retain p16 gene alterations more frequently (3). There is also evidence that p16 expression is down-regulated by *de novo* methylation of 5' CpG islands in the p16 promoter region (13,14).

In colorectal cancer (CRC) cell lines, expression of the p16 protein was reported to be undetectable and inactivation of p16 is thought to be a common alteration in CRC (15). It appears that deletion or loss of the p16 gene is rare in CRC (13,16,17) and the alternative pathway for inactivation of the p16 gene is thought to be methylation of its promoter (13,14). In CRC tissues, there is evidence that the p16 gene promoter is methylated in 29-55% of primary cancer tissues (13,18-22), whereas in non-neoplastic colonic mucosa the p16 promoter is hardly methylated (18,23). These findings invoke the notion that p16 gene inactivation via methylation may be involved in carcinogenesis of the colorectum.

However, recent immunohistochemical studies have shown that the majority of CRC tissues (64-82%) expressed the p16 protein, while normal mucosa displayed negative or very low expression (24,25). This overexpression of p16 in CRC tissues

autocrine stimulator of the ovarian surface epithelium which may be involved in the growth of ovarian tumors. Their immunohistochemical studies of FGF7 in borderline, stage I, and stage III human ovarian tumors revealed intense epithelial staining. The results of these immunohistochemical studies are consistent with our present results. Moreover, in 20 surgical specimens of ovarian cancer, we found that the incidence of FGF7 expression was higher in advanced ovarian cancers (stages III and IV) than in early ovarian cancers (stages I and II). Several different growth factors, including FGF, TGF-α, TGF-ß, bFGF, and FGF7, may be involved in controlling the normal and abnormal growth of ovarian surface epithelium. Expression of FGF7 by the ovarian surface epithelium suggest that this type of epithelial cell is normally in an altered state of differentiation which is susceptible to transformation.

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is somewhat paradoxical in view of the possible role of p16 as a tumor suppressor gene, indicating that the above scenario on human CRC tumorigenesis via p16 inactivation may not be quite as simple *in vivo*. The question then arises as to how methylation and overexpression of the p16 gene can coexist in a significant fraction of CRC tissues? One clue to this puzzle might be the difference in methylation density of the CpG island in the p16 promoter between cell culture systems and primary CRC tissues, since it is reported that the level of transcriptional repression is dependent on methylation density (26,27). Although many studies have examined the incidence of p16 methylation alone or p16 expression alone *in vivo* (18-23), no study has yet simultaneously examined the degree of methylation and p16 gene expression in CRC tissues. To address this question, we examined p16 methylation and p16 gene expression in CRC tissue samples together with control experiments using CRC cell lines. Real-time methylation-specific PCR (MSP) was also performed in methylation-positive CRC tissue samples. An extended immunohistochemical analysis was conducted to elucidate the functional significance of the p16 protein in CRC tissues. Our data provide a rationale for methylation-associated regulation of p16 expression in primary CRC tissues.

Materials and methods

Cell lines and tissues. The human breast epithelial cell line HBL100, human CRC cell lines HCT116, SW480, LoVo, HT29, DLD1, human breast cancer cell line MDAMB468, and human glioblastoma cell line T98G were obtained from the Japanese Cancer Research Resources Bank and ATCC (American Type Culture Collection). These cells were cultured in RPMI-1640 (Nissui, Tokyo, Japan) or DMEM medium (Nikken Bio Medical Laboratory, Kyoto, Japan), supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum at 37°C in 5% CO_2.

A total of 55 samples of CRC tissues together with adjacent non-neoplastic mucosa were obtained from patients who underwent surgery at the Department of Surgery and Clinical Oncology, Osaka University, between 2000 and 2001. The age of the patients ranged from 28 to 81 years (mean age: 61 years). The resected surgical specimens were fixed in 10% buffered formalin, dehydrated in graded ethanol, and embedded in paraffin. Tissue sample of sufficient quantity was frozen immediately in liquid nitrogen and stored at -80°C until use for reverse transcription-polymerase chain reaction (RT-PCR), methylation assay or immunoblotting. Samples of non-neoplastic mucosa were excised at least 5 cm lateral to the tumor. The study protocol was approved by the Human Ethics Review Committee of Osaka University, Graduate School of Medicine.

Immunostaining. The tissue specimens fixed in 10% buffered formalin were sliced into 4-µm thick sections, deparaffinized in xylene and rehydrated with graded concentrations of ethanol. Immunostaining was performed using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA) after boiling for antigen retrieval, as described in our previous studies (28,29). Anti-$p16^{INK4}$ polyclonal antibody, which was raised against a full-length recombinant GST-p16 fusion protein, was purchased from PharMingen (San Diego, CA). The primary antibody was applied to the sections at a dilution of 1:400. The human breast epithelial cell line HBL100, which expresses a high level of p16, and the glioblastoma cell line T98G in which the p16 gene is inactivated by homozygous deletion, were used as positive and negative controls, respectively (30). For the absorption test, the immunogen was obtained from PharMingen.

Evaluation of immunohistochemistry. Inflammatory cells served as positive internal controls and nuclear staining for p16 was considered positive as reported previously (30-32). Ten fields in each specimen were randomly selected and examined under high power magnification and >500 cells were counted in order to determine the labeling index (LI), which represented the percentage of cells that were p16-positive. The samples were then classified into three groups according to the value of LI: group A consisted of tissue samples that contained >50% p16-positive cells, group B contained 10-50% p16-positive cells, and group C contained <10% p16-positive cells. Staining was repeated at least twice to eliminate possible technical errors, and the results were reproducible.

Western blot analysis. Approximately $5x10^6$ cells or 100 mg of tissue were homogenized and lysed in 1.0 ml of lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP40, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin, and then placed on ice for 10 min. The lysates were clarified by centrifugation at 15,000 x g for 25 min at 4°C. The protein samples were subjected to SDS-PAGE (15% gels) and immunoblotting was performed as described in our previous studies (28-30). The final dilution of the primary antibody was 1:1,000.

RNA extraction and RT-PCR analysis. Total RNA was extracted with a single-step method using TRIzol reagent (Invitrogen Corp., Carlsbad, CA), and cDNA was generated using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). Semi-quantitative analysis for expression of p16 mRNA was performed by the multiplex RT-PCR technique, using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the internal standard (33,34). To minimize the inter-PCR difference, PCR was performed with GAPDH and p16 primers in identical tubes under unsaturated conditions, as described in our previous studies (33,35,36). PCR reactions were performed in a total volume of 25 µl of reaction mixture containing 2 µl of cDNA template, 1X Universal PCR buffer, 2 mM deoxynucleotide triphosphates, 20 pmol of primer for p16, 4 pmol of each primer for GAPDH, and 1 unit of Taq DNA Polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc., Alameda, CA). The primer set for p16 was designed to be localized in exon 1 and exon 2 of the p16 gene, flanking intron 1 and tested to ensure amplification of cDNA only, so that amplification of potentially contaminating genomic DNA could be avoided. The sequences of these PCR primers were as follows: p16 sense primer, 5'-AGC CTT CGG CTG ACT GGC TGG-3'; p16 antisense primer, 5'-CTG CCC ATC ATC ATG ACC TGG A-3'. The primers for GAPDH were synthesized as described previously (34). The sizes of the

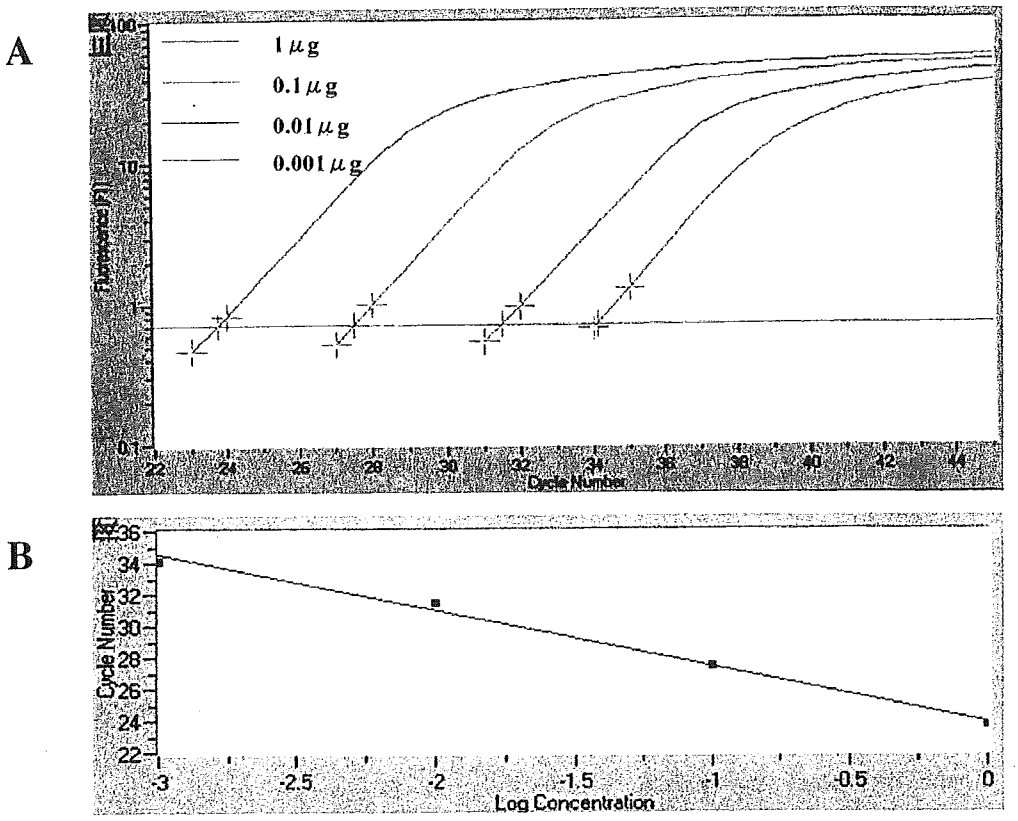

Figure 1. Methylation-specific PCR (MSP) using serial dilutions of methylation control DNA. (A). MSP was performed using 10-fold serial dilutions of methylation control genomic DNA from 2 ng to 2 μg. The PCR products were monitored by the fluorescence of dsDNA-specific dye SYBR Green I. (B), Based on the amplification results of (A), a standard curve was drawn using LightCycler software. Similar curves were drawn for unmethylated p16 DNA products, using bisulfite-converted unmethylated control genomic DNA (data not shown).

amplicons for p16, and GAPDH were 139 and 181 bp, respectively. The PCR conditions were as follows: a) initial denaturing at 95°C for 12 min; b) 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and c) a final extension at 72°C for 10 min. Each PCR product (10 μl) was electrophoresed on 2% agarose gels and stained with ethidium bromide. The PCR products were assayed by densitometry.

Methylation-specific PCR (MSP). To detect methylation at the 5' CpG island in the p16 promoter region, MSP was performed using the CpGenome™ DNA Modification kit (Chemicon International, Inc., Temecula, CA), as recommended by the supplier (30). Briefly, genomic DNA was modified by treatment with sodium bisulfite, which converts all unmethylated cytosines to uracils while 5-methylcytosines remain unaltered. PCR amplification was then carried out using primers specific for either methylated or unmethylated DNA (CpG WIZ™ Amplification Kit, Chemicon International, Inc.). The PCR mixture contained Gene-Amp 10X Universal PCR buffer, 2.5 μl of 2.5 mM dNTPs, 1.0 μl of primer pairs, 2 μl of template DNA, and 0.2 μl of AmpliTaq Gold (Roche Molecular Systems, Inc., Alameda, CA) for a final volume of 25 μl. Amplification was performed in a temperature cycler (Takara, Shiga, Japan) for 35 cycles (45 sec at 95°C, then at annealing temperature for 45 sec, and finally 60 sec at 72°C), followed by a final 5-min extension at 72°C. The PCR samples were loaded onto a 2% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination.

Quantitative real-time MSP using LightCycler™. Quantitative PCR was performed using LightCycler system (Idaho Technology Inc., Salt Lake City, UT), as described in our previous studies (36,37). Briefly, 10 μl PCR reaction contained 0.2 μM of each primer, LightCycler-DNA Master SYBR Green I (Roche Molecular Systems, Inc.), 4 mM $MgCl_2$ and 2 μl of template DNA. PCR conditions were set up as follows: one cycle of denaturing at 95°C for 2 min, followed by 50 cycles of 95°C for 0.1 sec, 62°C for 5 sec and 72°C for 18 sec. Fluorescence was acquired at the end of each 72°C extension phase. The melting curves of the final PCR products were analyzed after 50 cycles of PCR amplification by cooling samples to 65°C, increasing the temperature up to 99°C at a rate of 0.1°C/sec, and monitoring fluorescence at each 0.1°C. Quantification data from each sample were analyzed using LightCycler™ analysis software. Two real-time quantitative PCR reactions were performed for the detection and quantitation of the bisulfite-unconverted methylated version of the p16 gene and the bisulfite-converted unmethylated version of the p16 gene. Serial dilutions of methylated or unmethylated control genomic DNAs (CpG WIZ™ Amplification kit, Chemicon International, Inc.) were used for constructing the standard curves (Fig. 1).

The methylation index (MI) in each sample was calculated using the following equation (38):

$$\text{Methylation index} = \frac{M}{M+U} \times 100$$

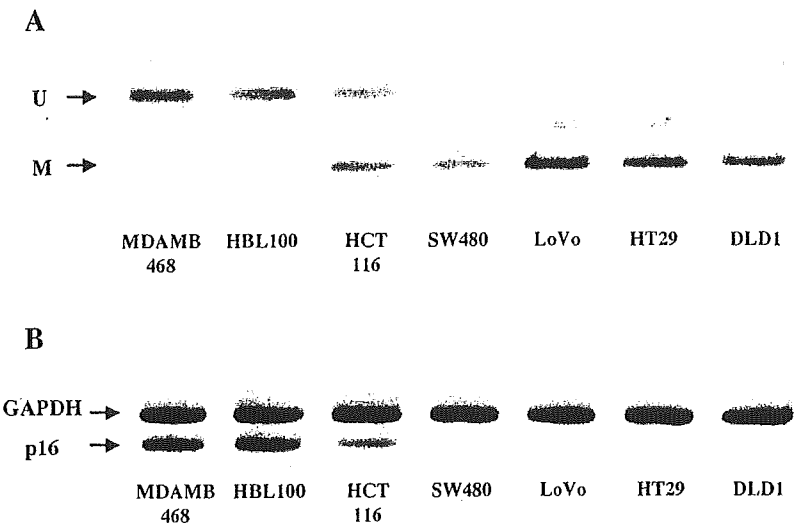

Figure 2. (A), Methylation of the p16 promoter region in cell lines. The breast cancer cell line MDAMB468 and the HBL100 breast epithelial cell line displayed a band when amplified with primers for unmethylated DNA (lanes 1 and 2). The HCT116 CRC cell line displayed bands of both unmethylated DNA and methylated DNA (lane 3). The CRC cell lines, SW480, LoVo, HT29 and DLD1 yielded a band of methylated DNA (lanes 4-7). U, unmethylated DNA; M, methylated DNA. (B), p16 mRNA expression by RT-PCR assay in cell lines. The MDAMB468 and the HBL100 cell lines expressed an intense band for p16 mRNA (lanes 1 and 2). HCT116 cells expressed a modest band for p16 mRNA (lane 3). The SW480, LoVo, HT29 and DLD1 cells lines did not express p16 mRNA (lanes 4-7). GAPDH served as internal control in duplex PCR.

Statistical analysis. Contingency tables were used to determine correlations between the expression of p16 protein and clinicopathologic parameters. Statistical association was determined by Fisher's exact test and mean values were compared by the Mann-Whitney U test using the Statview J-5.0 program (SAS Institute Inc., Cary, NC).

Results

Methylation and p16 gene expression in cell lines. Seven cell lines were examined for the presence of methylation in the p16 promoter region using MSP. The breast cancer cell line MDAMB468, which retains only the unmethylated p16 gene, was used as a negative control, while the SW480 CRC cell line served as a positive control for hypermethylation of the p16 gene, as described previously (30). The HBL100 breast epithelial cell line and the MDAMB468 cell line displayed a band only when amplified with primers for unmethylated DNA (Fig. 2A, lanes 1 and 2). By contrast, like the SW480 cell line, the CRC cell lines, LoVo, HT29 and DLD1 displayed a clear band of methylated DNA but not unmethylated DNA (Fig. 2A, lanes 4-7). The HCT116 cell line alone displayed bands of both methylated DNA and unmethylated DNA (Fig. 2A, lane 3).

The MDAMB468 and the HBL100 cell lines expressed an intense band for p16 mRNA (Fig. 2B, lanes 1 and 2), but CRC cell lines SW480, LoVo, HT29, and DLD1 did not express p16 mRNA (Fig. 2B, lanes 4-7). In HCT116 cells, a modest band for p16 mRNA was detected (Fig. 2B, lane 3).

Methylation and p16 gene expression in surgical specimens. Twenty-one paired normal and cancer tissues were then examined for the presence of p16 gene methylation using MSP. All surgical specimens displayed a band of unmethylated DNA that could be derived from unmethylated DNA of normal colonic cells and cancer cells as well as normal constituents in the stroma such as vascular endothelial cells, smooth muscles, fibroblasts, and inflammatory cells (Fig. 3A). A band of methylated DNA was found in 2 normal mucosa samples (9.5%) and 9 (42.9%) cancer tissues.

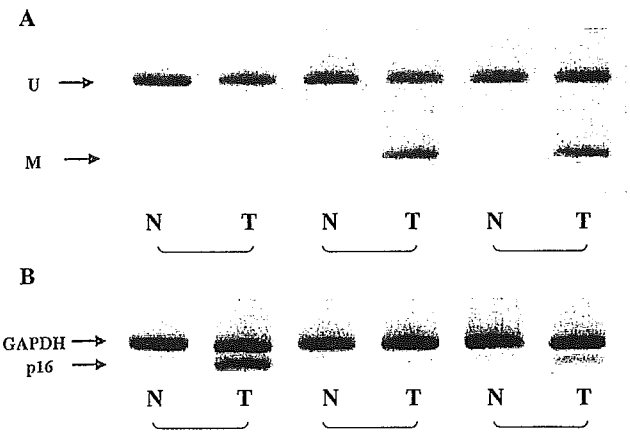

Figure 3. (A), Methylation of the p16 promoter region in surgical specimens. All surgical specimens displayed a band of unmethylated DNA. A band of methylated DNA was found in 2 of 21 normal mucosa samples (9.5%) and 9 of 21 cancer tissues (42.9%). Three representative pairs of normal and cancer tissues are shown here. U, unmethylated DNA; M, methylated DNA. (B), p16 mRNA expression in surgical specimens. p16 mRNA was undetectable in 12 of 21 normal mucosa samples (57.1%) (lanes 1, 3 and 5). On the other hand, p16 mRNA was detected in 10 of 12 methylation-minus CRC tissues (lane 2) and 5 of 9 methylation-plus CRC tissues (lane 6). GAPDH served as an internal control in duplex PCR. N, normal mucosa; T, CRC tumor tissue.

To confirm the conversion efficiency of bisulfite from unmethylated cytosines to uracils, a mixing experiment was performed. Prior to bisulfite treatment, a cancer DNA sample displaying both methylated and unmethylated bands was diluted serially with increasing amounts of unmethylated DNA from HBL100 cells. As expected, methylated band gradually decreased, resulting in an increasing ratio of unmethylated to methylated product in the mixture (Fig. 4).

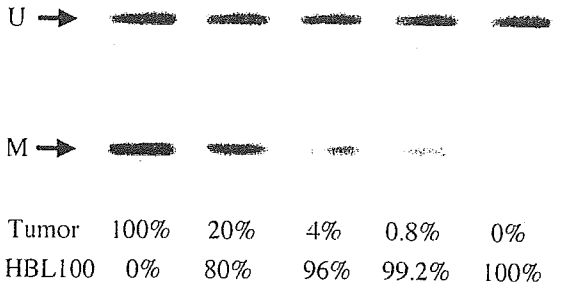

Figure 4. Mixing experiment. Prior to bisulfite treatment, a cancer DNA sample (1 μg) displaying both methylated and unmethylated bands (lane 1) was diluted serially (every 5-fold, lanes 2-4) with increasing amounts of unmethylated DNA from HBL100 cells. Then, PCR was performed. Unmethylated DNA remained unchanged, while methylated DNA product decreased in the mixture, enduring high conversion efficiency of bisfulfite from unmethylated cytosines to uracils. U, unmethylated DNA; M, methylated DNA.

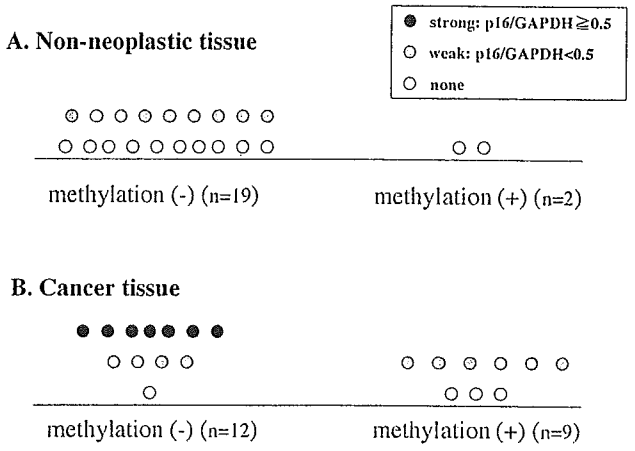

Figure 5. p16 mRNA expression stratified by the presence of methylation in non-neoplastic tissue (A) and cancer tissue (B). When high expression of p16 mRNA was defined as an intensity ratio of the p16 mRNA band to the GAPDH mRNA band of ≥0.5, 7 CRC tissues without methylation belonged to this category (solid circles). There were no high expressors of p16 in non-neoplastic tissues. Nine normal tissues devoid of methylation expressed weak p16 mRNA expression (intensity ratio of p16 mRNA band to GAPDH mRNA band <0.5, grey circles). Open circles indicate samples that did not express p16.

p16 mRNA was undetectable in 12 of 21 normal mucosa samples (57.1%) (Fig. 3B, lanes 1, 3 and 5). p16 expression was detected in 9 normal mucosa samples, all of which were devoid of p16 methylation; the expression level was generally weak (intensity ratio of p16 band to GAPDH band <0.5). On the other hand, p16 mRNA was detected in 11 of 12 methylation-negative CRC tissues (Fig. 3B, lane 2) and 6 of 9 methylation-positive CRC tissues (Fig. 3B, lane 6). Strong p16 mRNA expression (intensity ratio ≥0.5) was detected in 7 CRC tissues only in the methylation-negative group (results summarized in Fig. 5). When expression of p16 mRNA was compared between paired normal mucosa and tumor samples, only 1 of 21 cases (4.7%) showed a decrease in p16 mRNA expression during carcinogenesis (from normal mucosa to cancer tissue).

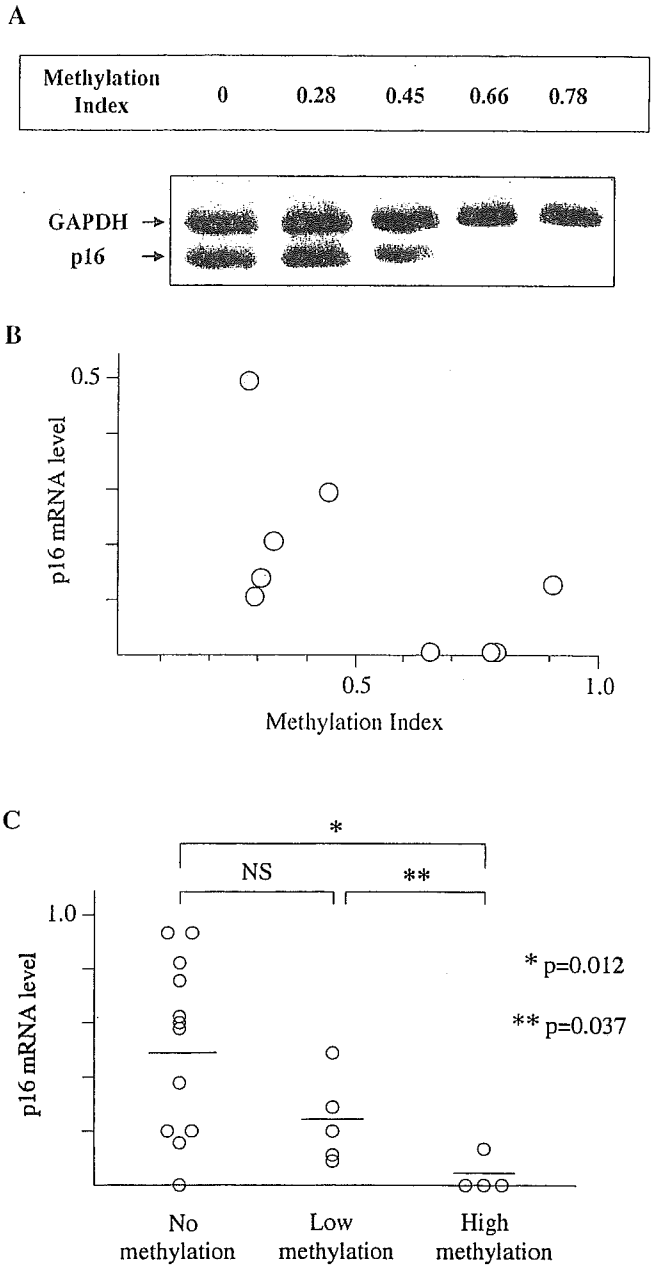

Figure 6. (A), Representative results of duplex RT-PCR for p16 mRNA expression and methylation index (MI). Methylation-specific real-time PCR was performed using methylation-positive CRC tissues. The MI varied as follows: 0.28, 0.29, 0.30, 0.32, 0.45, 0.66, 0.78, 0.79 and 0.91. Methylation-negative CRC tissues were regarded as MI 0. Representative CRC samples are shown. (B), Relationship between p16 mRNA level and MI. The MI value and level of p16 mRNA expression of methylation-positive CRC samples were plotted. (C), Association of p16 mRNA level and methylation extent. CRC cases were classified into three categories; no-methylation (MI ≥0), low-methylation (0< MI <0.5), high-methylation (MI ≥0.5). The mean expression levels of p16 mRNA were 0.518±0.322, 0.25±0.15, and 0.037±0.075, respectively. NS, not significant. The units ascribed to p16 mRNA level represent the ratio of the p16 to GAPDH RT-PCR products.

Methylation-specific real-time PCR. We then measured the extent of p16 methylation in methylation-positive CRC tissues (n=9) by a methylation-specific real-time PCR method. The MI varied widely as follows: 0.28, 0.29, 0.30, 0.32, 0.45, 0.66, 0.78, 0.79, and 0.91. The MI values were then compared with p16 mRNA expression (Fig. 6A) and plotted (Fig. 6B). CRC tissues with high methylation had relatively low p16

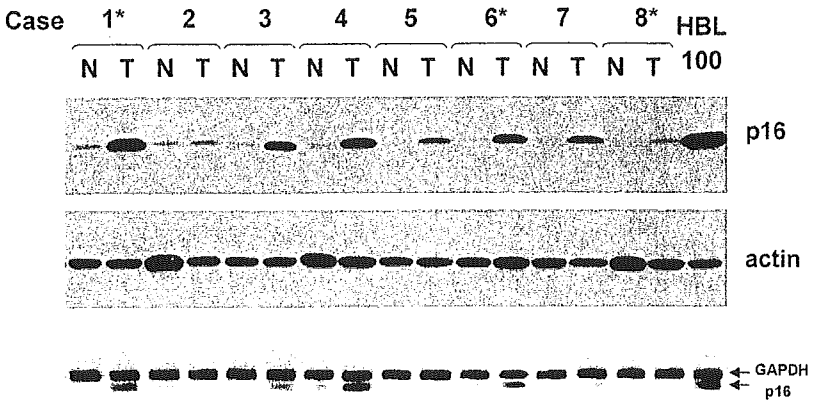

Figure 7. Western blot and RT-PCR analysis. The positive control HBL100 cells displayed a band for the p16 protein. Cancer tissue expressed a clear band for p16 to various extents, while non-neoplastic tissue generally expressed none or scarce band (upper panel). Actin served as loading control of equal amount of the protein (middle panel). RT-PCR assay for p16 mRNA expression in the same series of tissue sample (lower panel). Asterisk indicates cases with p16 methylation in cancer tissue. N, non-neoplastic tissue; T, tumor tissue.

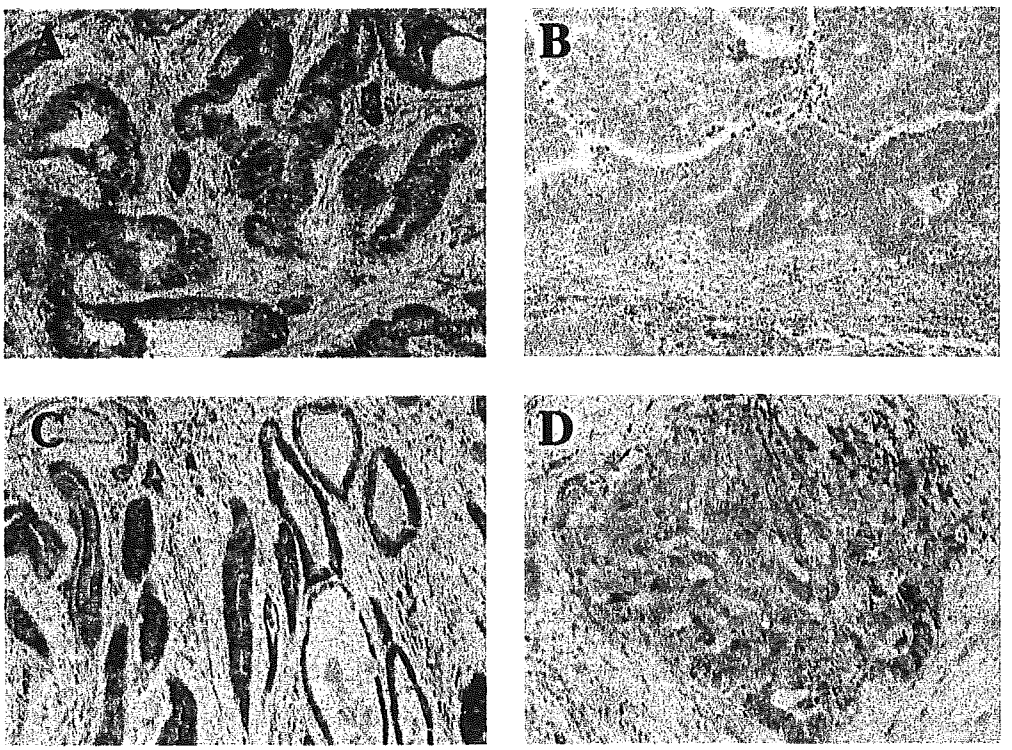

Figure 8. Immunostaining of p16 in colon cancer. In a colon cancer tissue the p16 protein was expressed in the nuclei and cytoplasm. Representative samples of methylation-minus and plus cancer tissues are shown. Methylation-minus groups: (A), high expression; (B), scarce expression. Methylation-plus groups: (C), high expression; (D), faint expression. Magnification, x150.

expression. When CRC cases were classified into three categories according to MI; no-methylation (MI = 0), low-methylation (0< MI <0.5), high-methylation (MI ≥0.5), there was a significant difference in p16 mRNA expression between the high-methylation group and the no-methylation group or low-methylation group (p=0.012, p=0.037, respectively, Fig. 6C).

Expression of p16 ^{INK4} protein in surgical specimens. Western blotting of 8 pairs of the non-neoplastic colonic mucosa and their corresponding CRC tissues showed that the intensity of the p16 band was scarcely detected in the non-neoplastic mucosa and an increased level of p16 expression was noted to various extent in cancer tissues (Fig. 7). RT-PCR indicated that p16 mRNA expression correlated well with p16 protein expression (Fig. 7). Immunostaining of the corresponding tissue samples showed that p16 quantity determined by Western blot was correlated with p16 protein expression in cancer tissues, but not in stromal cells (Fig. 8). The non-immune rabbit serum and antibodies with preabsorbed immunogen

Table I. Relationship between p16 expression and clinicopathologic parameters in colorectal cancer.

Clinicopathologic parameter	Labelling index of p16 staining (%)		
	Group A[a]	Group B[b]	Group C[c]
Gender			
Male	5	27	1
Female	5	16	1
Site			
Colon	6	23	1
Recrum	4	20	1
Dukes'			
A, B	8	21	1
C, D	2[d]	22	1
Histological type			
Well	6	22	0
Moderate/poor	4	21	2
Nodal involvement[e]			
Negative	10	21	1
Positive	0	22	1
Depth of invasion			
~mp	4	14	0
ss~	6	29	2
Distant metastasis			
Negative	8	41	2
Positive	2	2	0
Age (years)	56.7±13.1	62.6±10.3	60.5±20.5
Size (cm)[f]	3.5±1.8	4.8±2.2	8.1±1.6

[a]More than 50% of the cancer cells positive for p16. [b]Ten to 50% of the cells positive. [c]Less than 10% of the cells positive. [d]These 2 cases had no lymph node metastasis but displayed distant metastasis. [e]p=0.003. [f]p=0.048. mp, muscularis propria; ss, subserosa.

yielded no staining in the samples and the HBL100 cells displayed a positive staining and the T98G cells had no staining (data not shown).

In total, 55 paired samples were immunohistochemically stained with the anti-p16 antibody. In the normal colonic mucosa, clear nuclear p16 was detected only occasionally and the LI was <5% in all 55 cases tested (data not shown). In contrast, p16 was abundantly expressed in the majority of CRC tissues, and was localized in the nuclei and cytoplasm. When CRC cases were classified into three groups according to their LI, 10 cases (19%) were classified into group A (LI >50%), 43 (77%) into group B (LI 10-50%) and 2 (4%) into group C (LI <10%). In at least 53 cases (96%), overexpression of p16 was noted in CRC tissue when compared to the corresponding non-neoplastic mucosa. In the 21 CRC tissues utilized for methylation assay, the results of p16 staining correlated well with p16 mRNA expression (data not shown).

Relationship between p16 ^{INK4} expression and clinicopathologic parameters. The relationship between the level of staining of p16 in CRC samples and several clinicopathologic parameters was examined. These parameters included gender, tumor site, disease stage, histological grade, lymph node metastasis, depth of invasion, distant metastasis, age and size (Table I). High p16 expression level was associated with a low incidence of lymph node metastasis (group A vs. group B and C, p=0.003), and p16 expression was associated with small tumor size (p=0.048). There was no correlation between p16 expression and other clinicopathologic parameters.

Discussion

Dysregulation of CKIs, negative regulators of the cell cycle, is often found in human malignancies. The expression of $p21^{Waf1}$ is reduced in aberrant crypt foci with dysplasia and carcinomas of the colon and its high expression is related to a better prognosis in gastric cancer (39-41). A decrease in $p27^{Kip1}$ is associated with poor prognosis or high grade tumors in various types of human cancer (29,42). Loss or decreased expression of p16 is found in a subset of primary cancers (8-12). These lines of evidence indicate that inactivation of CKI may play a role in carcinogenesis and/or tumor progression of various types of human cancers. However, it appears to be unlikely that loss of p16 plays a central role in tumorigenesis of the colorectum *in vivo*. In this study we found that p16 gene expression was often upregulated in primary CRC tissues compared with non-neoplastic mucosa at both the mRNA and protein levels, though p16 mRNA was lost in most CRC cell lines. These findings are consistent with previous reports (15,24,25) and suggest that the regulatory mechanism of p16 gene expression may be quite distinct between cell lines and *in vivo* tissues of the colorectum.

Loss of p16 mRNA in CRC cell lines is understandable since a band corresponding to the methylated p16 gene was generally present in all CRC cell lines, and hypermethylation, as well as gene alterations, is known to be a major mechanism for inactivation of p16 gene transcription (13,14). On the other hand, a methylated p16 band was detected in 42.9% (9/21) of surgically resected CRC tissues, consistent with other reports that p16 methylation is present in 29-55% of CRC tissues (18-22). However, unlike cell lines, such methylation-positive CRC tissues frequently still expressed p16 mRNA. One clue to explain the differential regulatory mechanisms between CRC cell lines and CRC tissues may be methylation density in the p16 promoter region, rather than the presence of methylation *per se* (26,27). In support of this hypothesis on regulation of the p16 transcript, even cell lines exhibited varying extent of p16 methylation, which could affect p16 gene expression (38,43). We also confirmed that the HCT116 cell line whose one allele of p16 is methylated and silenced and the other is mutated and unmethylated (44), did display both a methylated and an unmethylated p16 band, leading to only modest expression of p16 mRNA (Fig. 2). Other CRC cell lines displaying only the methylated p16 band (MI: 100%) exhibited no p16 mRNA expression. Although methylation density of the p16 gene has been measured in several tumor cell lines, the extent of methylation in CRC tissues has yet not been clarified.

We performed quantitative MSP, using CRC tissues that exhibited p16 gene methylation. Our findings showed that there was a significant decrease in p16 mRNA level in the high-methylation group compared to the no-methylation or low-methylation groups (Fig. 6C). These findings suggest that a hyper-methylation density would facilitate transcriptional repression of the p16 gene in CRC tissues, but that low-methylation may only partially repress p16 gene expression. It was of interest that MI was variable (range: 0.28-0.91) and this could be a characteristic of *in vivo* p16 gene methylation. Concordant p16 expression at protein and RNA level (Fig. 7) suggest that p16 expression may be regulated at transcriptional level. Immunohistochemistry revealed that low level of the p16 protein represented reduced expression within neoplastic epithelium, but not in stromal cells (Fig. 8). In the no-methylation group, CRC tissues exhibited a wide range of p16 mRNA levels (Fig. 6C). This suggests that the basal p16 mRNA level could be also variable in the methylation-positive group, and therefore p16 expression level may not be determined by methylation extent alone. This might account for a few exceptional CRC samples that expressed some p16 mRNA even with the highest MI (0.91) or displayed relatively low p16 mRNA expression despite a low MI (0.29, 0.3) (Fig. 6B).

It remains controversial whether p16 expression is predictive of clinical events. Inactivation of the p16 gene is associated with aggressive disease phenotype including lymph node metastasis, shorter survival in esophageal cancer and advanced stage of hepatocellular carcinomas (45,46). Conversely, it has been reported that high levels of p16 are associated with poor prognosis in carcinomas of the ovary, prostate, and breast (47-49). In the latter tumor types, increased p16 expression might in turn reflect upregulated activity of the cell cycle positive regulators. Our immunohistochemical analysis showed that low p16 expression in CRC tissues correlated with larger tumor size and lymph node metastasis. Other investigators have also shown that p16 expression in CRC tissues was associated with low proliferative activity (24,25) and hypermethylation of the p16 gene was associated with advanced tumor stage and shorter survival (20,22). Taken together, these findings suggest that p16, despite its upregulation, may act as a tumor suppressor gene in CRC tissues, and it is likely that methylation would at least in part contribute to reduction in p16 expression, resulting in facilitation of tumor expansion and enlargement of CRC.

Overexpression of the p16 tumor suppressor gene in CRC tissues appeared to be paradoxical and we are not yet certain of the reason for this. However, overexpression of p16 is not limited to CRC, but is also seen in other gastrointestinal tumors, such as gastric and intestinal cancers (24,25,30,50). Moreover, studies have shown that several human cancers display increased expression of negative cell cycle regulators, although no mutations of the $p27^{Kip1}$ and $p21^{Waf1}$ genes have been reported for human cancer tissues. High levels of $p21^{Waf1}$ expression are seen in glial tumors, non-small cell lung cancer, and breast cancer (51-53). $p27^{Kip1}$ expression is increased in a subset of cancers, including breast and colon cancers (29,54). The precise reason for why negative regulators of the cell cycle often display increased expression is unknown at present. One explanation proposed by Weinstein is that multistage carcinogenesis is not simply a summation of the individual effects of oncogene activation and tumor suppressor gene inactivation, but that the evolving cancer cell must maintain a state of homeostasis between positive- and negative-acting factors in order to maintain structural integrity, viability, and normal replication (55). In this context, we speculate that p16 gene methylation may break the homeostatic balance, thereby tipping the balance towards positive regulators.

In conclusion, our study demonstrated that CRC tissues exhibited a variable methylation density of the CpG island in the p16 gene promoter, suggesting that p16 expression may be repressed in a methylation-dependent manner. Our data suggest that the majority of CRC tissues overexpressed p16 protein, and that low p16 expression among CRC tissues, probably via hypermethylation, may contribute to tumor enlargement and lymph node metastasis.

Acknowledgements

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