Detection of colorectal cancer cells from feces using quantitative real-time RT-PCR for colorectal cancer diagnosis

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Early detection of colorectal cancer (CRC) is desired for reducing its mortality rate. Recently, the feasibility of a new method for isolating colonocytes from feces was demonstrated, followed by direct sequencing analysis for detecting colorectal cancer. In the present study, gene expression analysis was conducted using quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR). One hundred and sixty-six patients with CRC and 134 healthy volunteers were enrolled. Messenger RNA expressions of *CEA*, *MMP7*, *MYBL2*, *PTGS2* and *TP53* in the colonocytes isolated from feces were analyzed by quantitative real-time RT-PCR. Beta-2-microglobulin, used for internal control, could not be detected in approximately 25% each of the CRC patients (39/166) and healthy volunteers (33/134). *CEA* expression did not differ significantly between CRC patients and healthy volunteers ($P = 0.21$). *MMP7*, *MYBL2*, *PTGS2* and *TP53* gene expressions were significantly higher in CRC patients than in healthy volunteers ($P < 0.001$). The overall sensitivity and specificity using these gene expressions were 58.3% (74/127, 95% CI; 49.2–67.0) and 88.1% (89/101, 95% CI; 80.2–93.7), respectively. The sensitivity was dependent on the tumor location ($P = 0.01$) and tumor size ($P = 0.02$), but not the tumor depth ($P = 0.06$) or cancer stage ($P = 0.37$). Gene expression analysis of colonocytes isolated from feces may be a useful method for CRC screening, if the number of isolated colonocytes is sufficiently high for analysis by quantitative real-time PCR. Therefore, improvement of the colonocyte retrieval system from feces may be necessary for the technique to be developed for clinical use. (*Cancer Sci* 2008; 99: 1977–1983)

Colorectal cancer (CRC) is one of the most common malignancies worldwide. In the world, CRC is the third leading cause of cancer-related mortality and the second leading cause of cancer-related incidence.[1] Meanwhile, the survival rate from CRC is good if this cancer can be diagnosed early and resected surgically at an early stage.[2] Thus, to reduce the mortality rate of CRC, the development of a screening test by which the cancer can be diagnosed at an early stage is necessary.

To date, the fecal occult blood test (FOBT) has been used widely as the screening test for CRC.[3–5] However, three recent large-scale studies showed that the sensitivity of FOBT was not very high using total colonoscopy as a reference standard in all subjects.[6–8]

Therefore, numerous other methods have been reported for early detection of CRC in fecal samples, including those based on the detection of mutated DNA,[9–13] cancer-related methylation[14,15] and DNA integrity.[16,17] However, these methods are time consuming and not sufficiently sensitive. The major reason for this inaccuracy is that the 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 the feces may be as low as 1% at the most.[18] This makes the application of gene detection methods difficult in clinical practice.

Use of quantitative real-time RT-PCR has been reported to detect colorectal cancer cells in the peripheral blood,[19–23] mesenteric venous blood,[20] peritoneal lavage fluid[20–23] or lymph nodes.[24,25] However, in the early stage of CRC, the cancer cells rarely invade the blood or peritoneal cavity; therefore, it is difficult to diagnose early CRC by gene expression analysis using these samples. Meanwhile, there have been a few studies that have attempted to detect CRC by utilizing RT-PCR in fecal samples.[26,27]

Previously, we reported the presence of viable cancer cells in the feces, which could be isolated from naturally evacuated feces using cell isolation methods.[28,29] We developed a new method for the detection of early CRC, wherein the extracted DNA from the cells isolated from feces was examined for CRC-related gene mutations. Our new method for the diagnosis of CRC was found to have a sensitivity of 71% (82/116) and a specificity of 88% (73/83).[29]

Our cell isolation method using immunomagnetic beads is relatively feasible for cytological or molecular biological analyses to detect colorectal cancer.[29] However, direct sequencing analysis involves many steps and it is difficult to conduct analysis for the point mutations. A simple method is necessary to analyze a large number of samples, so we attempted a gene expression analysis method in fecal samples for the detection of CRC in the present study.

Materials and Methods

Patients with CRC and healthy volunteers. From August 2003 to September 2004, 166 patients with histologically confirmed colorectal cancer and 134 healthy volunteers were enrolled in the present study. The healthy volunteers consisted of 59 men and 75 women with no evident abnormalities, such as adenoma or carcinoma (including hyperplastic polyps), as determined by total colonoscopy performed at the National Cancer Center Research Center for Cancer Prevention and Screening. The median age of the volunteers was 60 years (range, 40–70 years). The characteristics of these patients are summarized in Table 1. All the patients had undergone surgical resection of their primary cancer at the National Cancer Center Hospital, Tokyo, Japan.

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Table 1. Characteristics of patients and healthy volunteers

Characteristics	Patients (*N* = 166)	Healthy volunteers (*N* = 134)
Age, years		
Median	63	60
Range	32–83	40–70
Sex, no (%)		
Male	108 (65.1)	59 (44.0)
Female	58 (34.9)	75 (56.0)
Tumor location, no (%)		
Cecum	7 (4.2)	
Ascending colon	29 (17.5)	
Transverse colon	7 (4.2)	
Descending colon	7 (4.2)	
Sigmoid colon	34 (20.5)	
Rectum	82 (49.4)	
Tumor size, mm		
Median	38	
Range	10–160	
Histology, no (%)		
W/D	89 (53.6)	
M/D	67 (40.4)	
P/D	4 (2.4)	
Mucinous carcinoma	5 (3.0)	
Carcinoid tumor	1 (0.6)	
Tumor depth, no (%)		
T1	18 (10.8)	
T2	39 (23.5)	
T3	106 (63.9)	
T4	3 (1.8)	
Dukes' stage, no (%)		
A	46 (27.7)	
B	41 (24.7)	
C	66 (39.8)	
D	13 (7.8)	

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

The median age of the patients was 63 years (range, 32–83 years). There were 108 male and 58 female patients. The primary tumor was located at the following sites: rectum, 82 patients; sigmoid colon, 34 patients; descending colon, 7 patients; transverse colon, 7 patients; ascending colon, 29 patients; and cecum, 7 patients. The median diameter of the primary tumor was 38 mm (range, 10–160). The clinical stage of the patients according to Dukes' classification was as follows: Dukes' stage A, 46 patients; stage B, 41 patients; stage C, 66 patients; and stage D, 13 patients. All patients were provided detailed information about the study and gave written consent for participating in the study, which was approved by the Institutional Review Board of the National Cancer Center, Japan.

Immunomagnetic beads. Dynabeads Epithelial Enrich (Dynal, Oslo, Norway), which are commercially available immunomagnetic beads conjugated with epithelial cell adhesion molecule antibodies (EpCAM Ab; Ber-EP4) measuring 4.5 μm in diameter, were used for the present study.

Fecal samples and isolation of exfoliated cells. Naturally evacuated fecal samples were obtained from 166 colorectal cancer patients before they underwent surgical resection. Fecal samples were also obtained from 134 healthy volunteers a few weeks after they had undergone a total colonoscopy. All patients and volunteers were instructed to evacuate at home into a disposable polystyrene tray (ASone, Osaka, Japan) that measured 5 × 10 cm in size 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 fecal samples were prepared for the next step immediately after they were brought to our laboratory.

The samples were processed as described previously.[29] Briefly, the fecal sample was homogenized with a buffer (40 mL) consisting of Hanks solution, 10% fetal bovine serum (FBS) and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.35) at 200 times per minute for 1 min using a Stomacher system (Seward, Thetford, UK). The homogenate was filtered through a nylon filter (pore size; 512 μm) and following the addition of 80 μL of the commercially available immunomagnetic beads (Dynabeads Epithelial Enrich; Dynal, Oslo, Norway), the sample mixture was incubated for 30 min under a gentle rolling condition at room temperature. The mixture on the magnet was incubated on a shaking platform for 15 min at room temperature. The supernatant was then removed and the colonocytes in the pellet were stored at –80°C until RNA extraction.

Extraction of total RNA. Total RNA was extracted from the colonocytes isolated from the fecal samples using an RNeasy mini kit (QIAGEN, Valencia, CA, USA) in accordance with the manufacturer's instructions. Each sample was eluted in 100 μL of RNase-free water.

Complementary DNA synthesis. Complementary DNA (cDNA) was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA) in accordance with the manufacturer's instructions. The reaction mixture consisted of 10 μL of total RNA, 2 μL of 10 × reverse transcription (RT) buffer, 2 μL of 10 × random primer, 0.8 μL of 25 × deoxyribonucleotide triphosphates (dNTP) (100 mM), 1 μL of RNase Inhibitor (20 U/μL) and 1 μL of MultiScribe Reverse Transcriptase (50 U/μL) in a final reaction volume of 20 μL.

Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis. For gene expression analysis of the total RNA obtained, we targeted six genes: *CEA* (carcinoembryonic antigen), *MMP7* (matrix metallopeptidase 7), *MYBL2* (myeloblastosis viral oncogene homolog like 2), *PTGS2* (prostaglandin-endoperoxide sythase 2) and *TP53* (tumor protein p53), using *B2M* (beta-2-microglobulin) as the internal control gene. For all of these genes, we used commercially available TaqMan primers and probe mixture (Applied Biosystems). The reporter dye at the 5′-end of the probe was FAM™, and the quencher dye at the 3′-end was minor groove binder (MGB).

The reaction mixture for the analysis of the total RNA consisted of 4 μL of a template cDNA, 10 μL of TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and 1 μL of 20 × primers/probe mixture in a total reaction volume of 20 μL. Real-time PCR was performed with precycling heat activation at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s, and annealing/extension at 62°C for 30 s, in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). The relative quantification of the total RNA in each sample was conducted using the comparative Ct (threshold cycle) method. In this analysis, the formulae for the relative quantification of each of the genes were as follows: (dCt of each gene) = (Ct of each gene) – (Ct of *B2M*), and (Relative Quantification of each gene) = $2^{-(\text{dCt of each gene})}$. A negative control (without template) was run in each reaction plate.

Statistical analysis. The detection rate of the target genes and the sensitivity of detection of the tumor location, tumor size, tumor depth and Dukes' stage were analyzed using two-sided Fisher's exact test. The differences in the relative quantification of the target genes in both the patients and volunteers were analyzed by two-sided Mann–Whitney's *U*-tests. Statistical analyses were performed using StatView ver. 5 for Windows (Abacus Concepts, Berkeley, CA, USA). $P < 0.05$ was considered statistically significant.

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Table 2. Detection rate of target genes

Target genes	Patient (N = 166)	Healthy volunteer (N = 134)	P-value
B2M			
Detection, no (%)	127 (76.5)	101 (75.4)	
Median Ct	30.541	32.194	0.89
CEA			
Detection, no (%)	125 (75.3)	98 (73.1)	
Median Ct	30.229	32.023	0.69
MMP7			
Detection, no (%)	39 (23.5)	6 (4.5)	
Median Ct	34.381	37.612	<0.0001
MYBL2			
Detection, no (%)	40 (24.1)	1 (0.7)	
Median Ct	33.949	33.708	<0.0001
PTGS2			
Detection, no (%)	84 (50.6)	16 (11.9)	
Median Ct	33.529	34.892	<0.0001
TP53			
Detection, no (%)	49 (29.5)	7 (5.2)	
Median Ct	35.467	36.361	<0.0001

B2M, beta-2-microglobulin; *CEA*, carcinoembryonic antigen; *MMP7*, matrix metallopeptidase 7; *MYBL2*, myeloblastosis viral oncogene homolog like 2; *PTGS2*, prostaglandin-endoperoxide sythase 2; *TP53*, tumor protein p53. *P*-value analyzed by Fisher's exact test; $P < 0.05$ was considered statistically significant.

Table 3. Mean values of relative expression of target genes compared with a housekeeping gene, *B2M*

Target genes	Patient (N = 127)	Healthy volunteer (N = 101)	P-value
CEA			
RQ, mean	1.706	1.290	
Range	0–20.224	0–12.527	0.21
MMP7			
RQ, mean	0.008	0.0003	
Range	0–0.166	0–0.021	0.0009
MYBL2			
RQ, mean	0.023	9.648×10^{-8}	
Range	0–1.728	0–0.000009	<0.0001
PTGS2			
RQ, mean	0.137	0.015	
Range	0–2.539	0–0.409	<0.0001
TP53			
RQ, mean	0.003	0.0002	
Range	0–0.048	0–0.010	<0.0001

CEA, carcinoembryonic antigen; *MMP7*, matrix metallopeptidase 7; *MYBL2*, myeloblastosis viral oncogene homolog like 2; *PTGS2*, prostaglandin-endoperoxide sythase 2; *TP53*, tumor protein p53; RQ, relative quantification. *P*-value analyzed by Mann–Whitney test; $P < 0.05$ was considered statistically significant.

Results

Detection rate of each gene using real-time RT-PCR. The detection rate of *B2M* was 76.5% (127/166) in the patients with CRC and 75.4% (101/134) in the healthy volunteers (Table 2); there was no significant difference in the detection rate between the patients and volunteers ($P = 0.89$). On the other hand, the median Ct value of *B2M* was 30.541 (range, 21.060–36.719) in the CRC patients and 32.194 (16.231–38.488) in the volunteers, and the difference in the median Ct value between the two groups was significant ($P < 0.0001$). This result implies that the number of colonocytes in the 2-gram fecal samples obtained from the patients with CRC was three times higher than that in the samples of the same weight obtained from the healthy volunteers.

The detection rate of *CEA* was 75.3% (125/166) in the patients with CRC and 73.1% (98/134) in the healthy volunteers. There was no significant difference in the detection rate between the patients and volunteers ($P = 0.69$).

The detection rates of the *MMP*, *MYBL2*, *PTGS2* and *TP53* genes in the CRC patients were 23.5% (39/166), 24.1% (40/166), 50.6% (84/166) and 29.5% (49/166), respectively, and the corresponding rates in the healthy volunteers were 4.5% (6/134), 0.7% (1/134), 11.9% (16/134) and 5.2% (7/134), respectively. There were significant differences in the detection rates of these genes between the patients and volunteers ($P < 0.0001$).

Relative quantification of each target gene by normalizing the values to the *B2M* gene expression level. The relative expression level of each gene as compared with that of *B2M*, a housekeeping gene, was determined for 127 of the 166 CRC patients and 101 of the 134 healthy volunteers. The mean relative expression levels of *CEA*, *MMP7*, *MYBL2*, *PTGS2* and *TP53* in the CRC patients were 1.706 (range, 0–20.224), 0.008 (0–0.166), 0.023 (0–1.728), 0.137 (0–2.539) and 0.003 (0–0.048), respectively, and the corresponding relative expression levels in the healthy volunteers were 1.290 (range, 0–12.527), 0.0003 (0–0.021), 9.648×10^{-8} ($0–9.744 \times 10^{-6}$), 0.015 (0–0.409) and 0.0002 (0–0.010), respectively. Although there was no significant difference in the relative expression level of *CEA* between the CRC patients and volunteers ($P = 0.21$), significant differences were observed in the relative expression levels of *MMP7*, *MYBL2*, *PTGS2* and *TP53* between the patient and volunteer groups ($P < 0.001$) (Table 3). Thus, it was determined that these genes could be used for the detection of CRC by quantitative real-time RT-PCR analysis.

Threshold of each gene to detect CRC. To determine the suitable threshold for the detection of CRC, the numbers of CRC patients and healthy volunteers were calculated using the relative quantification of each gene and plotted into histograms (Fig. 1). To obtain a specificity of *CEA* detection of 95% for the diagnosis of CRC, the threshold required >2.8; however, at this threshold, a sensitivity of only 14.2% was obtained. Using these histograms, the suitable thresholds for *MMP7*, *MYBL2*, *PTGS2* and *TP53* were determined to be >0.0003, >0.00001, >0.06 and >0.001, respectively.

Sensitivity and specificity of the gene expression analysis. According to the gene expression analysis of all four genes, the overall sensitivity of patients and specificity of healthy volunteers were 58.3% (74/127, 95% CI; 49.2–67.0) and 88.1% (89/101, 95% CI; 80.2–93.7), respectively (Table 4). The sensitivities using *MMP7*, *MYBL2*, *PTGS2* and *TP53* were 30.7% (39/127, 95% CI; 22.8–39.5), 31.5% (40/127, 95% CI; 23.6–40.3), 33.9% (43/127, 95% CI; 25.7–42.8) and 29.1% (37/127, 95% CI; 21.4–37.8), respectively. The specificities using *MMP7*, *MYBL2*, *PTGS2* and *TP53* were 95.1% (96/101, 95% CI; 88.8–93.7), 100% (101/101, 95% CI; 96.4–100), 94.1% (95/101, 95% CI; 87.5–97.8) and 94.1% (95/101, 95% CI; 87.5–97.8), respectively.

Using combined markers, the sensitivities of detection of patients with cancers located on the right side of the colon and those cancers located on the left side of the colon were 40.5% (15/37, 95% CI; 24.8–58.0) and 65.6% (59/90, 95% CI; 54.8–75.3), respectively. The sensitivities of detection of patients with tumors less than 35 mm in diameter and those with tumors more than 35 mm in diameter were 46.7% (28/60, 95% CI; 33.7–60.0) and 68.7% (46/67, 95% CI; 56.2–79.5), respectively. The sensitivities of detection of patients with Dukes' stage A and B and those with Duke's stage C and D were 63.6% (42/66, 95% CI; 50.9–75.1) and 55.7% (34/61, 95% CI; 42.5–68.5), respectively.

Table 4. Sensitivity and specificity of the present gene expression analysis

	Target genes	Patients		Healthy volunteer	
		Sensitivity (%)		Specificity (%)	
		No.	(95% CI)	No.	(95% CI)
Overall	Combined markers	74	58.3 (49.2–67.0)	89	88.1 (80.2–93.7)
Patients (*N* = 127)	*MMP7*	39	30.7 (22.8–39.5)	96	95.1 (88.8–93.7)
Healthy volunteer (*N* = 101)	*MYBL2*	40	31.5 (23.6–40.3)	101	100 (96.4–100)
	PTGS2	43	33.9 (25.7–42.8)	95	94.1 (87.5–97.8)
	TP53	37	29.1 (21.4–37.8)	95	94.1 (87.5–97.8)

	Combined markers			*MMP7*			*MYBL2*			*PTGS2*			*TP53*		
	No.	Sensitivity (%)	*P*-value	No.	Sensitivity (%)	*P*-value	No.	Sensitivity (%)	*P*-value	No.	Sensitivity (%)	*P*-value	No.	Sensitivity (%)	*P*-value
Tumor location			0.01			0.002			0.0003			0.02			0.3
Right colon (*N* = 37)	15	40.5 (24.8–58.0)		4	10.8 (3.0–25.4)		3	8.1 (1.7–21.9)		7	18.9 (8.0–35.2)		8	21.6 (9.8–38.3)	
Left colon (*N* = 90)	59	65.6 (54.8–75.3)		35	38.9 (28.8–49.7)		37	41.1 (30.9–52.0)		36	40.0 (29.8–50.9)		29	32.2 (22.7–42.9)	
Tumor size			0.02			0.007			0.03			0.3			0.2
≤35 mm (*N* = 60)	28	46.7 (33.7–60.0)		11	18.3 (9.5–30.4)		13	21.7 (12.1–34.2)		17	28.3 (17.5–41.5)		14	23.3 (13.4–36.1)	
>35 mm (*N* = 67)	46	68.7 (56.2–79.5)		28	41.8 (29.9–54.5)		27	40.3 (28.5–53.0)		26	38.8 (27.2–51.5)		23	34.3 (23.1–46.9)	
Tumor depth			0.06			0.06			0.4			0.4			0.2
T1 and T2 (*N* = 42)	19	45.2 (29.8–61.3)		8	19.0 (8.6–34.1)		11	26.2 (13.9–42.0)		12	28.6 (15.7–44.6)		9	21.4 (10.3–36.9)	
T3 and T4 (*N* = 85)	55	64.7 (53.6–74.8)		31	36.5 (26.3–47.6)		29	34.1 (24.2–45.3)		31	36.5 (26.3–47.6)		28	32.9 (23.1–44.0)	
Dukes' stage			0.4			0.4			1			0.09			1
Stage A and B (*N* = 66)	42	63.6 (50.9–75.1)		18	27.3 (17.0–39.7)		21	31.8 (20.9–44.5)		27	40.9 (29.0–53.7)		19	28.8 (18.3–41.3)	
Stage C and D (*N* = 61)	34	55.7 (42.5–68.5)		21	34.4 (22.8–47.7)		19	31.1 (19.9–44.3)		16	26.2 (15.8–39.1)		18	29.5 (18.5–42.5)	

MMP7, matrix metallopeptidase 7; *MYBL2*, myeloblastosis viral oncogene homolog like 2; *PTGS2*, prostaglandin-endoperoxide sythase 2; *TP53*, tumor protein p53; Threshold, *MMP7* (>0.0003), *MYBL2* (>0.00001), *PTGS2* (>0.06) and *TP53* (>0.001); 95% CI, 95% confidence interval. *P*-value analyzed by Fisher's exact test; $P < 0.05$ was considered statistically significant.

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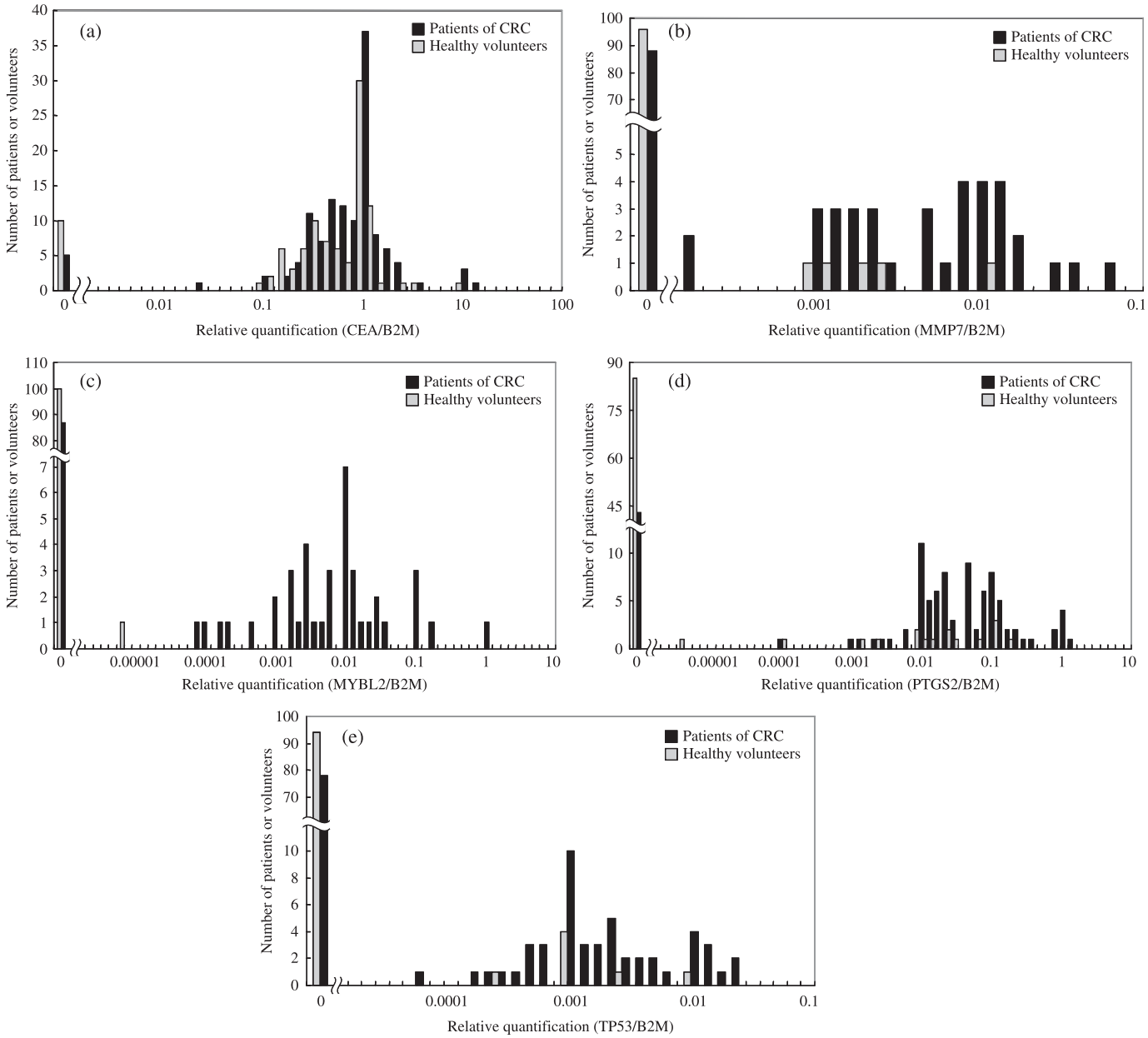

Fig. 1. Histogram of colorectal cancer (CRC) patients and healthy volunteers using relative quantification of each gene. (a) Histogram of CRC patients and healthy volunteers using relative quantification of the *CEA* gene. At the suitable threshold (>2.8), the sensitivity of *CEA* detection for the diagnosis of CRC was only 14% at the specificity of 95%. (b) Histogram of CRC patients and healthy volunteers using relative quantification of the *MMP7* gene. At the suitable threshold (>0.0003), the sensitivity of *MMP7* detection for the diagnosis of CRC was 31% at the specificity of 95%. (c) Histogram of CRC patients and healthy volunteers using relative quantification of the *MYBL2* gene. At the suitable threshold (>0.00001), the sensitivity of *MYBL2* detection for the diagnosis of CRC was 32% at the specificity of 100%. (d) Histogram of CRC patients and healthy volunteers using relative quantification of the *PTGS2* gene. At the suitable threshold (>0.06), the sensitivity of *PTGS2* detection for the diagnosis of CRC was 34% at the specificity of 95%. (e) Histogram of CRC patients and healthy volunteers using relative quantification of the *TP53* gene. At the suitable threshold (>0.06), the sensitivity of *TP53* detection for the diagnosis of CRC was 29% at the specificity of 95%.

The sensitivities of detection of patients with T1 and T2 cancers and those with T3 and T4 cancers were 45.2% (19/42, 95% CI; 29.8–61.3) and 64.7% (55/85, 95% CI; 53.6–74.8), respectively. Significant differences were seen among the sensitivities depending on the location of the primary tumor ($P = 0.01$) and the size of the primary tumor ($P = 0.02$). On the other hand, there were no significant differences in the sensitivity of detection among tumors of varying invasion depth ($P = 0.06$) and the Dukes' stage classification ($P = 0.37$).

Discussion

A suitable screening method must not only show high sensitivity and specificity, but also be simple to perform and cheap. Indeed, the cytological analysis, reported previously,[29] was conducted by two experienced cytotechnologists and the identification of colorectal cancer cells was not simple. The procedure for detection of genetic alterations by direct sequencing analysis involves many steps and is expensive. Meanwhile, the

cost of gene expression analysis by quantitative real-time RT-PCR is only about one-fourth of that for direct sequencing analysis. Colorectal cancers and breast cancers accumulate about 90 mutant genes[30] and the mutation sites differ among individual tumors. Thus, the detection of genetic alterations can hardly be considered as a suitable screening method for CRC.

CRC develops from the colorectal mucosa, and since feces would be in contact with the lesion from an early stage, it was considered that naturally evacuated fecal samples might be useful to detect colorectal cancer cells even from an early stage.[28,29] The gene expression analysis based on mRNA was one of the newly used detection methods using fecal samples. The target gene was *COX2* (same as *PTGS2*) and the authors suggested that gene expression analysis may be a promising approach for the detection of CRC. The problems with these studies were: the study population was too small, they used fecal RNA, not RNA from colonocytes isolated from feces and they used non-quantitative RT-PCR analysis, the sensitivity and specificity of which depended on the template RNA concentration.[26,27]

In the present study, we conducted gene expression analysis based on mRNA detection in isolated colonocytes from naturally evacuated feces of 166 patients with CRC and 134 healthy volunteers using quantitative real-time RT-PCR. The detection rate of *B2M*, which was used as the internal control gene, was about 75% (228 out of 300) in all subjects, including patients and volunteers (Table 2). This result implies that gene expression could not be analyzed in 25% of the total examinees enrolled in this study owing to the insufficient number of isolated colonocytes from the 2-gram fecal samples. To improve the detection rate of *B2M*, suitable immunomagnetic beads were considered to be necessary to increase the number of isolated colonocytes from the 2-gram fecal samples. Alternatively, colonocyte isolation methods capable of handling higher amounts of fecal samples would be required. The median Ct value of the gene for the CRC patients was 1.6 cycles lower than that for the healthy volunteers, which indicates that the number of colonocytes in the 2-gram fecal samples obtained from the patients with CRC was 3-times higher than that in the samples of the same weight obtained from the healthy volunteers. Consistent with our previous data, this result indicates that normal mucosal cells may die and be exfoliated during turnover and that the exfoliated cancer cells are more likely to survive for prolonged periods of time in the feces.[28,29,31–33]

Because the number of colonocytes contained in 2-gram fecal samples is small, the target genes investigated should not only be cancer specific, but also expressed at high levels. Thus, we decided to analyze five genes: *CEA*, *MMP7*, *MYBL2*, *PTGS2* and *TP53*. The gene expression level of *CEA* was not significantly different between the CRC patients and healthy volunteers (Table 3). Therefore, we concluded that the *CEA* gene was not suitable for diagnosing CRC by gene expression analysis of isolated colonocytes from feces using quantitative real-time RT-PCR.

In the gene expression analysis using quantitative real-time RT-PCR, it is necessary to define the threshold for drawing a sharp line between patients with CRC and healthy volunteers. In the present study, the sensitivity and specificity were 58.3% and 88.1% as per the defined threshold. These results were inferior to the those of direct sequencing analysis reported previously by us.[28] However, the cost of gene expression analysis was substantially lower than that of the direct sequencing analysis.

We could detect cases with resectable CRC as well as those with advanced CRC (Table 4). Detection at an early stage is important for reducing the mortality from CRC. Meanwhile, the detection rates of patients with the primary tumor located on the right side of the colon and with a small size of the tumor were lower than those of patients with the primary tumor located on the left side of the colon and with tumors of larger diameters. It is especially difficult to detect cancer located on the right side of the colon because it is associated with few symptoms and is relatively difficult to detect by colonoscopy.[34–36] The reason for the lower sensitivity of detection of cancer on the right side of the colon is that the exfoliated cancer cells from the right colon are exposed to the feces for a longer time than those from left colon cancer, resulting in a possibly reduced number of surviving cells in the feces. Also, one reason for the low sensitivity of detection of tumors of small size is that the number of exfoliated cancer cells from small-size tumors would be fewer than that from tumors of larger size. Thus, it is necessary to establish a new screening test for detecting CRC, including that located on the right side of colon.

In the present study, we conducted gene expression analysis of colonocytes isolated from feces for the detection of CRC using quantitative real-time RT-PCR. The *CEA* gene and *KRT20* gene, commonly used for the detection of CRC, were found to be not useful. The sensitivity and the specificity of the analysis were 58.3% and 88.1% for the combination of *MMP7*, *MYBL2*, *PTGS2* and *TP53*. We, however, believe that gene expression analysis of colonocytes isolated from feces offers promise of becoming a mass screening method for CRC in the future after several improvements are implemented, such as establishment of more powerful immunomagnetic beads and colonocyte recovery methods capable of dealing with higher amounts of feces.

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References

1 World Health Organization (WHO). World Health Report 2004. Available from URL: www.who.int/healthinfo/bodgbd2002revised/en/index.html
2 Bosman FT. Prognostic value of pathological characteristics of colorectal cancer. *Eur J Cancer* 1995; **31A**: 1216–21.
3 Towler B, Irwig L, Glasziou P, Kewenter J, Weller D, Silagy C. A systematic review of the effects of screening for colorectal cancer using the faecal occult blood test, hemoccult. *BMJ* 1998; **317**: 559–65.
4 Winawer S, Fletcher R, Rex D *et al*. Colorectal cancer screening and surveillance: clinical guidelines and rationale-update based on new evidence. *Gastroenterology* 2003; **124**: 544–60.
5 Mandel JS, Church TR, Bond JH *et al*. The effect of fecal occult-blood screening on the incidence of colorectal cancer. *N Engl J Med* 2000; **343**: 1603–7.
6 Lieberman DA, Weiss DG. One-time screening for colorectal cancer with combined fecal occult-blood testing and examination of the distal colon. *N Engl J Med* 2001; **345**: 555–60.
7 Sung JJ, Chan FK, Leung WK *et al*. Screening for colorectal cancer in Chinese: comparison of fecal occult blood test, flexible sigmoidoscopy, and colonoscopy. *Gastroenterology* 2003; **124**: 608–14.
8 Imperiale TF, Ransohoff DF, Itzkowitz SH, Turnbull BA, Ross ME. Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. *N Engl J Med* 2004; **351**: 2704–14.
9 Sidransky D, Tokino T, Hamilton SR *et al*. Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. *Science* 1992; **256**: 102–5.
10 Ahlquist DA, Skoletsky JE, Boynton KA *et al*. Colorectal cancer screening by detection of altered human DNA in stool: feasibility of a multitarget assay panel. *Gastroenterology* 2000; **119**: 1219–27.

doi: 10.1111/j.1349-7006.2008.00954.x

11 Rengucci C, Maiolo P, Saragoni L, Zoli W, Amadori D, Calistri D. Multiple detection of genetic alterations in tumors and stool. *Clin Cancer Res* 2001; **7**: 590–3.
12 Traverso G, Shuber A, Olsson L *et al*. Detection of proximal colorectal cancers through analysis of faecal DNA. *Lancet* 2002; **359**: 403–4.
13 Traverso G, Shuber A, Levin B *et al*. Detection of APC mutations in fecal DNA from patients with colorectal tumors. *N Engl J Med* 2002; **346**: 311–20.
14 Itzkowitz SH, Jandorf L, Brand R *et al*. Improved fecal DNA test for colorectal cancer screening. *Clin Gastroenterol Hepatol* 2007; **5**: 111–7.
15 Zou H, Harrington J, Rego RL, Ahlquist DA. A novel method to capture methylated human DNA from stool: implications for colorectal cancer screening. *Clin Chem* 2007; **53**: 1646–51.
16 Boynton KA, Summerhayes IC, Ahlquist DA, Shuber AP. DNA integrity as a potential marker for stool-based detection of colorectal cancer. *Clin Chem* 2003; **49**: 1058–65.
17 Zou H, Harrington JJ, Klatt KK, Ahlquist DA. A sensitive method to quantify human long DNA in stool: relevance to colorectal cancer screening. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1115–19.
18 Hasegawa Y, Takeda S, Ichii S *et al*. Detection of K-ras mutations in DNAs isolated from feces of patients with colorectal tumors by mutant-allele-specific amplification (MASA). *Oncogene* 1995; **10**: 1441–5.
19 Guadagni F, Kantor J, Aloe S *et al*. Detection of blood-borne cells in colorectal cancer patients by nested reverse transcription–polymerase chain reaction for carcinoembryonic antigen messenger RNA longitudinal analyses and demonstration of its potential importance as an adjunct to multiple serum markers. *Cancer Res* 2001; **61**: 2523–32.
20 Guller U, Zajac P, Schnider A *et al*. Disseminated single tumor cells as detected by real-time quantitative polymerase chain reaction represent a prognostic factor in patients undergoing surgery for colorectal cancer. *Ann Surg* 2002; **236**: 768–75; discussion 75–6.
21 Schuster R, Max N, Mann B *et al*. Quantitative real-time RT-PCR for detection of disseminated tumor cells in peripheral blood of patients with colorectal cancer using different mRNA markers. *Int J Cancer* 2004; **108**: 219–27.
22 Guo J, Xiao B, Jin Z *et al*. Detection of cytokeratin 20 mRNA in the peripheral blood of patients with colorectal cancer by immunomagnetic bead enrichment and real-time reverse transcriptase-polymeras chain reaction. *J Gastroenterol Hepatol* 2005; **20**: 1279–84.
23 Lloyd JM, McIver CM, Stephenson SA, Hewett PJ, Rieger N, Hardingham JE. Identification of early-stage colorectal cancer patients at risk of relapse post-resection by immunobead reverse transcription-PCR analysis of peritoneal lavage fluid for malignant cells. *Clin Cancer Res* 2006; **12**: 417–23.
24 Oberg AN, Lindmark GE, Israelsson AC, Hammarstrom SG, Hammarstrom ML. Detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR for *CEA* and *CK20* mRNAS. *Int J Cancer* 2004; **111**: 101–10.
25 Ohlsson L, Hammarstrom ML, Israelsson A *et al*. Biomarker selection for detection of occult tumour cells in lymph nodes of colorectal cancer patients using real-time quantitative RT-PCR. *Br J Cancer* 2006; **95**: 218–25.
26 Kanaoka S, Yoshida K, Miura N, Sugimura H, Kajimura M. Potential usefulness of detecting cyclooxygenase 2 messenger RNA in feces for colorectal cancer screening. *Gastroenterology* 2004; **127**: 422–7.
27 Leung WK, To KF, Man EP *et al*. Detection of hypermethylated DNA or cyclooxygenase-2 messenger RNA in fecal samples of patients with colorectal cancer or polyps. *Am J Gastroenterol* 2007; **102**: 1070–6.
28 Yamao T, Matsumura Y, Shimada Y *et al*. Abnormal expression of *CD44* variants in the exfoliated cells in the feces of patients with colorectal cancer. *Gastroenterology* 1998; **114**: 1196–205.
29 Matsushita H, Matsumura Y, Moriya Y *et al*. A new method for isolating colonocytes from naturally evacuated feces and its clinical application to colorectal cancer diagnosis. *Gastroenterology* 2005; **129**: 1918–27.
30 Sjoblom T, Jones S, Wood LD *et al*. The consensus coding sequences of human breast and colorectal cancers. *Science* 2006; **314**: 268–74.
31 Matsumura Y, Tarin D. Significance of *CD44* gene products for cancer diagnosis and disease evaluation. *Lancet* 1992; **340**: 1053–8.
32 Matsumura Y, Hanbury D, Smith J, Tarin D. Non-invasive detection of malignancy by identification of unusual *CD44* gene activity in exfoliated cancer cells. *Bmj* 1994; **308**: 619–24.
33 Onouchi S, Matsushita H, Nomura S, Minowa T, Matsumura Y. PCR-based assessment of the recovery rate of exfoliated colonocytes or cancer cells from fecal samples depends on the storage conditions after defecation. *J Gastrointestin Liver Dis* 2007; **16**: 369–72.
34 Regula J, Rupinski M, Kraszewska E *et al*. Colonoscopy in colorectal-cancer screening for detection of advanced neoplasia. *N Engl J Med* 2006; **355**: 1863–72.
35 Barclay RL, Vicari JJ, Doughty AS, Johanson JF, Greenlaw RL. Colonoscopic withdrawal times and adenoma detection during screening colonoscopy. *N Engl J Med* 2006; **355**: 2533–41.
36 Shah HA, Paszat LF, Saskin R, Stukel TA, Rabeneck L. Factors associated with incomplete colonoscopy: a population-based study. *Gastroenterology* 2007; **132**: 2297–303.