Research Article

Cancer Prevention Research

MicroRNA Expression Profiling of Exfoliated Colonocytes Isolated from Feces for Colorectal Cancer Screening

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Abstract

To reduce the colorectal cancer (CRC) mortality rate, we have reported several CRC screening methods using colonocytes isolated from feces. Expression analysis of oncogenic microRNA (miRNA) in peripheral blood was recently reported for CRC detection. In the present study, we conducted miRNA expression analysis of exfoliated colonocytes isolated from feces for CRC screening. Two hundred six CRC patients and 134 healthy volunteers were enrolled in the study. miRNA expression of the miR-17-92 cluster, miR-21, and miR-135 in colonocytes isolated from feces as well as frozen tissues was analyzed by quantitative real-time PCR. The expression of the miR-17-92 cluster, miR-21, and miR-135 was significantly higher in CRC tissues compared with normal tissues. The exfoliated colonocytes of 197 CRC patients and 119 healthy volunteers were analyzed because of the presence of sufficient miRNA concentration. miR-21 expression did not differ significantly between CRC patients and healthy volunteers ($P = 0.6$). The expression of miR-17-92 cluster and miR-135 was significantly higher in CRC patients than in healthy volunteers ($P < 0.0001$). The overall sensitivity and specificity by using miRNA expression was 74.1% (146/197; 95% confidence interval, 67.4-80.1) and 79.0% (94/119; 95% confidence interval, 70.6-85.9), respectively. Sensitivity was dependent only on tumor location ($P = 0.0001$). miRNA was relatively well conserved in exfoliated colonocytes from feces both of CRC patients and healthy volunteers. miRNA expression analysis of the isolated colonocytes may be a useful method for CRC screening. Furthermore, oncogenic miRNA highly expressed in CRC should be investigated for CRC screening tests in the future. *Cancer Prev Res; 3(11); 1435–42. ©2010 AACR.*

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality (639,000 deaths in 2004) and cancer-related incidence (1,080,000 new cases in 2004) worldwide (1). Yet, CRC is curable by surgical resection if diagnosed at an early stage. Thus, to reduce the mortality rate of CRC, it is necessary to develop a screening test by which the cancer can be diagnosed at an early stage. To date, the fecal occult blood test has been widely used as the main screening test for CRC (2–4). However, large-scale studies have shown that the sensitivity of fecal occult blood test is not very high, using total colonoscopy as a reference standard in all subjects (5–8). Therefore, other methods have been reported for early detection of CRC, including those based on the detection of mutated DNA (9, 10), cancer-related methylation analysis (11, 12), and DNA integrity (13, 14) in fecal samples. A combination of these analyses has been reported as the stool DNA test (7). Guidelines for screening and monitoring for the early detection of adenomatous polyps and CRC in average-risk adults were recently updated (15). In these guidelines, the stool DNA test was recommended as a CRC screening method; however, the sensitivity and specificity of the stool DNA test was insufficient compared with fecal occult blood test (16). Several studies have reported an attempt to detect CRC by using reverse transcriptase-PCR in fecal samples (17–19). However, there is no evidence that the stool RNA test is useful for CRC screening. The major reason for these inaccuracy is that the nucleic acids in feces are derived from an enormous number and variety of bacteria.

Previously, we reported the presence of viable cancer cells in the feces and that viable cancer cells can be isolated from naturally evacuated feces by using cell isolation methods (20, 21). Moreover, we reported several methods for the detection of early CRC, detection of CRC-related gene mutation using direct sequence analysis (21) and single-strand conformational polymorphism analysis (22), and analysis of CRC-related gene expression (23). The gene expression analysis based on real-time reverse transcriptase-PCR was simple and cost-effective; however,

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~25% of the CRC patients and healthy volunteers could not be detected because of the low concentration or degradation of mRNA.

MicroRNAs (miRNA), which are small (18-25 nucleotides in size) noncoding RNA molecules, regulate the activity of specific mRNA targets and play a major role in cancer. The function of miRNA is the downregulation of multiple target gene expression by degrading the mRNA or blocking its translation into protein through RNA interference (24, 25). Several miRNAs, such as miRNA-21 (miR-21), miR-17-92 cluster, and miR-135, were highly expressed in CRC tissues (26–29). Several recent studies reported that the circulating miRNA in plasma was a potential marker for CRC detection (30, 31) and was remarkably stable in plasma and protected from endogenous RNase activity (32).

To the best of our knowledge, there have been no other studies investigating miRNA expression profiling of fecal-based RNA. In the present study, we analyzed the miRNA expression profile in CRC tissue and exfoliated colonocytes and investigated the potential of miRNA expression analysis of exfoliated colonocytes as a new method for early detection of CRC.

Materials and Methods

CRC patients and healthy volunteers

From August 2003 to March 2007, 206 patients with histologically confirmed CRC and 134 healthy volunteers were enrolled in the present study. The healthy volunteers consisted of 59 men and 75 women without any symptoms and evident abnormalities, such as adenoma or carcinoma (including hyperplastic polyps) as determined by screening colonoscopy done at the Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo. The median age of the volunteers was 60 (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. The median age of the patients was 63 (range 32-83 years). There were 138 men and 68 women patients. The primary tumor was located at the following sites: rectum, 101 patients; sigmoid colon, 45 patients; descending colon, 9 patients; transverse colon, 11 patients; ascending colon, 31 patients; cecum, 9 patients. The median diameter of the primary tumor was 38 mm (range 7-160 mm). The clinical stage of the patients according to Dukes' classification was as follows: stage A, 57 patients; stage B, 56 patients; stage C, 79 patients; stage D, 14 patients. All participants were provided with detailed information about the study, and each 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), which are commercially available immunomagnetic beads conjugated with EpCAM monoclonal antibody (clone Ber-EP4) and measure 4.5 μm in diameter, were used in the present study. JSR beads (JSR), which are immunomagnetic beads conjugated with EpCAM monoclonal antibody (clone B8-4) and measure 3.0 μm in diameter and reported as optimal immunomagnetic beads for colonocyte isolation from stool (33), were also used in the present study.

Fecal samples and isolation of exfoliated cells

Naturally evacuated fecal samples were obtained from 206 CRC 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

Table 1. Characteristics of patients and healthy volunteers

Characteristics	Patients (n = 206)	Healthy volunteers (n = 134)
Age, y		
Median	63	60
Range	32-83	40-70
Sex, no. (%)		
Male	138 (67.0)	59 (44.0)
Female	68 (33.0)	75 (56.0)
Tumor location, no. (%)		
Cecum	9 (4.4)	
Ascending colon	31 (15.0)	
Transverse colon	11 (5.3)	
Descending colon	9 (4.4)	
Sigmoid colon	45 (21.8)	
Rectum	101 (49.0)	
Tumor size, mm		
Median	38	
Range	7-160	
Histology, no. (%)		
W/D	115 (55.8)	
M/D	79 (38.3)	
P/D	5 (2.4)	
Mucinous carcinoma	6 (2.9)	
Carcinoid tumor	1 (0.5)	
Tumor depth, no. (%)		
T_1	23 (11.2)	
T_2	46 (22.3)	
T_3	133 (64.6)	
T_4	4 (1.9)	
Dukes' stage, no. (%)		
A	57 (27.7)	
B	56 (27.2)	
C	79 (38.3)	
D	14 (6.8)	

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

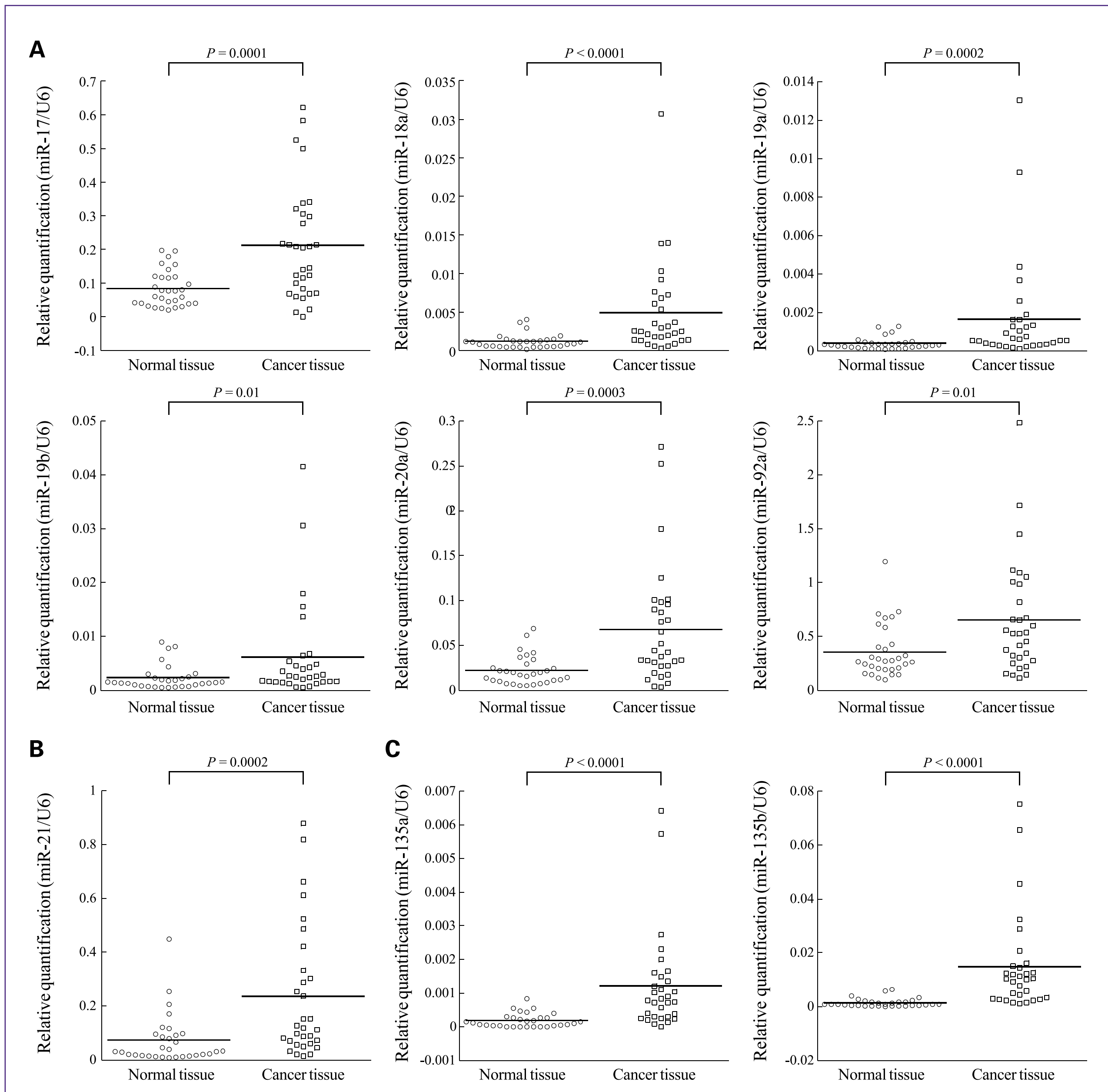

Fig. 1. miRNA expression profile in cancer and normal tissues. A, expression of the miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a) is significantly high in CRC tissues compared with normal tissues. B, expression of miR-21 is significantly high in CRC tissues compared with normal tissues. C, expression of miR-135 (miR-135a and miR-135b) is significantly high in CRC tissues compared with normal tissues. Each miRNA is normalized by U6 snRNA, an internal control miRNA. Horizontal bars represent the mean quantification of target miRNAs. The differences in the relative quantification of the target miRNAs in CRC tissues and normal tissues were analyzed by a two-sided Mann-Whitney's *U* test. $P < 0.05$ denotes statistical significance.

to evacuate the bowel at home, place the sample into a disposable 5 × 10-cm polystyrene tray (AsOne), 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 (21). Briefly, the fecal sample was homogenized with a buffer (40 mL) consisting of Hanks' solution, 10% fetal bovine serum, and 25 mmol/L HEPES buffer (pH 7.35) at 200 rpm for 1 minute using a Stomacher system (Seward). The homogenate was filtered through a nylon filter (pore size 512 μm). After the addition of 80 μL of the immunomagnetic beads, the sample mixture was incubated for 30 minutes under gentle rolling conditions at room temperature. The mixture on the magnet was incubated on

a shaking platform for 15 minutes at room temperature (25°C). The supernatant was then removed and the colonocytes in the pellet were stored at −80°C until RNA extraction.

Expression of oncogenic miRNA in CRC and adjacent normal colorectal mucosa

To clarify the difference of the expression of several miRNA between CRC and normal colorectal tissue, fresh tissue samples were obtained from the surgically resected specimens of 31 patients. The samples were frozen in liquid nitrogen within 20 minutes of their arrival at the pathologic specimen reception area and stored in liquid nitrogen 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), in accordance with the manufacturer's instructions. Each sample was eluted in 100 μL of RNase-free water. Fresh tissue samples were placed in tubes containing ceramic beads and were homogenized using a Precellys 24 device (Bertin Technologies) at 6,500 rpm for 50 seconds. Total RNA was also extracted from each homogenized tissue sample by using an RNeasy Mini Kit.

miRNA expression analysis

For miRNA expression analysis, we targeted 10 miRNAs: miR-17-92 cluster (includes miR-17, miR-18a, miR-19a, miR -19b, miR-20a, and miR-92a) and miR-21, miR-135 (miR-135a and miR-135b), and U6 snRNA as an internal control. For all of these miRNAs, we used the commercially available TaqMan MicroRNA Assay (Applied Biosystems).

cDNA was synthesized using the TaqMan MicroRNA RT Kit (Applied Biosystems), in accordance with the manufacturer's instructions. The reaction mixture consisted of 2 μL of total RNA, 0.5 μL of 10× reverse transcriptase buffer, 1 μL of 5× specific primer, 0.05 μL of deoxynucleotide triphosphates (100 mmol/L), 0.06 μL of RNase inhibitor (20 U/μL), and 0.33 μL of MultiScribe Reverse Transcriptase (50 U/μL) in a final reaction volume of 5 μL. The reaction mixture for the analysis consisted of 4 μL of a template cDNA, 10 μL of TaqMan Fast Universal PCR Master Mix (Applied Biosystems), and 1 μL of 20× primer/probe mixture in a total reaction volume of 20 μL. Real-time

Table 2. Detection rate of target miRNA

miRNA	Patients (*n* = 206)	Healthy volunteers (*n* = 134)
miR-17-92 cluster		
miR-17		
Detection, no. (%)	193 (93.7)	104 (77.6)
miR-18a		
Detection, no. (%)	164 (79.6)	41 (30.6)
miR-19a		
Detection, no. (%)	157 (76.2)	56 (41.8)
miR-19b		
Detection, no. (%)	192 (93.2)	96 (71.6)
miR-20a		
Detection, no. (%)	190 (92.2)	83 (61.9)
miR-92a		
Detection, no. (%)	201 (97.6)	121 (90.3)
miR-21		
Detection, no. (%)	199 (96.6)	131 (97.8)
miR-135		
miR-135a		
Detection, no. (%)	72 (35.0)	17 (12.7)
miR-135b		
Detection, no. (%)	115 (55.8)	27 (20.1)
U6 snRNA		
Detection, no. (%)	200 (97.1)	131 (97.8)

Table 3. Mean values of relative expression of target miRNA compared with an internal control, U6 snRNA

miRNA	Patients (*n* = 197)	Healthy volunteers (*n* = 119)	*P*
miR-17-92 cluster			
miR-17			<0.0001
RQ, mean	5.60	1.06	
Range	0-357.05	0-33.11	
miR-18a			<0.0001
RQ, mean	0.43	0.06	
Range	0-38.40	0-3.29	
miR-19a			<0.0001
RQ, mean	1.11	0.34	
Range	0-58.97	0-10.45	
miR-19b			<0.0001
RQ, mean	4.01	1.65	
Range	0-185.72	0-49.87	
miR-20a			<0.0001
RQ, mean	2.44	0.31	
Range	0-148.57	0-10.05	
miR-92a			<0.0001
RQ, mean	9.38	1.13	
Range	0-460.16	0-42.61	
miR-21			0.6
RQ, mean	25.26	11.59	
Range	0-1206.82	0-484.38	
miR-135			
miR-135a			<0.0001
RQ, mean	0.02	1.26×10^{-9}	
Range	0-2.46	0-5.07×10^{-7}	
miR-135b			<0.0001
RQ, mean	0.29	0.02	
Range	0-28.54	0-1.01	

NOTE: *P* values were analyzed by Mann-Whitney test. $P < 0.05$ was considered statistically significant.
Abbreviation: RQ, relative quantification.

PCR was done with precycling heat activation at 95°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 3 seconds, and annealing/extension at 62°C for 30 seconds, in an Applied Biosystems 7500 Fast Real-Time PCR System.

Statistical analysis

The miRNA expression analysis 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 miRNA) = (Ct of each miRNA) – (Ct of U6 snRNA), and (relative quantification of each miRNA) = $2^{-(\text{dCt of each miRNA})}$. Differences in relative quantification of the target miRNAs in tissue RNA or colonocyte RNA were analyzed by two-sided Mann Whitney's *U* test. Sensitivity of detection of the tumor location, tumor size, tumor depth, and Dukes' stage was analyzed by a two-sided Fisher's exact test. Statistical analyses were done using StatView Ver. 5 for Windows (Abacus Concepts). $P < 0.05$ was considered statistically significant.

Results

Expression of oncogenic miRNAs in CRC and normal colorectal tissue

The expression of the miR-17-92 cluster, considered as oncogenic miRNA including miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a, was significantly higher in CRC tissue compared with normal mucosal tissue (Fig. 1A). The mean expression level of each miRNA in CRC tissue and normal tissue was as follows: miR-17, 0.21 (0-0.62) in CRC tissue and 0.083 (0.020-0.198) in normal tissue ($P = 0.0001$); miR-18a, 0.0049 (0.0003-0.0307) in CRC tissue and 0.0012 (0-0.0040) in normal tissue ($P < 0.0001$); miR-19a, 0.0017 (0.0001-0.0130) in CRC tissue and 0.0004 (0-0.0013) in normal tissue ($P = 0.0002$); miR-19b, 0.0061 (0.0005-0.0416) in CRC tissue and 0.0023 (0.0005-0.0090) in normal tissue ($P = 0.01$); miR-20a, 0.068 (0.004-0.272) in CRC tissue and 0.022 (0.005-0.069) in normal tissue ($P = 0.0003$); and miR-92a, 0.65 (0.11-2.48) in CRC tissue and 0.36 (0.10-1.20) in normal tissue ($P = 0.01$), respectively. Also, the expression of miR-21 and miR-135 (miR-135a and miR-135b), considered as oncogenic miRNA, was significantly higher in CRC tissue compared with normal tissue (Fig. 1B and C). The mean expression level of each miRNA in CRC tissue and normal tissue were as follows: miR-21, 0.23 (0.01-0.88) in CRC tissue and 0.072 (0.008-0.448) in normal tissue ($P = 0.0002$); miR-135a, 0.0012 (0-0.0064) in CRC tissue and 0.0002 (0-0.0008) in normal tissue ($P < 0.0001$); and miR-135b, 0.015 (0.001-0.075) in CRC tissue and 0.0014 (0-0.0064) in normal tissue ($P < 0.0001$).

Detection rate of each miRNA expression in exfoliated colonocytes in feces

The detection rate of U6 snRNA was 97.1% (200/206) in the CRC patients and 97.8% (131/134) in the healthy volunteers (Table 2). The detection rate of miR-92a and miR-21 in the CRC patients and healthy volunteers exceeded 90%. Although the detection rate of miR-17, miR-19b, and miR-20a in the CRC patients exceeded 90%, the rate in the healthy volunteers was 60% to 80%. The detection rate of miR-18a, miR-19a, miR-135a, and miR-135b in healthy volunteers was <50%.

Relative quantification of each miRNA by normalizing the values to U6 snRNA expression level

Of the 206 CRC patients and 134 healthy volunteers enrolled in this study, six patients and three volunteers were excluded because of undetected U6 snRNA. When the Ct value of U6 snRNA was more than 36 due to the small amount of RNA, such samples were not used for analysis of miRNA expression. Three patients and 12 volunteers

Table 4. Sensitivity and specificity of miRNA expression

miRNA	Threshold	Patients (n = 197)		Healthy volunteers (n = 119)	
		No.	Sensitivity, % (95% CI)	No.	Specificity, % (95% CI)
Combined markers		146	74.1 (67.4-80.1)	94	79.0 (70.6-85.9)
miR-17-92 cluster		137	69.5 (62.6-75.9)	97	81.5 (73.3-88.0)
miR-17	0.77	31	15.7 (10.9-21.6)	106	89.1 (82.0-94.0)
miR-18a	0.0001	93	47.2 (40.1-54.5)	112	94.1 (88.3-97.6)
miR-19a	0.0001	105	53.3 (46.0-60.4)	106	89.1 (82.0-94.0)
miR-19b	0.6	31	15.7 (10.9-21.6)	108	90.8 (84.1-95.3)
miR-20a	0.19	36	18.3 (13.1-24.4)	109	91.6 (85.1-95.9)
miR-92a	1.3	43	21.8 (16.3-28.3)	108	90.8 (84.1-95.3)
miR-21	5.8	29	14.7 (10.1-20.5)	109	91.6 (85.1-95.9)
miR-135		91	46.2 (39.1-53.5)	113	95.0 (89.3-98.1)
miR-135a	0.000001	30	15.2 (10.5-21.0)	119	100 (97.0-100)
miR-135b	0.0001	90	45.7 (38.6-53.0)	113	95.0 (89.3-98.1)

were excluded from the miRNA expression analysis. Finally, the relative expression level of each miRNA compared with that of the U6 snRNA internal control was determined for 197 CRC patients and 119 healthy volunteers. The mean relative expression level of miR-17, miR-18a, miR-19a, miR-19b, miR-20a, miR-92a, miR-21, miR-135a, and miR-135b in the CRC patients was 5.60 (range 0-357.05), 0.43 (0-38.40), 1.11 (0-58.97), 4.01 (0-185.72), 2.44 (0-148.57), 9.38 (0-460.16), 25.26 (0-1206.82), 0.02 (0-2.46), and 0.29 (0-28.54), respectively, and the corresponding relative expression level in the healthy volunteers was 1.06 (range 0-33.11), 0.06 (0-3.29), 0.34 (0-10.45), 1.65 (0-49.87), 0.31 (0-10.05), 1.13 (0-42.61), 11.59 (0-484.38), 1.26×10^{-9} ($0\text{-}5.07 \times 10^{-7}$), and 0.02 (0-1.01), respectively. Although there was no significant difference in the relative expression level of miR-21 between the CRC patients and healthy volunteers ($P = 0.6$), significant differences were observed on the relative expression level of the miR-17-92 cluster and miR-135 between the patient and volunteer groups ($P < 0.001$; Table 3).

Sensitivity and specificity of the miRNA expression analysis

According to the expression analysis of nine miRNAs, the overall sensitivity of patients and specificity of healthy volunteers was 74.1% [146/197; 95% confidence interval (95% CI), 67.4-80.1] and 79.0% (94/119; 95% CI, 70.6-85.9), respectively (Table 4). The sensitivity obtained using the miR-17-92 cluster, miR-21, and miR-135 was 69.5% (137/197; 95% CI, 62.6-75.9), 14.7% (29/197; 95% CI, 10.1-20.5), and 46.2% (91/197; 95% CI, 39.1-53.5), respectively. The specificity obtained using the miR-17-92 cluster, miR-21, and miR-135 was 81.5% (97/119; 95%

Table 5. Sensitivity of miRNA expression analyzed by patient characteristics

miRNA	Tumor location				*P*
	Right colon (*n* = 51)		Left colon (*n* = 146)		
	No.	Sensitivity, % (95%CI)	No.	Sensitivity, % (95% CI)	
Combined markers	27	52.9 (38.4-67.1)	119	81.5 (74.3-87.4)	0.0001
miR-17-92 cluster	26	51.0 (36.6-65.3)	111	76.0 (68.3-82.7)	0.001
miR-21	5	9.8 (3.3-21.4)	24	16.4 (10.8-23.5)	0.36
miR-135	6	11.8 (4.4-23.8)	85	58.2 (49.7-66.3)	<0.0001
	Tumor size				
	<35 mm (*n* = 76)		≧35 mm (*n* = 121)		
Combined markers	53	69.7 (58.1-79.7)	93	76.9 (68.3-84.0)	0.32
miR-17-92 cluster	49	64.5 (52.6-75.1)	88	72.7 (63.9-80.4)	0.27
miR-21	14	18.4 (10.5-28.9)	15	12.4 (7.1-19.7)	0.30
miR-135	28	36.8 (26.1-48.8)	63	52.1 (42.8-61.3)	0.04
	Tumor depth				
	T_1 and T_2 (*n* = 67)		T_3 and T_4 (*n* = 130)		
Combined markers	47	70.1 (57.8-80.7)	99	76.2 (67.9-83.2)	0.39
miR-17-92 cluster	45	67.2 (54.6-78.1)	92	70.8 (62.2-78.4)	0.63
miR-21	13	19.4 (10.7-30.9)	16	12.3 (7.2-19.2)	0.21
miR-135	25	37.3 (25.8-50.0)	66	50.8 (41.9-59.6)	0.10
	Dukes' stage				
	Stage A and B (*n* = 110)		Stage C and D (*n* = 87)		
Combined markers	78	70.9 (61.5-79.2)	68	78.2 (68.0-86.3)	0.26
miR-17-92 cluster	74	67.3 (57.7-75.9)	63	72.4 (61.8-81.4)	0.53
miR-21	19	17.3 (10.8-25.6)	10	11.5 (5.7-20.1)	0.31
miR-135	46	41.8 (32.5-51.6)	45	51.7 (40.8-62.6)	0.20

NOTE: The miR-17-92 cluster contained miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a; miR-135 contained miR-135a and miR-135b. *P* values were analyzed by Fisher's exact test. $P < 0.05$ was considered statistically significant.

CI, 83.3-88.0), 91.6% (109/119; 95% CI, 85.1-95.9), and 95.0% (113/119; 95% CI, 89.3-98.1), respectively.

The sensitivity of detection of patients with cancers located on the right side of the colon and those located on the left side of the colon was 52.9% (27/51; 95% CI, 38.4-67.1) and 81.5% (119/146; 95% CI, 74.3-87.4), respectively (Table 5). There was a significant difference in sensitivity between the right and left side of the colon ($P = 0.0001$). On the other hand, there were no significant differences in sensitivity of detection among tumors of varying size ($P = 0.32$), invasion depth ($P = 0.39$), and Dukes' stage classification ($P = 0.26$).

Discussion

In the present study, we reported that the sensitivity and specificity of miRNA expression assay was 74% and 79%, respectively (Table 4). To date, we have reported several CRC screening methods using colonocytes isolated from feces (21–23). The sensitivity and specificity of the cancer-related gene mutation analysis were relatively high (71% and 88%, respectively); however, the cost of direct sequencing analysis was expensive and the procedure was complicated. On the other hand, the cost of real-time reverse transcriptase-PCR was inexpensive and the procedure was simple; however, ~25% of the subjects were excluded from gene expression analysis due to small concentration of total RNA. In the present miRNA expression assay, ~97% of all subjects were detected (Table 2). This means that small RNAs, including miRNAs, in exfoliated colonocytes may be conserved in greater number compared with mRNA in exfoliated colonocytes, and the miRNA expression assay may be more useful as a CRC screening method compared with the gene expression assay.

Recently, several oncogenic miRNAs and tumor-suppressor miRNAs were reported. let7, miR-34 family, miR-126, miR-143, miR-145, and miR-200 family are considered to be tumor-suppressor miRNAs in CRC (34), inhibiting several oncogenes (35–38). The expression level of tumor-suppressor miRNAs in cancer tissue was lower than that in normal tissue. On the other hand, the miR-17-92 cluster, miR-21, and miR-135 are considered to be oncogenic miRNAs in CRC (34), inhibiting several tumor-suppressor genes (29, 39–42). The expression level of the oncogenic miRNAs in cancer tissue was higher than that in normal tissue. Although the detection of oncogenic miRNAs in serum could be used for CRC biomarkers (30, 43), this would not be possible for cancer screening because the miRNAs secreted from cancer cells were very few in serum. In addition, the optimal internal control miRNA of serum circulating miRNA has not yet been determined. In our miRNA expression assay, exfoliated colonocytes were used and three internal control miRNAs—U6 snRNA, RNU6B, and miR-16—were analyzed. Subsequently, we concluded that U6 snRNA was the optimal internal control miRNA for colonocytes isolated from feces (data not shown).

The controls were obtained from asymptomatic patients at screening colonoscopy; therefore, one drawback of this study is that patients with inflammatory conditions have not been tested to evaluate potential false positives. However, the sensitivity of miRNA expression assay was 74% and specificity was <80% (Table 4). These oncogenic miRNAs were highly expressed not only in CRC patients but also in healthy volunteers because they are related to carcinogenesis but not to cancer. The reason for the low specificity is difficult to explain. There is a possibility that these oncogenic miRNAs were already highly expressed in healthy subjects; thus, the specificity of miRNA expression assay was relatively lower. Another possible reason may be that there are some high-risk groups for CRC among the healthy volunteers. We have to perform follow-up on these false-positive healthy subjects to monitor for newly developed CRC.

We were able to detect cases with resectable CRC as well as those with advanced CRC (Table 5). Detection at an early stage is important in reducing the rate of mortality from CRC. Meanwhile, the detection rate for patients with the primary tumor located on the right side of the colon was lower than that for patients with the primary tumor located on the left side of the colon. A possible reason for this is that the exfoliated cancer cells from the right colon are exposed to the feces for a longer time than those from the left colon, resulting in a reduced number of surviving cells in the feces. Given that colon cancer incidence is increasing on the right side, we need to develop a new method that improves the recovery of exfoliated cancer cells even from cancer on the right side of the colon.

In this study, we reported a new CRC screening method using miRNA expression profiling of exfoliated colonocytes. In the next step, we have to detect more useful oncogenic miRNAs by analyzing exfoliated colonocytes in feces from CRC patients or healthy volunteers. Our miRNA expression assay using exfoliated colonocytes should be further evaluated for future CRC screening.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. The global burden of disease: 2004 update. World Health Organization; 2008.
2. Mandel JS, Church TR, Ederer F, Bond JH. Colorectal cancer mortality: effectiveness of biennial screening for fecal occult blood. J Natl Cancer Inst 1999;91:434–7.
3. Scholefield JH, Moss S, Sufi F, Mangham CM, Hardcastle JD. Effect of faecal occult blood screening on mortality from colorectal cancer: results from a randomised controlled trial. Gut 2002;50:840–4.
4. Jorgensen OD, Kronborg O, Fenger C. A randomised study of screening for colorectal cancer using faecal occult blood testing: results after 13 years and seven biennial screening rounds. Gut 2002;50:29–32.
5. 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.
6. 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.
7. 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.
8. Morikawa T, Kato J, Yamaji Y, Wada R, Mitsushima T, Shiratori Y. A comparison of the immunochemical fecal occult blood test and total colonoscopy in the asymptomatic population. Gastroenterology 2005;129:422–8.
9. Traverso G, Shuber A, Olsson L, et al. Detection of proximal colorectal cancers through analysis of faecal DNA. Lancet 2002;359:403–4.
10. 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.
11. Itzkowitz SH, Jandorf L, Brand R, et al. Improved fecal DNA test for colorectal cancer screening. Clin Gastroenterol Hepatol 2007;5: 111–7.
12. 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.
13. 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.
14. 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;15:1115–9.
15. Smith RA, Cokkinides V, Brawley OW. Cancer screening in the United States, 2009: a review of current American Cancer Society guidelines and issues in cancer screening. CA Cancer J Clin 2009; 59:27–41.
16. Ahlquist DA, Sargent DJ, Loprinzi CL, et al. Stool DNA and occult blood testing for screen detection of colorectal neoplasia. Ann Intern Med 2008;149:441–50, W81.
17. 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.
18. 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.
19. Takai T, Kanaoka S, Yoshida K, et al. Fecal cyclooxygenase 2 plus matrix metalloproteinase 7 mRNA assays as a marker for colorectal cancer screening. Cancer Epidemiol Biomarkers Prev 2009;18: 1888–93.
20. 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.
21. 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.
22. Onouchi S, Matsushita H, Moriya Y, et al. New method for colorectal cancer diagnosis based on SSCP analysis of DNA from exfoliated colonocytes in naturally evacuated feces. Anticancer Res 2008;28: 145–50.
23. Koga Y, Yasunaga M, Moriya Y, et al. Detection of colorectal cancer cells from feces using quantitative real-time RT-PCR for colorectal cancer diagnosis. Cancer Sci 2008;99:1977–83.
24. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
25. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. Nat Rev Cancer 2006;6:259–69.
26. Yamamichi N, Shimomura R, Inada K, et al. Locked nucleic acid *in situ* hybridization analysis of miR-21 expression during colorectal cancer development. Clin Cancer Res 2009;15:4009–16.
27. Monzo M, Navarro A, Bandres E, et al. Overlapping expression of microRNAs in human embryonic colon and colorectal cancer. Cell Res 2008;18:823–33.
28. Diosdado B, van de Wiel MA, Terhaar Sive Droste JS, et al. MiR-17-92 cluster is associated with 13q gain and c-myc expression during colorectal adenoma to adenocarcinoma progression. Br J Cancer 2009; 101:707–14.
29. Nagel R, le Sage C, Diosdado B, et al. Regulation of the adenomatous polyposis coli gene by the miR-135 family in colorectal cancer. Cancer Res 2008;68:5795–802.
30. Ng EK, Chong WW, Jin H, et al. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. Gut 2009;58:1375–81.
31. Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. Int J Cancer 2010;127:118–26.
32. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008;105:10513–8.
33. Koga Y, Yasunaga M, Katayose S, et al. Improved recovery of exfoliated colonocytes from feces using newly developed immunomagnetic beads. Gastroenterol Res Pract 2008;2008:605273.
34. Slaby O, Svoboda M, Michalek J, Vyzula R. MicroRNAs in colorectal cancer: translation of molecular biology into clinical application. Mol Cancer 2009;8:102.
35. Shi B, Sepp-Lorenzino L, Prisco M, Linsley P, deAngelis T, Baserga R. Micro RNA 145 targets the insulin receptor substrate-1 and inhibits the growth of colon cancer cells. J Biol Chem 2007;282: 32582–90.
36. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. Cell 2005;120:635–47.
37. Chen X, Guo X, Zhang H, et al. Role of miR-143 targeting KRAS in colorectal tumorigenesis. Oncogene 2009;28:1385–92.
38. Guo C, Sah JF, Beard L, Willson JK, Markowitz SD, Guda K. The noncoding RNA, miR-126, suppresses the growth of neoplastic cells by targeting phosphatidylinositol 3-kinase signaling and is frequently lost in colon cancers. Genes Chromosomes Cancer 2008;47: 939–46.
39. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 2007;133: 647–58.
40. Asangani IA, Rasheed SA, Nikolova DA, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 2008;27:2128–36.
41. Woods K, Thomson JM, Hammond SM. Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. J Biol Chem 2007;282:2130–4.
42. Dews M, Homayouni A, Yu D, et al. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. Nat Genet 2006;38: 1060–5.
43. Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. Int J Cancer 2010;127:118–26.