

Fecal miR-106a Is a Useful Marker for Colorectal Cancer Patients with False-Negative Results in Immunochemical Fecal Occult Blood Test

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Abstract

Background: Immunochemical fecal occult blood test (iFOBT) is widely used for colorectal cancer screening; however, its sensitivity is insufficient. We recently reported a fecal microRNA (miRNA) test (FmiRT) to detect colorectal cancer. In this study, we investigated a new colorectal cancer screening method combining iFOBT and FmiRT to improve the sensitivity compared with iFOBT alone.

Methods: In total, 117 colorectal cancer patients and 107 healthy volunteers were enrolled. Ten-milligram fecal samples were collected and iFOBT was conducted. Fecal RNA was extracted from residuum of iFOBT and then the expression of 14 kinds of miRNA was analyzed for the FmiRT using real-time reverse transcription PCR.

Results: Levels of fecal miR-106a expression in iFOBT+ patients and iFOBT- patients were significantly higher than in healthy volunteers ($P = 0.001$). The sensitivity and specificity of FmiRT using miR-106a were 34.2% and 97.2%, and those of iFOBT were 60.7% and 98.1%, respectively. The overall sensitivity and specificity of the new screening method combining iFOBT and FmiRT were 70.9% and 96.3%, respectively. One quarter of colorectal cancer patients with false-negative iFOBT seemed to be true positive upon adding FmiRT using fecal miR-106a.

Conclusions: Fecal miR-106a is a good molecular marker to identify colorectal cancer patients from among those with negative iFOBT results. FmiRT combined with iFOBT may improve the sensitivity to detect colorectal cancer.

Impact: We have shown the usefulness of fecal miR-106a to detect the colorectal cancer patients among those with negative iFOBT results. *Cancer Epidemiol Biomarkers Prev*; 22(10); 1844–52. ©2013 AACR.

Introduction

Cancer screening is important for various cancers, including colorectal cancer, to reduce the mortality rates. Fecal occult blood test (FOBT) has been widely used as a screening test for colorectal cancer (1–3) and is classified into two different methods: one is chemical FOBT, such as guaiac FOBT (gFOBT), and the other is immunochemical FOBT (iFOBT). The sensitivity of iFOBT to detect colorectal cancer and advanced adenoma is higher than that of gFOBT (4, 5). Several studies have investigated the effi-

cacy of FOBT using total colonoscopy (TCS) as a reference standard in all participants (6–9). Three studies were conducted on the basis of gFOBT and the other was on iFOBT. However, these large-scale studies showed that the sensitivity of FOBT is not very high. The problem with FOBT is that some colorectal cancer patients present with false-negative results whereas healthy individuals have false-positive ones.

Several attempts to use molecular biological methods for the early detection of colorectal cancer have been reported. In fecal DNA-based analysis, the stool DNA test (sDNA test; ref. 8) was recommended as one approach (10). We also reported several DNA-based methods for the detection of early colorectal cancer using direct sequence analysis (11) and single-strand conformation polymorphism analysis (12) in exfoliated colonocytes. However, the sensitivity and specificity of the sDNA test were insufficient compared with those of gFOBT (13). In fecal RNA-based analysis, several attempts to detect colorectal cancer by using reverse transcription PCR (RT-PCR) on fecal samples have been reported (14–16). However, no molecular biological method has proved to be superior to iFOBT in terms of sensitivity and specificity.

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MicroRNAs (miRNA) are small (18–25 nucleotides in size) noncoding RNA molecules that regulate the activity of specific mRNA targets and play various roles in cancer. Several recent studies have clarified that circulating miRNA in plasma is a potential marker for colorectal cancer detection (17, 18), and is remarkably stable in plasma due to its protection from endogenous RNase activity (19). miRNAs can be preserved in poor conditions, such as formalin-fixed paraffin-embedded sections stored for 20 years (20) and fecal samples (21). Thus, miRNAs could be worthy of investigation as tumor biomarkers. Recently, we have reported that miRNA could be extracted from the residuum of iFOBT at a level sufficient for analysis and that fecal miRNA could become a tool for the detection of colorectal cancer (22).

In this study, fecal miRNA extracted from the residuum of iFOBT for colorectal cancer patients and healthy volunteers with no abnormal lesions was assessed as a fecal miRNA test (FmiRT) to determine whether the combination of FmiRT with iFOBT can improve the rate of false-negative results compared with those obtained using iFOBT alone in colorectal cancer screening.

Materials and Methods

Study participants

From October 2009 to September 2012 and from October 2010 to December 2011, 117 patients with colorectal cancer and 107 healthy volunteers were enrolled in this study. Their characteristics are summarized in Table 1. All the patients had undergone surgical resection of their primary cancer at the National Cancer Center Hospital, Tokyo, Japan, or the National Cancer Center Hospital East, Kashiwa, Japan. The median age of the patients was 65 years. The locations of the primary tumor were right colon in 32 patients (27.4%) and left colon in 85 (72.6%). The median diameter of the primary tumor was 33 mm. The clinical stages of the patients were early stage (Dukes stage A and B) in 76 (65.0%) and advanced stage (Dukes stage C and D) in 41 (35.0%). All the healthy volunteers were shown to have no symptoms or evident abnormalities, such as adenoma or carcinoma (including hyperplastic polyps), by screening colonoscopy conducted at the Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo, Japan. The median age of the volunteers was 60 years. All participants were provided with detailed information about the study, and each gave written consent to participate in it, the protocol of which was approved by the Institutional Review Board of the National Cancer Center, Japan.

Collection of fecal samples and immunochemical fecal occult blood test

Naturally evacuated fecal samples were obtained from colorectal cancer patients before they underwent surgical resection. Fecal samples were also obtained from healthy

volunteers a few weeks after they had undergone screening colonoscopy. All participants were instructed to evacuate at home into a disposable 5 cm × 10 cm polystyrene tray (AsOne) and bring the sample to the outpatient clinic or the Cancer Prevention and Screening Center of the National Cancer Center, Japan. The fecal samples were prepared for the next step immediately after they were brought to our laboratory, and excess feces was stored at –80°C.

Ten-milligram fecal samples were transferred into the fecal sampling container provided with the immunochemical fecal occult blood test (iFOBT) kit, OC-Hemocatch (Eiken Chemical). iFOBT was immediately conducted using OC-Hemocatch in accordance with the manufacturer's instructions. Briefly, 100- μ L samples of the dissolved feces were incubated with the iFOBT kit reagents for 5 minutes at room temperature. After incubation, a blue line seemed in the control window if the iFOBT had been conducted correctly. iFOBT was designated as positive if a blue line also seemed in the test window, whereas the samples for which a line did not seem in the test window were designated as negative. The cut-off value for the concentration of hemoglobin in the iFOBT kit was 50 ng/mL.

Extraction of total RNA from residuum of iFOBT or directly from feces

The solution remaining after iFOBT analysis was collected into a 2-mL tube and centrifuged at $700 \times g$ for 5 minutes at 4°C, following which the fecal pellet was collected by removal of the supernatant. Total RNA was extracted using an miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, each fecal pellet was resuspended in 1 mL of QIAzol (Qiagen). Thereafter, 200 μ L of chloroform was added, the mixture was vortexed vigorously for 15 seconds and incubated for 3 minutes at room temperature, and then centrifuged at $13,000 \times g$ for 15 minutes at 4°C. The upper aqueous phase was transferred to a 1.5-mL tube, and 1.5 volumes of 100% ethanol were added. The solution was mixed thoroughly by pipetting and transferred to an miRNeasy spin column, and the columns were centrifuged at $7,000 \times g$ for 15 seconds at room temperature. After washing, total RNA was dissolved in 100 μ L of RNase-free water.

Total RNA extracted directly from feces was also obtained. Fecal samples were homogenized as described previously (21), and total RNA was extracted using an miRNeasy Mini Kit by almost the same procedure as described earlier. Briefly, 500 mg of feces was homogenized with 5 mL of QIAzol using an IKA Ultra-Turrax homogenizer (IKA-Werke) at 6,000 rpm for 2 minutes at room temperature. The homogenates were centrifuged at $13,000 \times g$ for 5 minutes at 4°C. The supernatants were then transferred to a new tube, and up to 5 mL more of QIAzol was added, followed by 1.5 mL of chloroform. The tubes were shaken vigorously for 30 seconds and centrifuged at $13,000 \times g$ for 15 minutes at 4°C. The aqueous

Table 1. Clinicopathologic characteristics of colorectal cancer patients and healthy volunteers

	All patients (N = 117)	iFOBT+ patients (N = 71)	iFOBT- patients (N = 46)	P value	Healthy volunteers (N = 107)
Age (median, range)	65 (30–84)	63 (32–84)	67 (30–83)	0.655	60 (40–78)
Gender (No., %)					
Male	69 (59.0%)	42 (59.2%)	27 (58.7%)	0.961	66 (61.7%)
Female	48 (41.0%)	29 (40.8%)	19 (41.3%)		41 (38.3%)
Pellet RNA (μg, median, range)	1.96 (0.33–5.64)	2.15 (0.42–4.88)	1.68 (0.33–5.64)	0.114	1.59 (0.33–6.00)
Direct RNA (μg, median, range)	53.35 (6.63–243.72)	63.31 (6.63–243.72)	39.79 (10.33–138.90)	0.031	43.04 (6.18–163.79)
Tumor size (mm, median, range)	33 (7–125)	37 (13–125)	21 (7–82)	<0.001	
Tumor location (No., %)					
Rt colon	32 (27.4%)	16 (22.5%)	16 (34.8%)	0.215	
Lt colon	85 (72.6%)	55 (77.5%)	30 (65.2%)		
Tumor depth (No., %)					
T1 and T2	53 (45.3%)	25 (35.2%)	28 (60.9%)	0.011	
T3 and T4	64 (54.7%)	46 (64.8%)	18 (39.1%)		
Dukes stage (No., %)					
A and B	76 (65.0%)	44 (62.0%)	32 (69.6%)	0.521	
C and D	41 (35.0%)	27 (38.0%)	14 (30.4%)		

Pellet RNA, total RNA extracted from residuum of an immunochemical fecal occult blood test; direct RNA, total RNA extracted directly from feces; Rt colon, tumor was located in cecum, ascending colon, and transverse colon; Lt colon, tumor was located in descending colon, sigmoid colon, and rectum; T1 and T2, tumor invaded up to the muscularis propria; T3 and T4, tumor invaded beyond the muscularis propria; iFOBT+ patients, colorectal cancer patients whose iFOBT was positive; iFOBT- patients, colorectal cancer patients whose iFOBT was negative; P value, differences between iFOBT+ patients and iFOBT- patients analyzed by a Mann-Whitney U test or χ^2 test, with $P < 0.05$ considered to denote statistical significance.

phase was then transferred into a new tube. One and a half volumes of 100% ethanol were added, and the solution was mixed thoroughly by pipetting. The mixture was then poured onto a miRNeasy spin column and the column was centrifuged at $7,000 \times g$ for 15 seconds at room temperature. After washing, total RNA was dissolved in 100 μ L of RNase-free water.

The concentrations of total RNA were determined using a NanoDrop UV spectrometer (LMS). The RNA samples were stored at -80°C until analysis. Total RNA extracted from residuum of iFOBT is referred to as pellet RNA and total RNA directly extracted from feces was named as direct RNA.

cDNA synthesis and miRNA expression analysis by real-time PCR

cDNA was synthesized using the TaqMan MicroRNA RT Kit (Applied Biosystems), in accordance with the manufacturer's instructions. The reaction mixture consisted of 5 ng of total RNA, 0.5 μ L of $10 \times$ RT buffer, 1 μ L of $5 \times$ specific primer, 0.05 μ L of dNTPs (100 mmol/L), 0.06 μ L of RNase inhibitor (20 U/ μ L), and 0.33 μ L of MultiScribe Reverse Transcriptase (50 U/ μ L) in a final reaction volume of 5 μ L. cDNA synthesis was conducted with incubation at 16°C for 30 minutes and 42°C for 30 minutes.

The reaction mixture for the real-time PCR analysis consisted of 4 μ L of template cDNA, 10 μ L of TaqMan Fast Universal PCR Master Mix (Applied Biosystems), and

1 μ L of $20 \times$ primer/probe mixture in a total reaction volume of 20 μ L. Real-time PCR was conducted with pre-cycling heat activation at 95°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 3 seconds and annealing/extension at 60°C for 30 seconds, in an Applied Biosystems 7500 Fast Real-Time PCR System. For miRNA expression analysis, we targeted 14 miRNAs: miR-15b, -16, -19a, -19b, -20b, -92a, -93, -106a, -132, -142-3p, -223, -223*, -342-3p, and -451, and miR-24 as an internal control. For all of these miRNAs, we used the commercially available TaqMan MicroRNA Assay (Applied Biosystems).

Statistical analysis

The correlations between "direct miRNA" and "pellet miRNA" were analyzed using Pearson's product-moment correlation coefficient. The miRNA expression analysis was conducted using the comparative Ct (threshold cycle) method (available at <http://pathmicro.med.sc.edu/pcr/realtime-home.htm>). In this analysis, the formulae for the relative quantification of each of the miRNAs were as follows: $(\text{dCt of each miRNA}) = (\text{Ct of each miRNA}) - (\text{Ct of miR-24})$, and $(\text{relative quantification of each miRNA}) = 2^{-(\text{dCt of each miRNA})}$. Differences in Ct values of the target miRNAs were analyzed by two-sided Mann-Whitney U test and differences in relative quantification of the target miRNAs between 2 subgroups of colorectal cancer patients and healthy volunteers were analyzed by Games-Howell test of

one-way ANOVA. Statistical analyses were conducted using SPSS Statistics Ver. 19 (IBM). $P < 0.05$ was considered statistically significant.

Results

Correlation of RNA extracted from residuum of iFOBT and directly from feces

The median amounts of pellet RNA (from 10 mg of feces) and direct RNA (from 500 mg of feces) in colorectal cancer patients were 1.96 μg (range, 0.33–5.64) and 53.35 μg (range, 6.63–243.72), respectively (Table 1). Those in healthy volunteers were also 1.59 μg (range, 0.33–6.00) and 43.04 μg (range, 6.18–163.79), respectively. The amount of direct RNA was dozens of times higher than that of pellet RNA because of a greater starting volume of fecal sample. The correlations between pellet RNA and direct RNA are shown in Fig. 1. Significant positive correlations were observed between direct RNA and pellet RNA in terms of miR-92a ($P < 0.001$, $r = 0.549$), miR-106a ($P < 0.001$, $r = 0.747$), and miR-24 ($P < 0.001$, $r = 0.684$). These results

show that pellet RNA could be applied to FmiRT as effectively as direct RNA.

Relative quantification of each miRNA normalized by miR-24

The Ct values of miR-92a, -106a, and -24 in all participants are shown in the box and whisker plots in Fig. 2. Their median values in pellet RNA from colorectal cancer patients were 29.65 (range, 22.65–40), 29.99 (22.18–35.95), and 28.31 (21.55–33.26); however, those in pellet RNA from healthy volunteers were 30.24 (25.99–40), 30.70 (27.60–33.61), and 28.32 (25.31–31.41). Between the colorectal cancer patients and healthy volunteers, significant differences were observed in the Ct values of miR-92a ($P = 0.016$) and miR-106a ($P < 0.001$); however, there was no significant difference in the Ct values of miR-24 ($P = 0.67$).

On the basis of the above results, miR-24 was used as an internal miRNA control in this study, as in our previous work (22, 23). The median relative expression levels of miR-15b, -16, -19a, -19b, -20b, -92a, -93, -106a, -132, -142-3p, -223, -223*, -342-3p, and -451 in the colorectal cancer

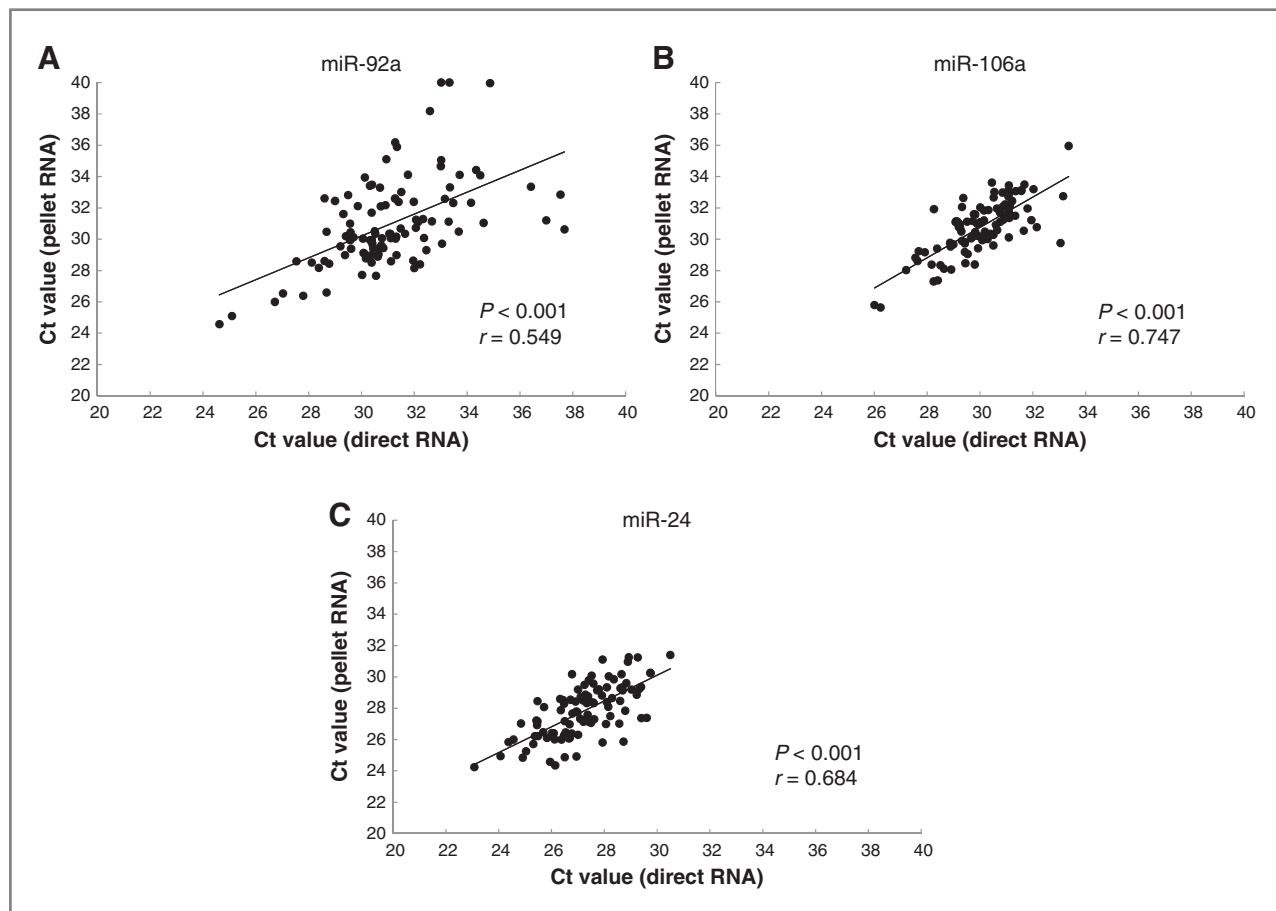


Figure 1. Correlation of direct RNA and pellet RNA for several miRNAs. Ct values of several miRNAs using RNA extracted directly from a fecal sample (direct RNA) and RNA extracted from residuum of an immunochemical fecal occult blood test (pellet RNA) in both colorectal cancer patients and healthy volunteers are plotted. A, a significant positive correlation was observed between direct RNA and pellet RNA for miR-92a ($P < 0.001$, $r = 0.549$). B, a significant positive correlation was observed between direct RNA and pellet RNA for miR-106a ($P < 0.001$, $r = 0.747$). C, a significant positive correlation was observed between direct RNA and pellet RNA for miR-24 ($P < 0.001$, $r = 0.684$).

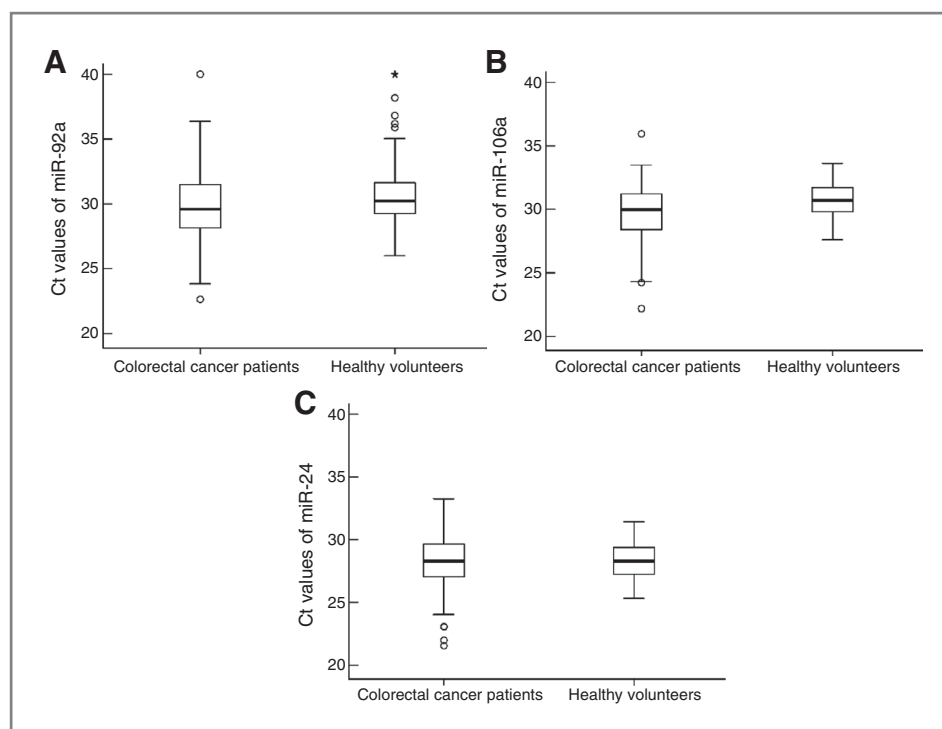


Figure 2. Ct values of each miRNA in colorectal cancer patients and healthy volunteers shown in box and whisker plots. A, median Ct values of miR-92a in colorectal cancer patients and healthy volunteers were 29.65 and 30.24, respectively, with a significant difference between them ($P = 0.016$). B, median Ct values of miR-106a in colorectal cancer patients and healthy volunteers were 29.99 and 30.70, respectively, with a significant difference between them ($P < 0.001$). C, median Ct values of miR-24 in colorectal cancer patients and healthy volunteers were 28.31 and 28.32, respectively, with no significant difference between them ($P = 0.67$). Open circles and asterisks denote outliers. Open circles imply 1.5–3 fold out from the interquartile range (IQR). Asterisks imply 3 fold out from the IQR. Differences in Ct values of the target miRNAs were analyzed by two-sided Mann–Whitney U test. $P < 0.05$ was considered statistically significant.

patients and the healthy volunteers were shown in Table 2. Significant differences were observed regarding the relative expression levels of miR-16, -19a, -92a, -106a, -132, -142-3p, -223, -223*, and -451 between the colorectal cancer patients and healthy volunteers ($P < 0.05$). Moreover, particularly significant differences were found for miR-16, -92a, -106a, -142-3p, -223, and -451 ($P < 0.01$).

Novel colorectal cancer screening method combining iFOBT with FmiRT

To improve the relatively low sensitivity of iFOBT, it is important to identify colorectal cancer patients with false-negative results correctly as positive. In this study, the numbers of true-positive colorectal cancer patients and false-negative colorectal cancer patients using iFOBT

Table 2. Relative quantification of each miRNA using RNA extracted from residuum of iFOBT

miRNA	Median RQ (range)		P value
	Colorectal cancer patients (N = 117)	Healthy volunteers (N = 107)	
miR-15b	0.057 (0.0005–1.704)	0.058 (0.008–0.402)	0.801
miR-16	2.918 (0.235–138.525)	2.342 (0.501–5.820)	<0.001
miR-19a	0.170 (0.008–3.138)	0.140 (0.025–0.378)	0.049
miR-19b	0.358 (0.013–5.869)	0.309 (0.070–71.556)	0.074
miR-20b	0.045 (0.001–1.061)	0.035 (0.007–1.201)	0.107
miR-92a	0.365 (0.001–6.845)	0.298 (0.0003–1.562)	0.003
miR-93	0.246 (0.006–2.413)	0.226 (0.019–0.748)	0.624
miR-106a	0.333 (0.023–4.411)	0.202 (0.034–0.515)	<0.001
miR-132	0.019 (0.0002–0.373)	0.014 (0.0003–0.219)	0.042
miR-142-3p	0.392 (0.001–5.645)	0.135 (0.018–2.422)	<0.001
miR-223	6.902 (0.275–38.774)	2.413 (0.249–17.244)	<0.001
miR-223*	0.020 (0.0001–1.963)	0.012 (0.0002–0.264)	0.047
miR-342-3p	0.027 (0.0001–4.721)	0.031 (0.0002–14.104)	0.099
miR-451	0.025 (0.0001–20.379)	0.010 (0.0001–0.217)	<0.001

Abbreviations: P value, analyzed by a Mann–Whitney U test, with $P < 0.05$ considered to denote statistical significance; RQ, relative quantification.

were 71 (60.7%) and 46 (39.3%), respectively (Table 1). The median tumor sizes of colorectal cancer patients with true-positive iFOBT (true-positive subgroup) and false-negative iFOBT (false-negative subgroup) were 37 mm (range, 13–125 mm) and 21 mm (7–82mm), respectively. There were significant differences between the true-positive subgroup and the false-negative subgroup regarding the tumor size ($P < 0.001$) and tumor depth ($P = 0.011$). However, no significant differences between the true-positive subgroup and the false-negative subgroup were observed for the tumor location and cancer stage ($P > 0.05$). The relative expression levels of 6 selected miRNAs (miR-16, -92a, -106a, -142-3p, -223, and -451) in the true-positive subgroup, the false-negative subgroup, and the healthy volunteer group are shown in Fig. 3. The expression of miR-451 in the true-positive subgroup was not significantly different from that in the healthy volunteer group ($P = 0.122$). However, significant differences were

observed in the expression of miR-16, -92a, -106a, -142-3p, and -223 between the true-positive subgroup and the healthy volunteer group ($P < 0.05$). A significant difference was also observed for only miR-106a between the false-negative subgroup and the healthy volunteer group ($P < 0.001$); however, no significant difference was observed between the false-negative subgroup and the true-positive subgroup ($P = 0.291$). Thus, miR-106a was used for FmiRT because its expression may reflect the presence of colorectal cancer cells, but not intestinal bleeding into feces.

The sensitivities of FmiRT using miR-106a expression in the true-positive subgroup and the false-negative subgroup were 39.4% [28 of 71; 95% confidence interval (CI), 28.0–51.7] and 26.1% (12 of 46; 95% CI, 14.3–41.1%) using a threshold of miR-106a calculated by volunteer's data that 97% of volunteers were diagnosed as negative result of FmiRT. The sensitivity and specificity of the

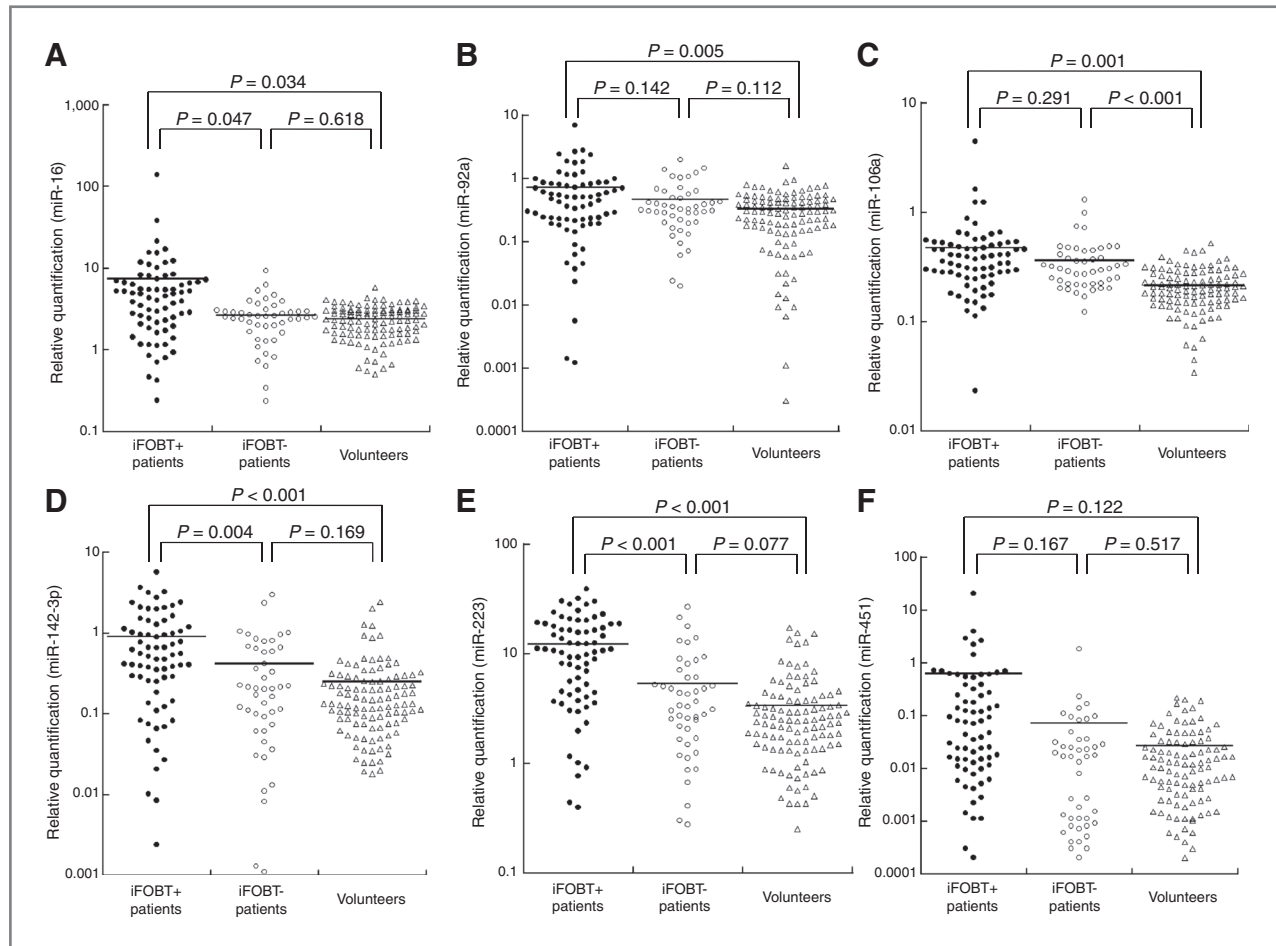


Figure 3. Relative quantification of each miRNA in the true-positive subgroup of colorectal cancer patients, the false-negative subgroup of colorectal cancer patients, and the healthy volunteer group. A, mean relative levels of miR-16 in the true-positive subgroup, the false-negative subgroup, and the healthy volunteer group were 7.467, 2.623, and 2.385, respectively. B, those of miR-92a were 0.709, 0.461, and 0.328, respectively. C, those of miR-106a were 0.467, 0.359, and 0.213, respectively. D, those of miR-142-3p were 0.906, 0.419, and 0.250, respectively. E, those of miR-223 were 12.181, 5.326, and 3.361, respectively. F, those of miR-451 were 0.613, 0.070, and 0.027, respectively. Differences in relative levels of the target miRNAs between the two subgroups of colorectal cancer patients and the healthy volunteer group were analyzed by Games–Howell test of one-way ANOVA. $P < 0.05$ was considered statistically significant.

Table 3. Sensitivity and specificity of FmiRT and iFOBT

Target	Threshold	All patients (N = 117)		iFOBT+ patients (N = 71)		iFOBT- patients (N = 46)		Healthy volunteers (N = 107)	
		No.	Sensitivity (%, 95% CI)	No.	Sensitivity (%, 95% CI)	No.	Sensitivity (%, 95% CI)	No.	Specificity (%, 95% CI)
FmiRT (miR-106a)	0.43	40	34.2 (25.6–43.6)	28	39.4 (28.0–51.7)	12	26.1 (14.3–41.1)	104	97.2 (92.0–99.4)
iFOBT	50 ng/mL	71	60.7 (51.2–69.6)					105	98.1 (93.4–99.8)
iFOBT + FmiRT		83	70.9 (61.8–79.0)					103	96.3 (90.7–99.0)

Abbreviations: iFOBT+ patients, colorectal cancer patients whose iFOBT was positive; iFOBT- patients, colorectal cancer patients whose iFOBT was negative; No., number of patients whose FmiRT or iFOBT was positive, and number of volunteers whose FmiRT or iFOBT was negative.

method combining iFOBT and FmiRT were 70.9% (83 of 117; 95% CI, 61.8–79.0) and 96.3% (103 of 107; 95% CI, 90.7–99.0), respectively (Table 3). One quarter of colorectal cancer patients (12 patients) with false-negative results of iFOBT seemed as true positive by adding FmiRT using fecal miR-106a.

Discussion

We previously reported the analysis of fecal miRNA using exfoliated colonocytes isolated from feces (23, 24) and several studies have reported that fecal miRNA could be useful for the diagnosis of colorectal cancer using RNA directly extracted from feces (25–27) because miRNA extracted directly from feces was more stable than that from the residuum of iFOBT. However, the sensitivity and specificity of an FmiRT are inferior to those of iFOBT in the several reports. We thought that the issue of iFOBT (the presence of false-negative colorectal cancer patients) could be resolved by combination of iFOBT and FmiRT, thus we used the residuum of iFOBT because "pellet miRNA" is more comparable than "direct miRNA." Very recently, we also reported that miRNA could be extracted at a level sufficiently for analysis from the residuum of iFOBT (22); however, there have been no studies comparing the miRNA expression between direct fecal RNA and pellet fecal RNA. In reality, we could show the positive correlation between "direct miRNA" and "pellet miRNA" in this study. miRNAs capsuled in exosome could be detected in the supernatant of fecal solution or cell culture as we reported previously (21). However, the fecal pellet contains exfoliated cancer cells, and we thought that colorectal cancer-related miRNAs could be extracted from fecal pellet more efficiently. In addition, our preliminary study revealed that PCR using 100 or 500 ng of fecal miRNA was not succeeded because of the presence of PCR inhibitory molecules contained in feces, and 5 ng of miRNA was enough for analysis of miRNA expression. Because FOBT is well known to produce a large number of false-positive results, Calistri and colleagues assessed whether the long DNA test could improve its specificity in a large series of consecutively enrolled iFOBT+ indi-

viduals by reducing false-positive results using residuum of iFOBT (28). They concluded that iFOBT and long DNA analysis were largely independent variables, and that an approach combining iFOBT and long DNA evaluation could help to reduce the rate of unnecessary TCS for false-positive healthy individuals. Thus, the numbers of false-negative colorectal cancer patients and false-positive healthy individuals might be reduced by applying molecular biological methods using fecal DNA or RNA extracted from the residuum of iFOBT.

In our preliminary study, the expressions of fecal miRNA (direct miRNA) from 10 patients of colorectal cancer and 10 healthy volunteers were analyzed using TaqMan Array Human MicroRNA Cards Set v3.0 (Applied Biosystems). A total of 22 miRNAs of 749 miRNAs were selected using following criteria: (i) the fecal miRNA expression in colorectal cancer patients was 2 times higher than that in healthy volunteers, (ii) the mean Ct value of each miRNA was less than 36, and (iii) miRNA was detected in 50% of patients and volunteers. Custom array card contained with these 22 miRNAs (Applied Biosystems) were obtained, and 14 miRNAs of 22 miRNAs were expressed significantly high in colorectal cancer patients compared to healthy volunteers. Thus, 14 miRNAs were used in this study.

The large numbers of false-negative colorectal cancer patients and false-positive healthy individuals in FOBT have long been a concern. In this study, we focused on reducing the size of the former group. It is important in colorectal cancer patients with false-negative results in iFOBT for this error to be corrected because a correct early diagnosis greatly increases the likelihood of curative treatment. In this study, no significant difference between the true-positive subgroup and the false-negative subgroup was observed regarding the expression of miR-106a in colorectal cancer patients. However, there was a significant difference for this marker between the false-negative subgroup in colorectal cancer patients and the healthy volunteer group. However, no significant differences were observed between the false-negative subgroup and the healthy volunteer group in terms of the expression of miR-16, miR-142-3p, and miR-223, whereas there were

significant differences between the true-positive and the false-negative subgroups. These results indicate that miR-16, miR-142-3p, and miR-223 may be diagnostic markers of intestinal bleeding, as reflected by the presence of factors such as granulocytes in the feces.

miR-106a is known to be expressed high in colorectal cancer tissue than in normal colorectal tissue, and to suppress several gene expressions. Recently, the expression of TGFBR2 could be suppressed by miR-106a resulting in the enhancement of the cancer invasion (29). Thus, we consider that miR-106a is a suitable marker for cancer screening. The usefulness of fecal miR-106a to detect colorectal cancer has been reported recently (25). However, it is difficult to diagnose the colorectal cancer using fecal miRNA alone. Thus, we conducted to combine iFOBT and fecal miR-106a. The fecal miRNA assay using residuum of iFOBT to detect colorectal cancer has been reported recently (22). The solution of iFOBT is consisted of PBS or HEPES buffer, thus, the exfoliated colorectal cancer cells are stable in the solution at 4°C up to 5 days. In this study, one quarter of colorectal cancer patients (12 patients) with false-negative results of iFOBT seemed as true positive using fecal miR-106a. We think that this is a novel finding. Thus, fecal miR-106a was shown to be a good molecular marker to identify colorectal cancer patients from among iFOBT-negative participants. Combinatory use of FmiRT and iFOBT may thus enhance the sensitivity to detect colorectal cancer.

References

- 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.
- 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.
- 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.
- Dancourt V, Lejeune C, Lepage C, Gailliard MC, Meny B, Faivre J. Immunochemical faecal occult blood tests are superior to guaiac-based tests for the detection of colorectal neoplasms. *Eur J Cancer* 2008;44:2254-8.
- Parra-Blanco A, Gimeno-Garcia AZ, Quintero E, Nicolas D, Moreno SG, Jimenez A, et al. Diagnostic accuracy of immunochemical versus guaiac faecal occult blood tests for colorectal cancer screening. *J Gastroenterol* 2010;45:703-12.
- Lieberman DA, Weiss DG, Veterans Affairs Cooperative Study G. 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.
- Sung JJ, Chan FK, Leung WK, Wu JC, Lau JY, Ching J, et al. Screening for colorectal cancer in Chinese: comparison of fecal occult blood test, flexible sigmoidoscopy, and colonoscopy. *Gastroenterology* 2003;124:608-14.
- Imperiale TF, Ransohoff DF, Itzkowitz SH, Turnbull BA, Ross MEC. Colorectal Cancer Study G. Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. *N Engl J Med* 2004;351:2704-14.
- 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.
- 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.
- Matsushita H, Matsumura Y, Moriya Y, Akasu T, Fujita S, Yamamoto S, 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.
- Onouchi S, Matsushita H, Moriya Y, Akasu T, Fujita S, Yamamoto S, 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.
- Ahliquist DA, Sargent DJ, Loprinzi CL, Levin TR, Rex DK, Ahnen DJ, et al. Stool DNA and occult blood testing for screen detection of colorectal neoplasia. *Ann Intern Med* 2008;149:441-50, W81.
- 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.
- Leung WK, To KF, Man EP, Chan MW, Hui AJ, Ng SS, 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.
- Takai T, Kanaoka S, Yoshida K, Hamaya Y, Ikuma M, Miura N, 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Koga, N. Yamazaki, Y. Yamamoto, Y. Matsumura

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17. Ng EK, Chong WW, Jin H, Lam EK, Shin VY, Yu J, 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.
18. 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.
19. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008;105:10513–8.
20. Bovell L, Shanmugam C, Katkooi VR, Zhang B, Vogtmann E, Grizzle WE, et al. miRNAs are stable in colorectal cancer archival tissue blocks. *Front Biosci (Elite edition)* 2012;4:1937–40.
21. Koga Y, Yasunaga M, Moriya Y, Akasu T, Fujita S, Yamamoto S, et al. Exosome can prevent RNase from degrading microRNA in feces. *J Gastrointest Oncol* 2011;2:215–22.
22. Yamazaki N, Koga Y, Yamamoto S, Kakugawa Y, Otake Y, Hayashi R, et al. Application of the fecal microRNA test to the residuum from the fecal occult blood test. *Jpn J Clin Oncol* 2013;43:726–33.
23. Murata S, Koga Y, Moriya Y, Akasu T, Fujita S, Yamamoto S, et al. Application of miRNA expression analysis on exfoliated colonocytes for colorectal cancer diagnosis. *Gastrointest Cancer* 2012; 2:11–8.
24. Koga Y, Yasunaga M, Takahashi A, Kuroda J, Moriya Y, Akasu T, et al. MicroRNA expression profiling of exfoliated colonocytes isolated from feces for colorectal cancer screening. *Cancer Prev Res* 2010;3:1435–42.
25. Link A, Balaguer F, Shen Y, Nagasaka T, Lozano JJ, Boland CR, et al. Fecal MicroRNAs as novel biomarkers for colon cancer screening. *Cancer Epidemiol Biomarkers Prev* 2010;19:1766–74.
26. Kalimutho M, Del Vecchio Blanco G, Di Cecilia S, Sileri P, Cretella M, Pallone F, et al. Differential expression of miR-144* as a novel fecal-based diagnostic marker for colorectal cancer. *J Gastroenterol* 2011; 46:1391–402.
27. Wu CW, Ng SS, Dong YJ, Ng SC, Leung WW, Lee CW, et al. Detection of miR-92a and miR-21 in stool samples as potential screening biomarkers for colorectal cancer and polyps. *Gut* 2012;61:739–45.
28. Calistri D, Rengucci C, Casadei Gardini A, Frassinetti GL, Scarpi E, Zoli W, et al. Fecal DNA for noninvasive diagnosis of colorectal cancer in immunochemical fecal occult blood test-positive individuals. *Cancer Epidemiol Biomarkers Prev* 2010;19:2647–54.
29. Feng B, Dong TT, Wang LL, Zhou HM, Zhao HC, Dong F, et al. Colorectal cancer migration and invasion initiated by microRNA-106a. *PLoS One* 2012;7:e43452.