Title: 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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Running title: FmiRT combined with iFOBT

Keywords: fecal miRNA, cancer screening, colorectal cancer, fecal occult blood test, early detection

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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 miRNA test (FmiRT) to detect colorectal cancer. In the present 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 performed. 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 RT-PCR.

Results: Levels of fecal miR-106a expression in iFOBT-positive patients and iFOBT-negative 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 of iFOBT appeared 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.
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 efficacy 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 while 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) (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 (SSCP) 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 utilizing reverse transcription polymerase chain reaction (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.

MicroRNAs (miRNAs) are small (18–25 nucleotides in size) non-coding 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 (FFPE) 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 the present 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 the present 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 performed 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 (iFOBT)

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 × 10-cm polystyrene tray (AsOne, Osaka, Japan) 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 iFOBT kit, OC-Hemocatch (Eiken Chemical, Tokyo, Japan). iFOBT was immediately performed using OC-Hemocatch in accordance with the manufacturer’s instructions. Briefly, 100-μL samples of the dissolved feces were incubated with the iFOBT kit reagents for 5 min at room temperature. After incubation, a blue line appeared in the control window if the iFOBT had been performed correctly. iFOBT was designated as positive if a blue line also appeared in the test window, while the samples for which a line did not appear 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 g for 5 min 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, Valencia, CA) according to the manufacturer’s instructions. Briefly, each fecal pellet was resuspended in 1 mL of QIAzol (Qiagen). Thereafter, 200 μL of chloroform was added, the mixture was vortexed vigorously for 15 s and incubated for 3 min at room temperature, and then centrifuged at 13,000 g for 15 min 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 g for 15 s at room temperature. After washing, total RNA was dissolved in 100 μL 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 above. Briefly, 500 mg of feces was homogenized with 5 mL of QIAzol using an IKA Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany) at 6,000 rpm for 2 min at room temperature. The homogenates were centrifuged at 13,000 g for 5 min 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 s and centrifuged at 13,000 g for 15 min at 4°C. The aqueous 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 an miRNeasy spin column and the column was centrifuged at 7,000 g for 15 s at room temperature. After washing, total
RNA was dissolved in 100 μL of RNase-free water.

The concentrations of total RNA were determined using a NanoDrop UV spectrometer (LMS, Tokyo, Japan). 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, Foster, CA), in accordance with the manufacturer’s instructions. The reaction mixture consisted of 5 ng of total RNA, 0.5 μL of 10 × RT buffer, 1 μL of 5 × specific primer, 0.05 μL of dNTPs (100 mM), 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. cDNA synthesis was performed with incubation at 16°C for 30 min and 42°C for 30 min.

The reaction mixture for the real-time PCR analysis consisted of 4 μL of 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 PCR was performed with pre-cycling heat activation at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30 s, 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: (dCt of each miRNA) = (Ct of each miRNA) − (Ct of miR-24), and (Relative quantification of each miRNA) = 2^{-(dCt of each miRNA)}. Differences in Ct values of the target miRNAs were analyzed by two-sided Mann-Whitney’s U-test and differences in relative quantification of the target miRNAs between two subgroups of colorectal cancer patients and healthy volunteers were analyzed by Games-Howell test of one-way ANOVA. Statistical analyses were performed using SPSS Statistics Ver. 19 (IBM, Armonk, NY). 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 Figure 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 Figure 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); on the other hand, 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 -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 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 the present study, the numbers of true-positive colorectal cancer patients and false-negative colorectal cancer patients using iFOBT 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 (7–82 mm), 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). On the other hand, 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 six 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 Figure 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% (28/71; 95% CI, 28.0–51.7) and 26.1% (12/46; 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 fecal miRNA test. The sensitivity and specificity of the method combining iFOBT and FmiRT were 70.9% (83/117; 95% CI, 61.8–79.0) and 96.3% (103/107; 95% CI, 90.7–99.0), respectively (Table 3). One-quarter of colorectal cancer patients (12 patients) with false-negative results of iFOBT appeared 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 a fecal miRNA test are inferior to those of iFOBT in the several reports. We thought that the issue of iFOBT (the presence of false-negative CRC patients) could be resolved by combination of iFOBT and fecal miRNA test, 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 CRC related miRNAs could be extracted from fecal pellet more efficiently. In addition, our preliminary study revealed that PCR using 100 ng 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 et al. assessed whether the long DNA test could improve its specificity in a large series of consecutively enrolled iFOBT-positive individuals 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 CRC and 10 healthy volunteers were analyzed using TaqMan Array Human MicroRNA Cards Set v3.0 (Applied Biosystems). 22 miRNAs of 749 miRNAs were selected using following criteria: 1) the fecal miRNA expression in CRC patients was two times higher than that in healthy volunteers, 2) the mean Ct value of each miRNA was less than 36, and 3) 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 CRC 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 the present 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 the present 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. On the other hand, 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 subgroup and the false-negative subgroup. 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 CRC 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 CRC 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 CRC cells are stable in the solution at 4°C up to 5 days. In the present study, one-quarter of colorectal cancer patients (12 patients) with false-negative results of iFOBT appeared 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.

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References


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

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

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 chi-square test, with P < 0.05 considered to denote statistical significance.
<table>
<thead>
<tr>
<th>miRNA</th>
<th>Median RQ (range)</th>
<th>CRC patients (N = 117)</th>
<th>Healthy volunteers (N = 107)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-15b</td>
<td>0.057 (0.0005—1.704)</td>
<td>0.058 (0.008—0.402)</td>
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<td>0.801</td>
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<tr>
<td>miR-16</td>
<td>2.918 (0.235—138.525)</td>
<td>2.342 (0.501—5.820)</td>
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<td>&lt;0.001</td>
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<td>miR-19a</td>
<td>0.170 (0.008—3.138)</td>
<td>0.140 (0.025—0.378)</td>
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<td>0.049</td>
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<td>miR-19b</td>
<td>0.358 (0.013—5.869)</td>
<td>0.309 (0.070—71.556)</td>
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<td>0.074</td>
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<tr>
<td>miR-20b</td>
<td>0.045 (0.001—1.061)</td>
<td>0.035 (0.007—1.201)</td>
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<td>0.107</td>
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<td>miR-92a</td>
<td>0.365 (0.001—6.845)</td>
<td>0.298 (0.0003—1.562)</td>
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<td>miR-93</td>
<td>0.246 (0.006—2.413)</td>
<td>0.226 (0.019—0.748)</td>
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<td>0.624</td>
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<tr>
<td>miR-106a</td>
<td>0.333 (0.023—4.411)</td>
<td>0.202 (0.034—0.515)</td>
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<td>&lt;0.001</td>
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<tr>
<td>miR-132</td>
<td>0.019 (0.0002—0.373)</td>
<td>0.014 (0.0003—0.219)</td>
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<td>0.042</td>
</tr>
<tr>
<td>miR-142-3p</td>
<td>0.392 (0.001—5.645)</td>
<td>0.135 (0.018—2.422)</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-223</td>
<td>6.902 (0.275—38.774)</td>
<td>2.413 (0.249—17.244)</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-223*</td>
<td>0.020 (0.0001—1.963)</td>
<td>0.012 (0.0002—0.264)</td>
<td></td>
<td>0.047</td>
</tr>
<tr>
<td>miR-342-3p</td>
<td>0.027 (0.0001—4.721)</td>
<td>0.031 (0.0002—14.104)</td>
<td></td>
<td>0.099</td>
</tr>
<tr>
<td>miR-451</td>
<td>0.025 (0.0001—20.379)</td>
<td>0.010 (0.0001—0.217)</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

RQ: relative quantification, P value: analyzed by a Mann-Whitney U test, with P < 0.05 considered to denote statistical significance.
<table>
<thead>
<tr>
<th>Target</th>
<th>Threshold</th>
<th>All patients (N = 117)</th>
<th>iFOBT+ patients (N = 71)</th>
<th>iFOBT- patients (N = 46)</th>
<th>Healthy volunteers (N = 107)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. Sensitivity (%)</td>
<td>No. Sensitivity (%)</td>
<td>No. Sensitivity (%)</td>
<td>No. Specificity (%)</td>
</tr>
<tr>
<td>FmiRT (miR-106a)</td>
<td>0.43</td>
<td>40</td>
<td>34.2 (25.6—43.6)</td>
<td>28</td>
<td>39.4 (28.0—51.7)</td>
</tr>
<tr>
<td>iFOBT</td>
<td>50 ng/mL</td>
<td>71</td>
<td>60.7 (51.2—69.6)</td>
<td>105</td>
<td>98.1 (93.4—99.8)</td>
</tr>
<tr>
<td>iFOBT + FmiRT</td>
<td>83</td>
<td>103</td>
<td>70.9 (61.8—79.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FmiRT: fecal miRNA test, iFOBT: immunochemical fecal occult blood test, 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, 95% CI: 95% confidence interval.
Figure legends:

**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). Differences in Ct values of the target miRNAs were analyzed by two-sided Mann-Whitney’s U-test. P < 0.05 was considered statistically significant.
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.
Figure 1

A) miR-92a

B) miR-106a

C) miR-24

P < 0.001
r = 0.549

P < 0.001
r = 0.747

P < 0.001
r = 0.684
Figure 2

A) Ct values of miR-92a

Colorectal cancer patients

Healthy volunteers

B) Ct values of miR-106a

Colorectal cancer patients

Healthy volunteers

C) Ct values of miR-24

Colorectal cancer patients

Healthy volunteers
Figure 3

A) $P = 0.034$

B) $P = 0.005$

C) $P = 0.001$

D) $P < 0.001$

E) $P < 0.001$

F) $P = 0.122$

Relative quantification (mRNA)

- iFOBT+ patients
- iFOBT- patients
- Volunteers

$P$ values indicate statistical significance.
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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