

Application of the Fecal MicroRNA Test to the Residuum from the Fecal Occult Blood Test

メタデータ	言語: English 出版者: 公開日: 2016-03-20 キーワード (Ja): キーワード (En): 作成者: 山崎, 信義 メールアドレス: 所属:
URL	https://jair.repo.nii.ac.jp/records/2001908

**Title: Application of the fecal micro RNA test to the residuum from the fecal occult
blood test**

Authors

Nobuyoshi Yamazaki^{1,2}, Yoshikatsu Koga³, Seiichiro Yamamoto⁴, Yasuo Kakugawa⁵,
Yosuke Otake⁵, Ryuichi Hayashi^{2,6}, Norio Saito¹, Yasuhiro Matsumura³

Author's affiliations

1: Department of Colorectal and Pelvic Surgery, National Cancer Center Hospital East

2: Center for Lifetime Cancer Education, Juntendo University Graduate School of
Medicine

3: Division of Developmental Therapeutics, Research Center for Innovative Oncology,
National Cancer Center Hospital East

4: Colorectal Surgery Division, National Cancer Center Hospital

5: Screening Technology and Development Division, Research Center for Cancer
Prevention and Screening, National Cancer Center

6: Division of Head and Neck Surgery, National Cancer Center Hospital East

Corresponding author: Yasuhiro Matsumura

Division of Developmental Therapeutics, Research Center for Innovative Oncology,

National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, 277-8577, Japan

Telephone and FAX: +81-4-7134-6857

E-mail: yhmatsum@east.ncc.go.jp

Running title: Fecal miRNA test using FOBT residuum

Abstract

Background: Though fecal occult blood test (FOBT) is used for colorectal cancer (CRC) screening worldwide, it does not have a particularly high sensitivity for detecting CRC. Here, we investigated the applicability of the fecal micro RNA (miRNA) test to fecal samples used for previous FOBT stored under various conditions.

Methods: Five CRC patients and 5 healthy volunteers were enrolled. Fecal samples were stored for 0–5 days at either 4°C, room temperature, or 37°C. Total RNA was extracted from FOBT residuum and miRNA expression was analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR).

Results: There were no remarkable differences in either CRC patients or controls in terms of concentration of RNA extracted from FOBT residuum in any of the storage groups compared with the samples prepared on day 0 (Group 0). Ribosomal RNA stored at room temperature or 37°C degraded rapidly. In contrast, ribosomal RNA stored at 4°C remained intact for at least 5 days. miRNAs in samples stored at 4°C and room temperature were conserved; however, miRNA stored at 37°C were significantly degraded compared with Group 0 ($P<0.05$). In residuum stored at 4°C up to 5 days, the relative quantification of miR-106a normalized to miR-24 in CRC patients was significantly higher than those in healthy volunteers ($P<0.05$). In contrast, the

quantification of normalized miR-106a was remarkably low in samples stored at room temperature and 37°C.

Conclusion: Sufficient quality of fecal miRNA for RT-PCR analysis was extracted from FOBT residuum stored at 4°C for up to 5 days.

Key words: colorectal cancer, fecal occult blood test, fecal miRNA, cancer screening

Mini-abstract

The present study demonstrates that fecal miRNA for RT-PCR analysis can be performed using FOBT residuum stored at 4°C for up to 5 days.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality worldwide (1). To reduce the mortality rate of CRC, the fecal occult blood test (FOBT) has been widely used as a screening test for CRC (2-4). However, large-scale studies have shown that the sensitivity of FOBT is not very high in all subjects using total colonoscopy as a reference standard (5-8). Therefore, several methods for the early detection of CRC have been developed.

MicroRNAs (miRNAs), which are small (18–25 nucleotides in size) noncoding RNA molecules, are known to regulate the activity of specific mRNAs and play a major role in cancer progression. The function of miRNAs is to downregulate the expression of multiple target genes by degrading their mRNAs or blocking their translation into protein through RNA interference (9, 10). Several recent studies have reported that fecal miRNA is relatively stable (11), and several fecal miRNAs, such as miRNA-21 (miR-21) (12, 13), miR-92a (13), miR-106a (12), and miR-144* (14), are expressed more highly in CRC patients than in healthy volunteers. In addition, we have developed a fecal miRNA test using colonocyte RNA (15).

Hemoglobin in fecal samples stored at 4°C remained stable (16, 17); however, hemoglobin degraded rapidly in feces stored at room temperature (RT) or 37°C (18).

Thus, the FOBT may be influenced by the storage temperature (19). In contrast, fecal miRNA stored at RT was stable for up to 72 hrs (13, 14), and may be useful for CRC screening. When FOBT is positive, we expect that an auxiliary test using same stool samples can improve the accuracy. Thus, we planned this pilot study.

In the present study, we investigated whether miRNA could be extracted from residual FOBT samples, and assessed the suitability of the FOBT samples stored in several conditions for the fecal miRNA test.

Methods

Study participants

In the present study, we enrolled 5 male patients with histologically confirmed CRC, and 5 healthy volunteers between May 2011 and December 2011 (Table 1). CRC patients with positive result of FOBT were selected. All CRC patients underwent surgical resection of their primary cancer at the National Cancer Center Hospital East, Kashiwa, Japan. The median age of the patients was 52 years (range, 39–68 years). The primary tumors were located in the sigmoid colon in 2 patients and the rectum in 3 patients. The median diameter of the primary tumors was 35 mm (range, 18–45 mm). The clinical stage of the patients according to Dukes' classification was stage A in 2

patients, stage B in 2 patients, and stage C in one patient. The healthy volunteers consisted of 2 men and 3 women without any symptoms or evidence of abnormalities such as adenoma or carcinoma (including hyperplastic polyp), as determined by screening colonoscopy performed at the Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo. The median age of the volunteers was 57 years (range, 41–73 years). All patients and all volunteers were provided with detailed information about this study and provided written consent for participating in the study, which was approved by the institutional review board of the National Cancer Center, Japan.

Fecal samples and storage conditions

Naturally evacuated fecal samples were obtained from the CRC patients prior to surgical resection. Fecal samples were also obtained from the healthy volunteers approximately 2 weeks after they underwent a total colonoscopy. All patients and volunteers were instructed to evacuate the feces into a disposable 5 × 10 cm polystyrene tray (AS ONE, Osaka, Japan). All fecal samples were immediately cooled at 4°C and delivered to our laboratory.

The fecal samples were transferred into the fecal sampling container provided

with the FOBT kit, OC-Hemocatch (EIKEN CHEMICAL, Tokyo, Japan). The fecal samples were collected from several sites of feces, because the feces were heterogeneity. The fecal samples were divided into 16 groups, one being prepared on the day (day 0) of arrival at the laboratory, and the others being stored for 1, 2, 3, 4, or 5 days at 4°C, RT, or 37°C. Thus, the storage conditions for the fecal fragments were as follows: Group 0 (processed on the day of arrival); Group 1A (4°C, 1 day); Group 1B (RT, 1 day); Group 1C (37°C, 1 day); Group 2A (4°C, 2 days); Group 2B (RT, 2 days); Group 2C (37°C, 2 days); Group 3A (4°C, 3 days); Group 3B (RT, 3 days); Group 3C (37°C, 3 days); Group 4A (4°C, 4 days); Group 4B (RT, 4 days); Group 4C (37°C, 4 days); Group 5A (4°C, 5 days); Group 5B (RT, 5 days); and Group 5C (37°C, 5 days) (Table 2).

Fecal occult blood test

Initially, immunochemical FOBT was performed using OC-Hemocatch in accordance with the manufacturer's instructions. Briefly, 100 µL samples of the dissolved feces were incubated with the FOBT kit reagents at RT for 5 min. After incubation, a blue line appeared in the control window if the FOBT was performed correctly. The samples in which a blue line appeared clearly in the test window were

designated as positive, and the samples in which a line did not appear in the test window were designated as negative. We defined the FOBT scores as positive = 1 and negative = 0. The cut-off value for the concentration of hemoglobin in the FOBT kit was 50 ng/mL.

Total RNA extraction from residuum of FOBT

The solution remaining after FOBT analysis was collected into a fresh 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 the 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 were added, the mixture was vortexed vigorously for 15 s and incubated for 3 min at RT, and then centrifuged at 13000 g for 15 min at 4°C. The upper aqueous phase was transferred to a fresh 1.5 mL tube, and 1.5 volumes of 100% ethanol were added. The solution was mixed thoroughly by pipetting and transferred to a miRNeasy Mini column. After washing with buffer RWT and buffer RPE, total RNA was extracted in 100 µL of RNase-free water. The miRNA was stored at -80°C until use.

The concentration of RNA was measured with a NanoDrop (Thermo

Scientific, Wilmington, DE), and the quality of RNA was measured with a 2100 Bioanalyzer (Aligent Technologies, Santa Clara, CA).

cDNA synthesis and real-time PCR

cDNA was synthesized using a High Capacity 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 μ L of 10 \times RT buffer, 1 μ L of 5 \times specific primer, 0.05 μ L of 25 \times dNTPs (100 mM), 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. The thermal cycling conditions were as follows: 16 $^{\circ}$ C for 30 min, 42 $^{\circ}$ C for 30 min, 85 $^{\circ}$ C for 5 min, followed by incubation at 4 $^{\circ}$ C.

We analyzed the expression of 3 miRNAs: miR-24, miR-92a, and miR-106a. For all of these miRNAs, we used the commercially available TaqMan MicroRNA Assay (Applied Biosystems). The reaction mixture for real-time PCR 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 primers and probe mixture in a total reaction volume of 20 μ L. Real-time PCR was performed with precycling heat activation at 95 $^{\circ}$ C for 20 s, followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 3 s and annealing/extension at 60 $^{\circ}$ C

for 30 s, using a 7500 Fast Real-time PCR System (Applied Biosystems).

Statistical analysis

The miRNA expression data were analyzed using the comparative Ct (threshold cycle) method. In this analysis, the formula for the relative quantification of each gene was as follows: (dCt of each miRNA) = (Ct of each miRNA) – (Ct of each miRNA in Group 0), and (relative quantification of each miRNA) = $2^{-(\text{dCt of each miRNA})}$.

Differences between the 2 groups were analyzed using Mann-Whitney *U* test or χ^2 test, whereas differences among more than 2 groups were analyzed using Dunnett's test.

Statistical analyses were performed using JMP 10 (SAS Institute Inc., Cary, NC). A *P* value of <0.05 was considered statistically significant.

Results

Determination of FOBT score in samples stored under various conditions

Initially, FOBT was performed with fecal samples stored under various conditions. The FOBT scores for samples in Group 0 from CRC patients and healthy volunteers were 0.73 ± 0.46 (mean \pm SD) and 0, respectively (Table 1). In CRC patients, the FOBT scores for fecal samples stored at 37°C for 1, 4, and 5 days were significantly

lower than the score for fecal samples prepared on the day of the arrival (day 0) ($P < 0.05$), while no significant differences were noted in FOBT scores for samples stored at 4°C and RT compared with Group 0 samples (Fig. 1). Thus, FOBT was stable in the samples stored at 4°C and RT at least for 5 days.

Total RNA extracted from FOBT residuum

Next, the concentration and quality of RNA extracted from FOBT residuum were analyzed. The median amounts of total RNA extracted from the FOBT residuum of Group 0 samples for CRC patients and healthy volunteers were 32.2 µg (range, 17.7–65.9 µg) and 24.1 µg (range, 6.0–74.4 µg), respectively (Table 1). In CRC patients, no significant differences in RNA concentration were noted in all groups compared with Group 0 samples (Fig. 2A). Moreover, no significant differences in RNA concentration were observed in healthy volunteers in all groups compared with Group 0 (Fig. 2A). Small RNAs, including miRNAs, and degraded RNA, and ribosomal RNA (rRNA) from enterobacteria including 16S and 23S rRNA, were detected in RNA extracted from fecal samples for both CRC patients and healthy volunteers (Fig 2B). RNA stored at 4°C for 5 days was stable compared with that of Group 0 samples. In contrast, 16S and 23S rRNA were not detected in RNA stored at RT or 37°C (Fig. 2B). These results

indicated that fecal RNA stored at 4°C was stable up to 5 days after evacuation, and that fecal RNA stored at RT and 37°C degraded rapidly.

Stability of miRNA in samples stored under various conditions

As discussed above, rRNA stored at RT or 37°C was degraded. We further analyzed the stability of miRNA by real-time PCR. All miRNAs in samples stored at 4°C and RT were conserved compared with those in Group 0 samples up to 5 days. However, miRNAs of CRC patients stored at 37°C were significantly degraded in Group 1C, 3C and 5C compared with those in Group 0 samples (Fig. 2C), and that of healthy volunteers were significantly degraded in Group 3C and 5C compared with those in Group 0 samples (Fig. 2D).

Relative quantification of miRNA by normalization to miR-24

When gene expression is analyzed by real-time PCR, the target genes are normalized to an internal control gene such as GAPDH or β -2-microglobulin (20). Similarly, miRNA should be normalized to an internal control when performing miRNA expression analysis. However, a universal miRNA suitable for use as an internal control has not yet been determined. In the present study, miR-24 was used as an internal control according to our previous study (15). The relative quantification of miR-92a in

fecal RNA extracted from CRC patients was not significant as compared with that from healthy volunteers at any storage conditions ($P = 0.13$) (Table 3 and Fig. 3A). On the other hands, the relative quantification of miR-106a in CRC patients was significantly higher than that in healthy volunteers at 4°C up to 5 days ($P < 0.05$). The difference at RT and 37°C was not significant between healthy volunteers and CRC patients (Table 3 and Fig. 3B).

Discussion

The use of fecal DNA testing to improve upon the unsatisfactory sensitivity of FOBT for CRC screening has been investigated since the 1990s (21, 22). A large-scale study using this methodology was reported in 2004 (5), yielding results from which the American Cancer Society subsequently updated 2008 guidelines to include the fecal DNA test as a recommended screening test for CRC (23). To date, the fecal DNA test is the only commercially available method of fecal analysis based on molecular biology techniques (24). Regarding the fecal RNA test, fecal COX2 analysis for detecting CRC was reported for the first time in 2004 (25). Recently, various studies in which CRC was detected using fecal miRNA have been reported (12-14, 26, 27). In Japan, immunochemical FOBT has been used for CRC screening and initially appeared to have

higher sensitivity and specificity than Guaiac FOBT; however, the sensitivity was reported as 26–69% (3, 6, 7, 28-30). Thus, to improve the sensitivity of FOBT, we evaluated the suitability of fecal miRNA extracted from FOBT residuum in this study. In the previous study, the detections of fecal miRNA expression were performed from stool samples or colonocytes (12-15, 20, 27, 31). In the present study, we examined the miRNA expression in total RNA extracted from residual FOBT samples.

Immunochemical FOBT has a high sensitivity for the detection of human hemoglobin in feces. However, the detection rate varies according to storage conditions; for example, rates are lower when samples are stored at 37°C than at 4°C or RT. Long RNAs, such as 16s and 23s rRNA degraded rapidly at RT and 37°C. In contrast, fecal miRNA was stable at 4°C and RT. Fecal miRNA degraded at 37°C; however, PCR amplification remained successful under these conditions. Indeed, it has been previously reported that miRNA extracted from formalin-fixed paraffin-embedded tissue stored for over 20 years can still be analyzed (32). The relative quantification of miRNAs normalized to an internal control in fecal samples stored at 4°C for up to 5 days was similar to those in Group 0. In contrast, in samples stored at RT and 37°C, the relative quantification of miRNA was significantly reduced significantly. Thus, fecal miRNA extracted from FOBT residuum should be stored at appropriate condition.

To improve upon the unsatisfactory sensitivity of FOBT, several tests, including fecal transferrin analysis, have been combined with FOBT. In the present study, the fecal miR-106a normalized with miR-24 in CRC patients was higher than that in healthy volunteers (Fig.3). Thus, the fecal miRNA test using total RNA extracted from FOBT residuum may potentially assist in improving the sensitivity of CRC screening.

To our knowledge, this is the first study indicating that it is possible to extract miRNA from residual samples after FOBT. In sub-optimal storage conditions, RNA degraded rapidly; but, we demonstrated that the fecal miRNA test using relative quantification was able to be performed efficiently. However, the present data are preliminary because only 5 CRC patients were analyzed. Therefore, further evaluation on a large scale must be needed.

Acknowledgments

This work was supported by a Grant-in-Aid for the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan (Y. Koga); Young Scientists (B) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Y. Koga); the

Innovation Promotion Program from the New Energy and Industrial Technology Development Organization of Japan (Y. Matsumura); the Japan Society for the Promotion of Science (JSPS) through the “Funding Program for World-Leading Innovative R & D on Science and Technology (FIRST Program)” initiated by the Council for Science and Technology Policy (CSTP) (Y. Matsumura); and National Cancer Center Research and Development Fund (Y. Matsumura).

We thank Ms. Noriko F. Abe and Ms. Masae Ohmaru for their technical assistance, and Ms. Kaoru Shiina for secretarial assistance.

References

1. The global burden of disease: 2004 update World Health Organization 2008.
2. 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.
3. Mandel JS, Church TR, Ederer F, et al. Colorectal cancer mortality: effectiveness of biennial screening for fecal occult blood. *J Natl Cancer Inst* 1999;91:434-7.
4. Scholefield JH, Moss S, Sufi F, et al. Effect of faecal occult blood screening on mortality from colorectal cancer: results from a randomised controlled trial. *Gut* 2002;50:840-4.
5. Imperiale TF, Ransohoff DF, Itzkowitz SH, et al. Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. *N Engl J Med* 2004;351:2704-14.
6. 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.
7. Morikawa T, Kato J, Yamaji Y, et al. A comparison of the immunochemical fecal occult blood test and total colonoscopy in the asymptomatic population. *Gastroenterology* 2005;129:422-8.
8. 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.

9. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281-97.

10. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* 2006;6:259-69.

11. Koga Y, Yasunaga M, Moriya Y, et al. Exosome can prevent RNase from degrading microRNA in feces. *Journal of gastrointestinal oncology* 2011;2:215-22.

12. Link A, Balaguer F, Shen Y, et al. Fecal MicroRNAs as novel biomarkers for colon cancer screening. *Cancer Epidemiol Biomarkers Prev* 2010;19:1766-74.

13. Wu CW, Ng SS, Dong YJ, 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.

14. Kalimutho M, Del Vecchio Blanco G, Di Cecilia S, et al. Differential expression of miR-144* as a novel fecal-based diagnostic marker for colorectal cancer. *J Gastroenterol* 2011;46:1391-402.

15. Murata S, Koga Y, Moriya Y, et al. Application of miRNA expression analysis on exfoliated colonocytes for diagnosis of colorectal cancer. *Gastrointestinal Cancer: Targets and Therapy* 2012:11.

16. Rozen P, Waked A, Vilkin A, et al. Evaluation of a desk top instrument for the automated

development and immunochemical quantification of fecal occult blood. *Med Sci Monit* 2006;12:MT27-32.

17. Vilkin A, Rozen P, Levi Z, et al. Performance characteristics and evaluation of an automated-developed and quantitative, immunochemical, fecal occult blood screening test. *Am J Gastroenterol* 2005;100:2519-25.

18. Levi Z, Hazazi R, Rozen P, et al. A quantitative immunochemical faecal occult blood test is more efficient for detecting significant colorectal neoplasia than a sensitive guaiac test. *Aliment Pharmacol Ther* 2006;23:1359-64.

19. van Rossum LG, van Rijn AF, van Oijen MG, et al. False negative fecal occult blood tests due to delayed sample return in colorectal cancer screening. *Int J Cancer* 2009;125:746-50.

20. 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.

21. Ahlquist DA, Skoletsy JE, Boynton KA, et al. Colorectal cancer screening by detection of altered human DNA in stool: feasibility of a multitarget assay panel. *Gastroenterology* 2000;119:1219-27.

22. 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.

23. Smith RA, Cokkinides V, Brawley OW. Cancer screening in the United States, 2008: a

review of current American Cancer Society guidelines and cancer screening issues. *CA Cancer J Clin* 2008;58:161-79.

24. 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.

25. Kanaoka S, Yoshida K-I, Miura N, et al. Potential usefulness of detecting cyclooxygenase 2 messenger RNA in feces for colorectal cancer screening. *Gastroenterology* 2004;127:422-7.

26. Ahmed FE, Jeffries CD, Vos PW, et al. Diagnostic microRNA markers for screening sporadic human colon cancer and active ulcerative colitis in stool and tissue. *Cancer genomics & proteomics* 2009;6:281-95.

27. Koga Y, Yasunaga M, Takahashi A, et al. MicroRNA expression profiling of exfoliated colonocytes isolated from feces for colorectal cancer screening. *Cancer Prev Res (Phila)* 2010;3:1435-42.

28. Allison JE, Feldman R, Tekawa IS. Hemoccult screening in detecting colorectal neoplasm: sensitivity, specificity, and predictive value. Long-term follow-up in a large group practice setting. *Ann Intern Med* 1990;112:328-33.

29. Ahlquist DA, Wieand HS, Moertel CG, et al. Accuracy of fecal occult blood screening for colorectal neoplasia. A prospective study using Hemoccult and HemoQuant tests. *JAMA* 1993;269:1262-7.

30. Allison JE, Tekawa IS, Ransom LJ, et al. A comparison of fecal occult-blood tests for colorectal-cancer screening. *N Engl J Med* 1996;334:155-9.
31. 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.
32. Bovell L, Shanmugam C, Katkooi VR, et al. miRNAs are stable in colorectal cancer archival tissue blocks. *Frontiers in bioscience (Elite edition)* 2012;4:1937-40.

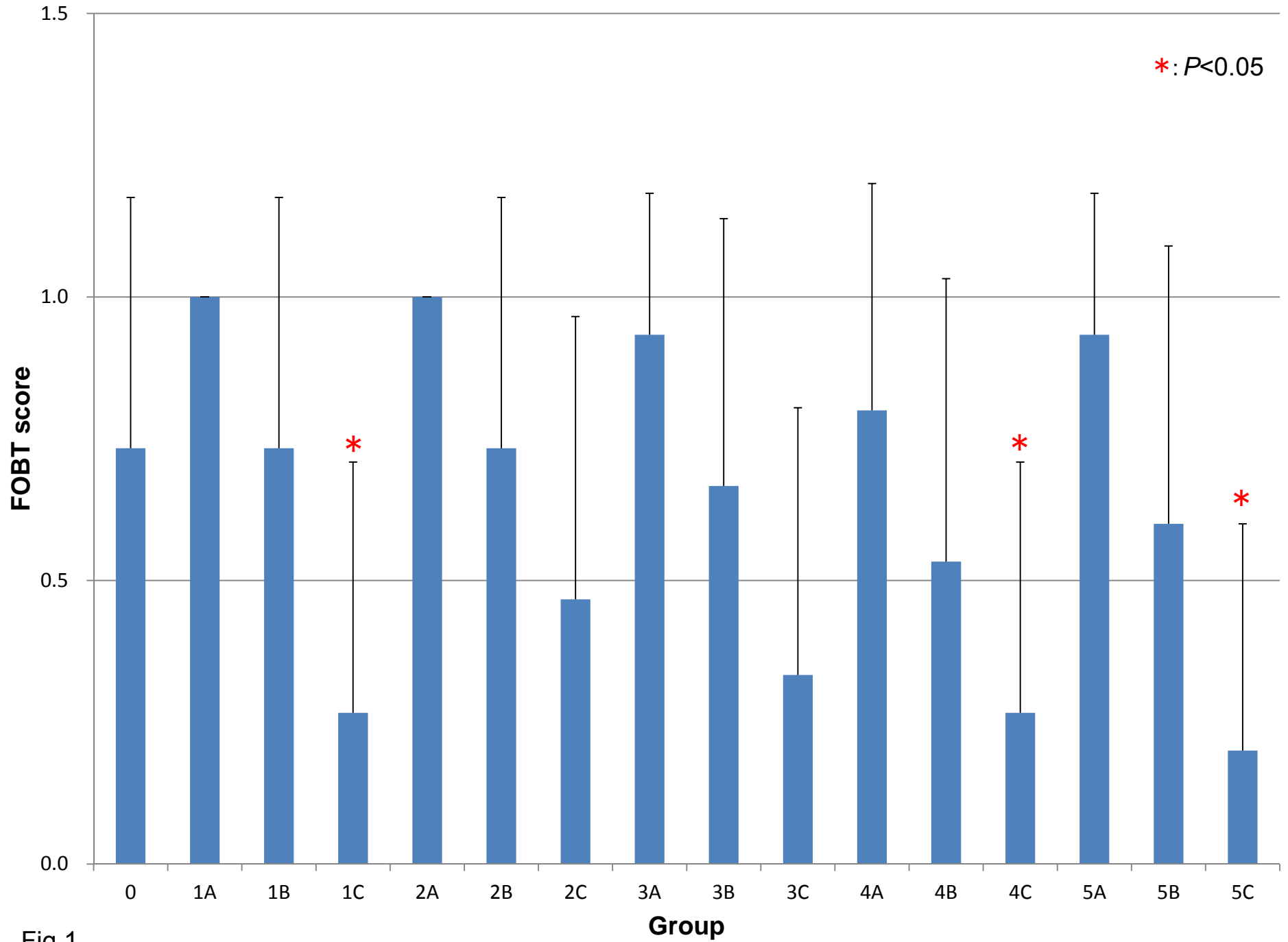


Fig.1

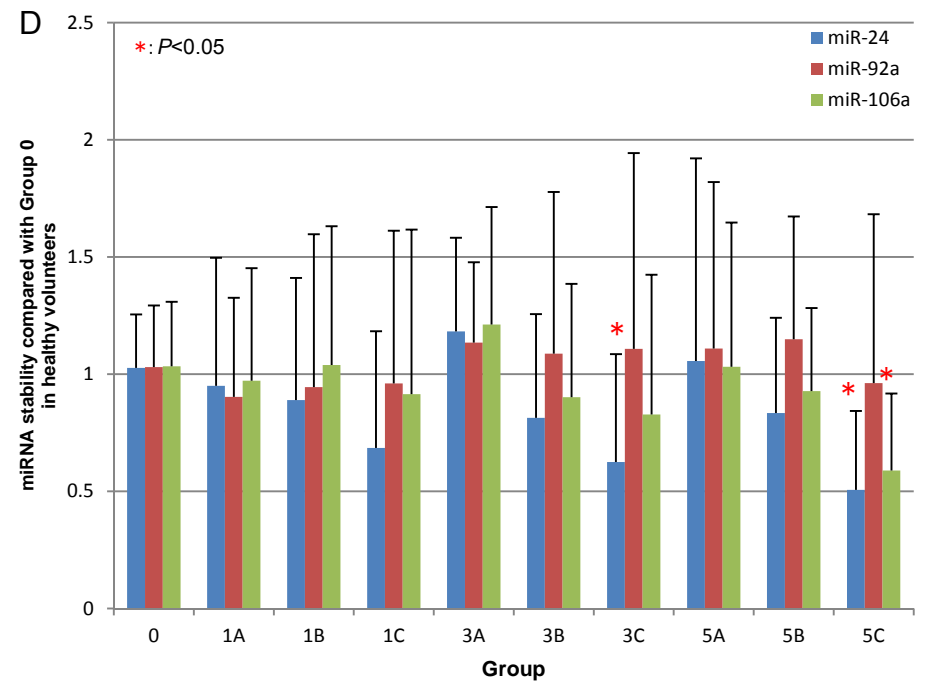
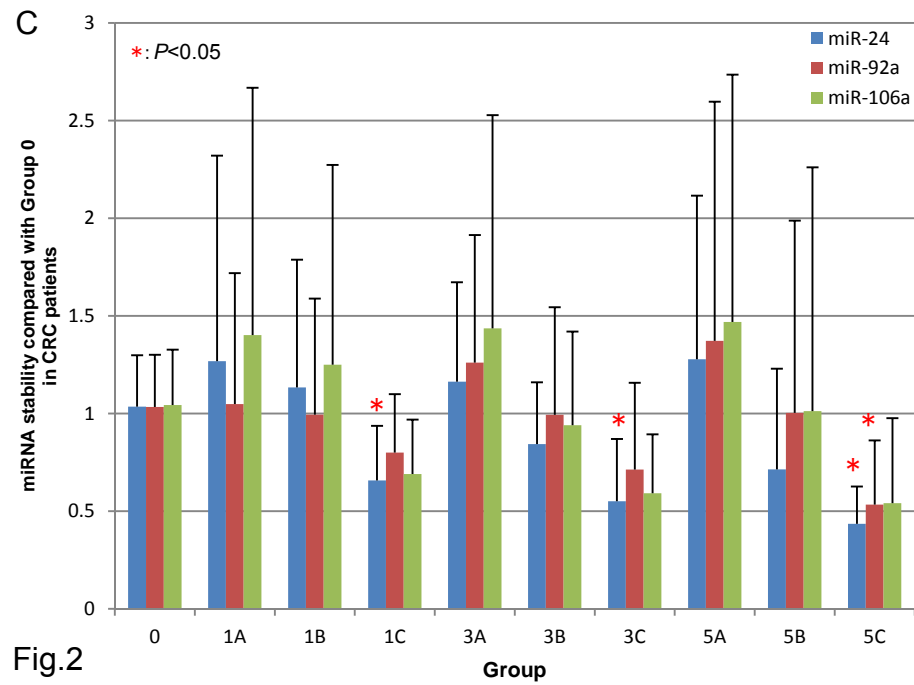
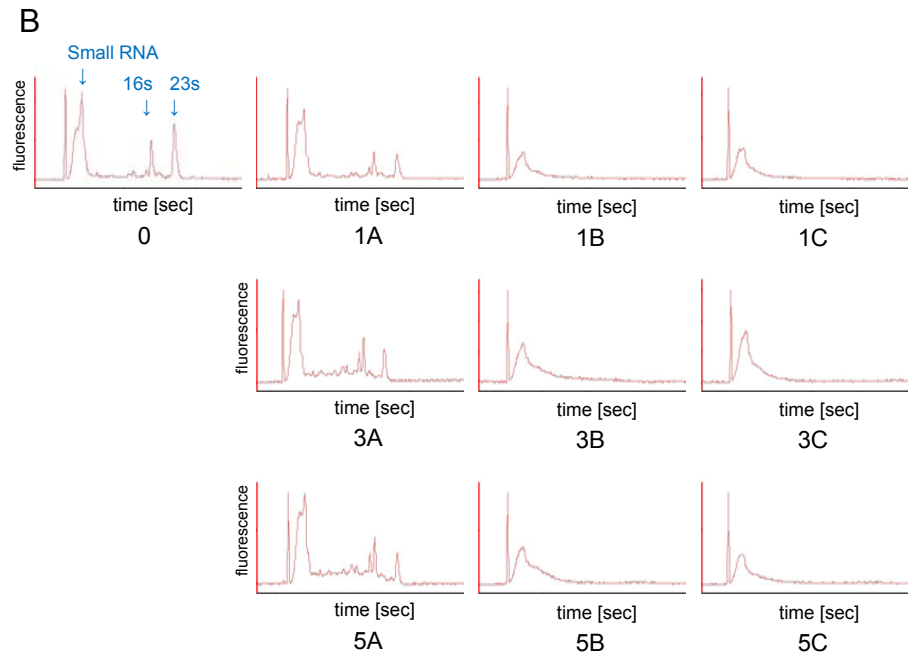
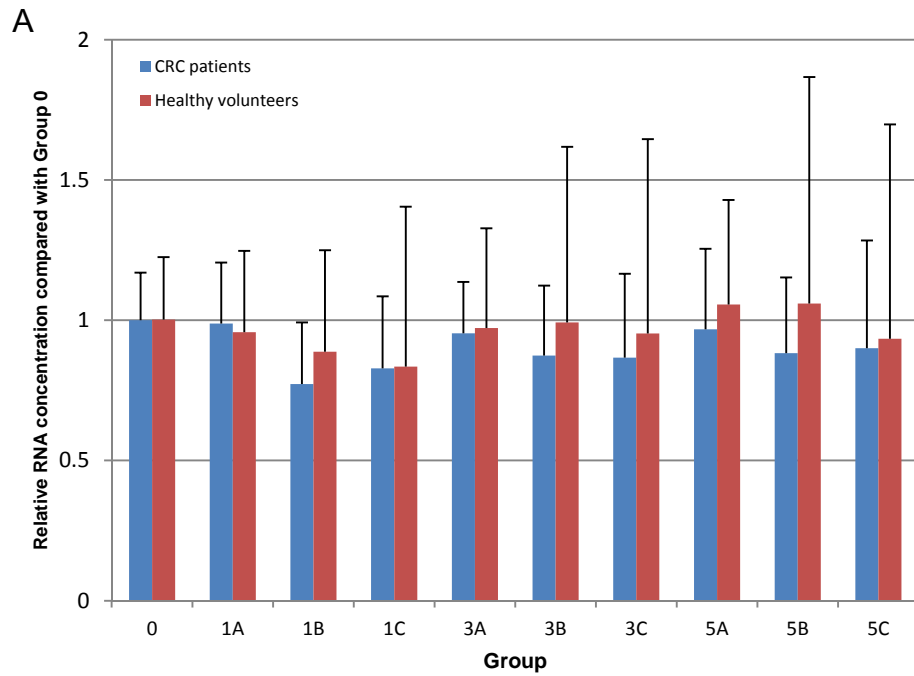


Fig.2

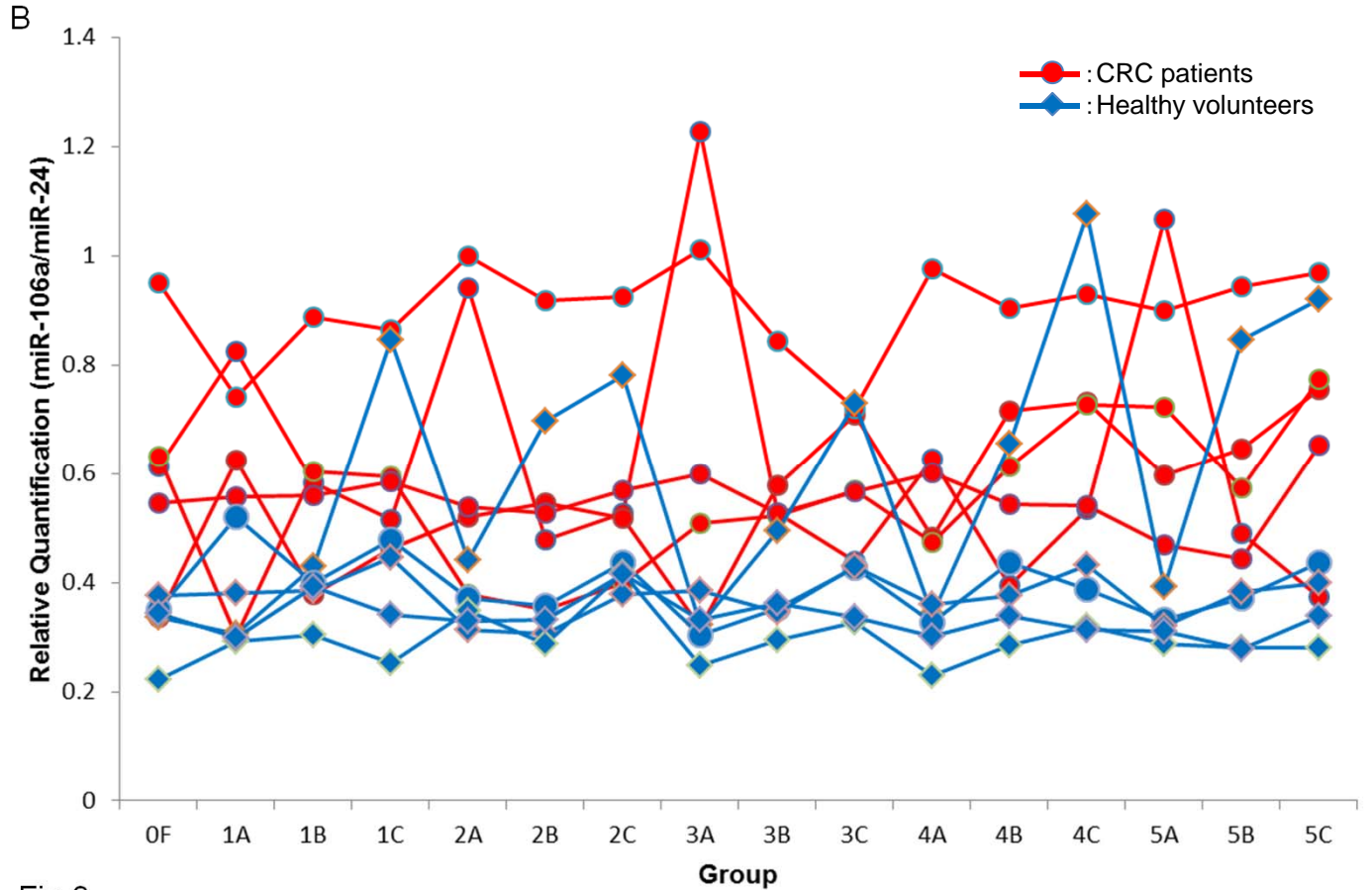
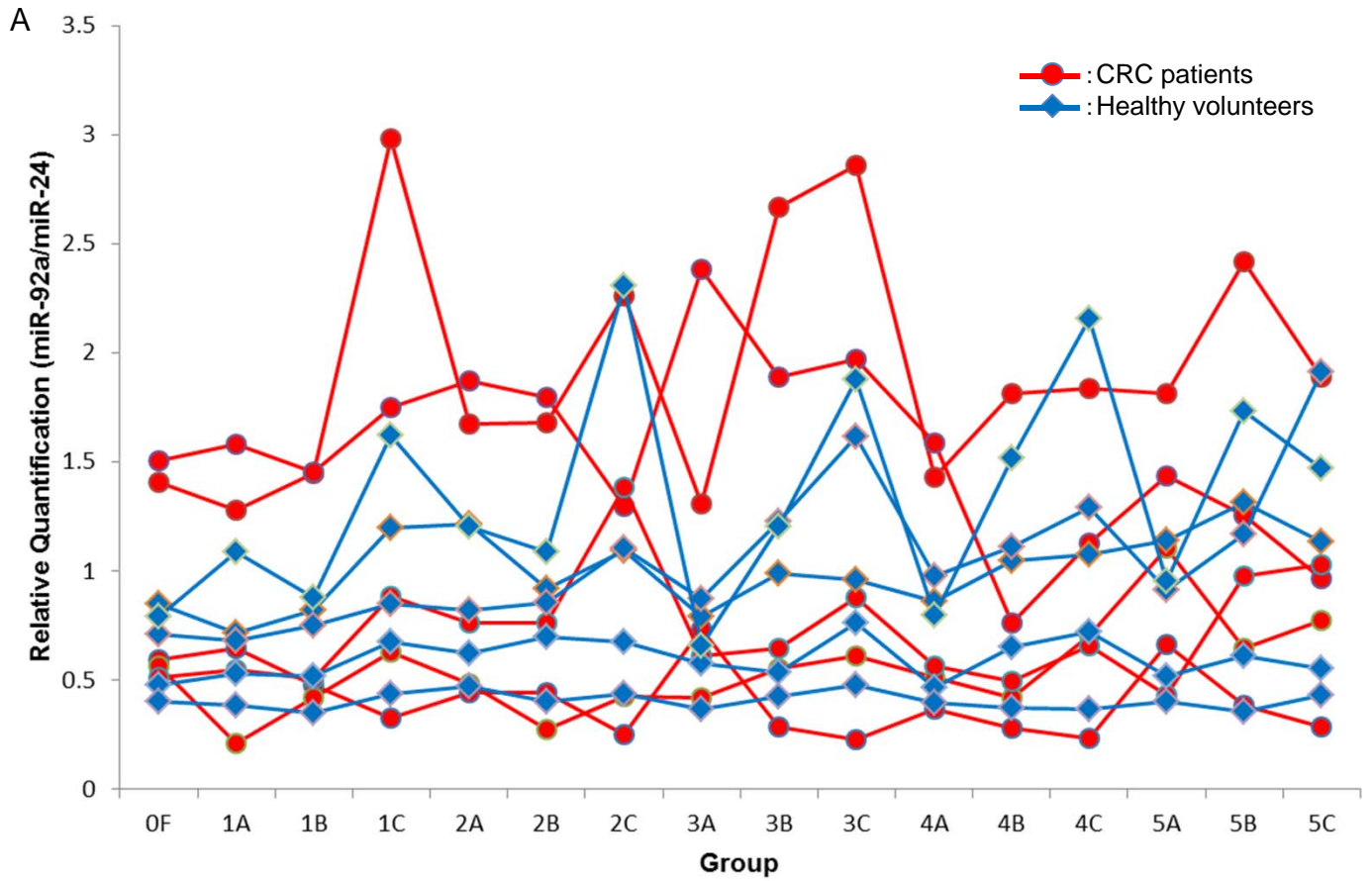


Fig.3

Figure Legends

Figure 1: FOBT scores determined using samples from CRC patients stored under various conditions. The FOBT positive and negative scores were 1 and 0, respectively. The differences were analyzed by Dunnett's test compared with Group 0. $P < 0.05$ denotes a statistically significant difference. *: $P < 0.05$.

Figure 2: RNA concentration and miRNA stability in samples stored under various conditions. A) Relative RNA concentration compared with Group 0. There were no differences between any of the groups. The differences were analyzed by Dunnett's test compared with Group 0. B) Quality of fecal RNA determined using the Bioanalyzer. Three peaks representing small RNAs, 16s rRNA, and 23s rRNA are observed. rRNAs were degraded rapidly when stored at RT and 37°C. C) miRNA stability compared with Group 0 in CRC patients. miRNA stored at 37°C was significantly more degraded compared with Group 0. The differences were analyzed by Dunnett's test compared with Group 0. $P < 0.05$ denotes a statistically significant difference. D) miRNA stability compared with Group 0 in healthy volunteers. miRNA stored at 37°C was significantly more degraded compared with Group 0. The differences were analyzed by Dunnett's

test compared with Group 0. $P < 0.05$ denotes a statistically significant difference.

Figure 3: Relative quantification of each miRNA. A) Relative quantification of miR-92a normalized to miR-24. The quantification of miR-92a in CRC patients was no significant difference compared with the quantification in healthy volunteers. B) Relative quantification of miR-106a normalized to miR-24. The quantification of miR-106a in CRC patients was significantly higher than the quantification in healthy volunteers. The differences were analyzed by Mann-Whitney U test. $P < 0.05$ denotes a statistically significant difference. Circle (●) and red line, CRC patients; rhombus (◆) and blue line, healthy volunteers. *: $P < 0.05$.

Table 1.
 Characteristics of patients and healthy volunteers

Characteristics	patients n=5	healthy volunteers n=5	P value
Age, y			
Median	54	57	0.17
Range	39-68	41-73	
Sex, no.(%)			0.038
M / F	5 / 0	2 / 3	
Tumor location, no. (%)			
Sigmoid colon	2 (40)		
Rectum	3 (60)		
Tumor size, mm			
Median	23		
Range	18-45		
Histology, no. (%)			
W/D	3 (60)		
M/D	2 (40)		
Tumor depth, no. (%)			
T1	3 (60)		
T2	1 (20)		
T3	1 (20)		
T4	0		
Dukes' stage, no. (%)			
A	2 (40)		
B	2 (40)		
C	1 (20)		
D	0		
FOBT, no.(%)			0.0016
+	5 (100)	0	
-	0	5 (100)	
RNA(μg)			0.089
Median	32.2	24.1	
Range	17.7-65.9	16.3-74.4	

W/D: well-differentiated adenocarcinoma, M/D: moderately differentiated adenocarcinoma, RNA: RNA extracted from FOBT residuum, The differences were analyzed by Mann-Whitney *U* test or χ^2 test. *P* < 0.05 denotes statistically significant difference.

Table 2
Storage condition of fecal samples

Storage durations after evacuation (day)						temperature
0	1	2	3	4	5	
Group 0	1A	2A	3A	4A	5A	4°C
	1B	2B	3B	4B	5B	RT
	1C	2C	3C	4C	5C	37°C

RT: room temperature

Table 3

Relative quantification of micro RNA (miRNA) normalized to miR-24 in Group 0

	CRC patients mean \pm SD	Healthy volunteers mean \pm SD	<i>P</i> value
miR-92a	0.92 \pm 0.48	0.65 \pm 0.23	0.13
miR-106a	0.59 \pm 0.18	0.33 \pm 0.09	0.0003

CRC: colorectal cancer, The differences were analyzed by Mann-Whitney *U* test or χ^2 test. *P* < 0.05 denotes statistically significant difference.