

Research Article

Fecal MicroRNAs as Novel Biomarkers for Colon Cancer Screening

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Abstract

Introduction: Colorectal cancer (CRC) is the second leading cause of cancer-related deaths, but currently available noninvasive screening programs have achieved only a modest decrease in mortality. MicroRNAs (miRNA) play an important role in a wide array of biological processes and are commonly dysregulated in neoplasia. We aimed to evaluate the feasibility of fecal miRNAs as biomarkers for colorectal neoplasia screening.

Materials and Methods: Total RNA was extracted from freshly collected stool samples from 8 healthy volunteers and 29 samples collected via fecal occult blood testing from subjects with normal colonoscopies, colon adenomas, and CRCs. miRNA expression analyses were done with TaqMan quantitative reverse transcription-PCR for a subset of miRNAs. Illumina miRNA microarray profiling was done to evaluate the differences in expression patterns between normal colonic mucosa tissues and stool samples from healthy subjects.

Results: We efficiently extracted miRNAs from stool specimens using our developed protocol. Data from independent experiments showed high reproducibility for miRNA extraction and expression. miRNA expression patterns were similar in stool specimens among healthy volunteers, and reproducible in stool samples that were collected serially in time from the same individuals. miRNA expression profiles from 29 patients showed higher expression of miR-21 and miR-106a in patients with adenomas and CRCs compared with individuals free of colorectal neoplasia.

Conclusion: Our data indicate that miRNAs could be extracted from stool easily and reproducibly. The stools of patients with colorectal neoplasms have unique and identifiable patterns of miRNA expression.

Impact: Fecal miRNAs may be an excellent candidate for the development of a noninvasive screening test for colorectal neoplasms. *Cancer Epidemiol Biomarkers Prev*; 19(7); 1766–74. ©2010 AACR.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer deaths in adults in the United States (1). Early detection of these neoplasms is critical because of a direct effect on prognosis. Therefore, new robust and reliable diagnostic approaches that could improve the existing screening strategies are urgently needed. Even though the need for such diagnostic tools has long been recognized, there has only been modest success in the development of novel and effective approaches to screening (2, 3).

Endoscopic procedures such as colonoscopy are very accurate and permit the removal of adenomas, which reduces cancer incidence. However, this procedure has several practical limitations, as it requires bowel preparation, sedation, is associated with a risk of serious medical complications, and is expensive. Clinical guidelines recommend screening colonoscopy beginning at age 50, but in >80% individuals, the procedure could be potentially spared because no important lesions are found (4, 5). Therefore, an optimal screening test might reduce the necessity for an invasive procedure, reduce

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org>).

A. Link, F. Balaguer, C.R. Boland, and A. Goel: study concept and design,

analyses and interpretation of data, drafting of manuscript, and obtained funding. A. Link and Y. Shen performed the experiments. J.J. Lozano, A. Link, and F. Balaguer performed statistical analyses. T. Nagasaka provided patient material.

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doi: 10.1158/1055-9965.EPI-10-0027

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the cost, have better screening compliance, and could more accurately select those individuals that require the colonoscopic removal of a neoplastic lesion. Although computed tomographic colonography fulfills some of these criteria, there is concern about the high radiation exposure that could itself be theoretically associated with cancer risk (6).

Most noninvasive molecular tests for CRC are based on the analysis of feces and/or blood. Guaiac-based fecal occult blood testing (FOBT) is the most commonly used test that detects blood in the stool. FOBT screening has been associated with a reduction in CRC-related mortality of 15% to 33% (7). However, this test has several limitations, including low specificity and sensitivity in the detection of CRCs (33-50%) and colon adenomas (11%; ref. 8). This limitation has been partially overcome through the development of fecal immunochemical occult blood tests, which detect human-specific globin (5). Both tests have shown potential for CRC detection in asymptomatic patients if done annually, and according to recent American Cancer Society guidelines, both assays are an acceptable option for CRC screening (5). Another promising approach for the identification of colorectal and other tumors is to assay stool or bodily fluids for molecular biomarkers that represent the spectrum of genetic and/or epigenetic alterations associated with cancer. Based on this paradigm, fecal DNA-based testing has been an area of active investigation since the early 1990s (2). There is constant sloughing and shedding of tumor cells into the stool from neoplastic tissues, which provides a substrate for the discovery of cancer-related genetic "signatures." Genetic markers for CRC have been based on the identification of alterations in a subset of genes including *APC*, *p53*, and *K-Ras* (2). Even though versions of these tests are being offered commercially, they are cumbersome to perform, and provide a modest diagnostic sensitivity of ~50% to 80% for invasive cancers, and 18% to 40% for advanced benign neoplasms (2, 8, 9). More recently, there has been growing interest in exploiting fecal-based testing for another DNA-based target, i.e., aberrant hypermethylation of CpG islands. In a cohort of patients with various gastrointestinal lesions, our group has recently shown that aberrant methylation of two genes significantly improved sensitivity and specificity for the detection of gastrointestinal neoplasia (10).

MicroRNAs (miRNA) are small noncoding transcripts that have recently been identified as a new class of cellular molecules with important diagnostic, prognostic, and therapeutic implications (11, 12). Cross-species comparisons show that miRNAs are evolutionarily conserved and play an important role in a wide range of physiologic and pathologic processes. Although the biology of miRNAs is still poorly understood, it is now known that each miRNA may control hundreds of mRNA targets and act as master regulators of gene expression. Moreover, miRNAs are involved in the pathogenesis of multiple types of cancers (12, 13). The pattern of miRNA expression

could be used to classify diverse types and subtypes of cancers (12). One of the most exciting biological features of miRNA compared with mRNA is that they are present in different tissues in a very stable form and, due to their small size, are remarkably well-protected from endogenous degradation (14-16). Although growing evidence suggests the potential of miRNA expression analysis in tumor tissues, serum, plasma, and urine as a promising approach for early tumor detection, limited data exists on the usefulness of fecal miRNAs for this purpose (17, 18).

In the present study, we show that miRNAs could be easily detected in stool specimens from healthy subjects and patients with colorectal disease. Pilot analyses of the stool specimens from patients with CRC and colonic adenomas suggest a potential role for fecal miRNAs as novel biomarkers in the early detection of colorectal neoplasia.

Materials and Methods

Stool samples from healthy subjects

We collected fresh stool samples from eight healthy individuals [four males and four females; mean age, 28.9 (21-41 years)]. The protocol was approved by the Institutional Review Board at Baylor University Medical Center and written informed consent was obtained from each volunteer. Stool samples were stored at -80°C after collection, and RNA isolation was done within 2 to 3 weeks.

Clinical samples

A total of 29 stool specimens collected in FOBT sample collection devices made by the Eiken Chemical Co. (Japan) were obtained from 10 individuals with normal colonoscopy, 9 patients with advanced and non-advanced colonic adenomas, and 10 patients with CRC. These samples were randomly selected from a larger collection of 303 fecal samples previously collected at the Okayama University Hospital, Okayama, Japan (10). The stool samples were kept either at 4°C or at -25°C for short-term or at -80°C for long-term storage. All patients provided written informed consent, and the study was approved by the institutional review board. Clinical and demographical data of the patients are presented in Table 1.

RNA isolation

Total RNA (including miRNAs) from fresh stool specimens and FOBT samples were extracted using Qiagen miRNAeasy Mini Kits (Qiagen) according to the instructions of the manufacturer with some modifications. Briefly, ~100 mg of stool was homogenized with RNase-free water and 150 μL of this homogenate was lysed in a proportion of 1:6 with QIAzol lysis reagent (Qiagen). Similarly, for the stool specimens collected in the FOBT kits, 150 μL of the diluted stools were processed for RNA extraction. After homogenization, RNA was precipitated with chloroform. The aqueous phase was mixed with 1.5 volumes of 100% ethanol. The concentration of

extracted RNA from stool samples was measured using RiboGreen RNA quantitation kits (Molecular Probes).

Direct miRNA analysis

To assess the feasibility of direct miRNA expression detection from fresh stool specimens without prior RNA extraction, we developed and optimized a new protocol, which we called direct miRNA analysis (DMA). Equal amounts of stool were diluted with either RNase-free water or normal saline (0.9%). Following centrifugation ($4,000 \times g$ at 4°C), supernatant was carefully collected and used for direct miRNA amplification.

MiRNA microarray expression profiling and data analysis

To explore the miRNA expression signature of fecal specimens and normal colonic mucosal tissues, we ana-

lyzed the miRNA expression profiles in five normal colonic mucosa tissues and one stool sample from a healthy individual. Total RNA from histologically normal colonic mucosa was extracted from formalin-fixed, paraffin-embedded tissues from patients undergoing colonic surgery for diverticulosis using the RecoverAll kit (Ambion, Inc.) following the instructions of the manufacturer. Total RNA from a stool sample from a healthy subject was done using Qiagen miRNAeasy Mini Kits (Qiagen) as described above. RNA was amplified and subsequently hybridized to the SAM-Bead microarray according to the instructions of the manufacturer (Illumina, Inc.). Microarray data processing and analysis were done using Illumina BeadStudio software. Data were processed and normalized using the Lumi Bioconductor software package (19). We used a conservative probe-filtering step, which excluded probes that did

Table 1. Clinicopathologic characteristics of patients with FOBT samples

ID	Diagnosis	Gender	Age (y)	TNM stage	Histology	Tumor location	Proximal/distal
N1	N	M	70	—	—	—	—
N2	N	F	79	—	—	—	—
N3	N	M	46	—	—	—	—
N4	N	M	76	—	—	—	—
N5	N	M	79	—	—	—	—
N6	N	M	82	—	—	—	—
N7	N	M	77	—	—	—	—
N8	N	F	79	—	—	—	—
N9	N	M	67	—	—	—	—
N10	N	F	72	—	—	—	—
A1	NAA	M	55	—	LGD	As/S	D
A2	NAA	F	76	—	LGD	As	P
A3	NAA	M	52	—	LGD	S	D
A4	NAA	F	74	—	LGD	S	D
A5	AA	F	41	—	LGD	D	D
A6	AA	M	85	—	LGD	R	D
A7	NAA	M	55	—	LGD	As	P
A8	NAA	M	75	—	LGD	T	P
A9	AA	M	69	—	LGD	As	P
C1	Cancer	F	75	4	IV	As	P
C2	Cancer	F	65	3	III	As	P
C3	Cancer	F	67	2	II	As	P
C4	Cancer	M	77	4	IV	S	D
C5	Cancer	M	75	4	IV	S	D
C6	Cancer	F	53	2	II	R	D
C7	Cancer	F	57	2	II	As	P
C8	Cancer	M	47	1	I	D	D
C9	Cancer	F	51	3	III	As	P
C10	Cancer	F	65	2	II	D	D

Abbreviations: M, male; F, female; N, normal; A, adenoma; AA, advanced adenoma; NAA, non-advanced adenoma; C, colorectal cancer; LGD, low-grade dysplasia; As, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum; TNM, tumor-node-metastasis.

not reach a detection value of $P < 0.05$. This analysis resulted in the reliable detection of 912 probes from 1,145 probes on the microarray chip. GeneSpring GX 7.3 software (Agilent Technologies) was used for data analysis and image generation.

MiRNA quantification by real-time reverse transcription-PCR

Quantification of miRNA was done using either TaqMan miRNA Assays (Applied Biosystems) or a SYBR Green method, with some modifications (16, 20). Briefly, ~20 ng of RNA was reverse-transcribed and real-time quantification was done using an Applied Biosystems 7300 Sequence detection system. All reactions were run in triplicate. Primer sequences for the reverse transcription-PCR (RT-PCR) assays are listed in the Supplementary Table S1. Selection of miRNAs was done based on the following criteria: (a) previously published with potential implications in CRC development (miR-21, -17, -25, -29b, -106a, and -143); and (b) differential expression between CRC tissues and normal colonic mucosa of miRNA profiling in these tissues (miR-654-3p, -622, -1238, and -938).⁵ Differences between the groups are presented as Δ Ct, indicating the difference between the Ct value of the miRNA of interest and the Ct value of the normalizer miRNA. Selection of the targets for normalization was carried out based on previous publications and on the coherence of endogenous Ct signals (21, 22).

Statistical analysis

Data analyses were done with GraphPad Prism 4.0 software. Differences between the two groups were analyzed using Student's *t* tests, whereas differences among more than two groups were analyzed using ANOVA or Kruskal-Wallis with appropriate post hoc tests. Correlation analyses were done using Spearman's or Pearson's test, where appropriate, and logarithmic regression was used to calculate the R^2 and to create the equation of the slope. Two-sided $P < 0.05$ were regarded significant.

Results

Fecal RNA: extraction and reproducibility

Given the fact that miRNAs have previously been shown to be present in other body fluids, we sought to evaluate the presence of miRNA in stool. Following optimization and modification of existing commercial kits recommended for total RNA extraction, we were able to isolate an adequate amount of total fecal RNA from eight healthy individuals. The RNA concentrations

in stool ranged from 622 to 2,475 ng/ μ L (Fig. 1A). To evaluate the integrity of small RNAs, we did quantitative RT-PCR analysis which robustly amplified RNU6b, a small nuclear ubiquitous RNA ~50 bp, which is commonly used as an endogenous control in miRNA studies (Fig. 1A). To assess the reproducibility of the miRNA extraction methodology, we repeated this procedure in a subset of samples. As shown in Fig. 1B and C, our results were highly reproducible, both in terms of total RNA concentrations ($R^2 = 0.998$, $P < 0.0001$) and RNU6b expression levels ($R^2 = 0.968$, $P = 0.0025$).

DMA

miRNAs have been shown to be present in blood both as intracellular entities and as extracellular content of exosomes (15, 16). To evaluate the feasibility of detecting extracellular miRNAs in stool, we developed a new method called DMA, which obviates the need for RNA extraction prior to expression analysis. We then compared the expression levels of different miRNAs in healthy subjects using both RNA extraction with Qiagen kits and DMA (Fig. 1D). Although the miRNA concentrations were lower using DMA, we found a significant correlation between Ct values using both methods.

Fecal miRNA: reliable normalization to housekeeping miRNAs

Several reports (15, 16) have clearly shown that, in contrast with mRNAs, miRNAs are remarkably stable at high temperatures and are minimally affected by RNase-induced degradation. RNU6b, as previously mentioned, is commonly used as an endogenous control in miRNAs studies; however, unlike mRNAs, its stability and significance as an endogenous normalization control has been questioned (15, 20). In this study, we found the total RNA concentrations in stool did not correlate with RNU6b expression (data not shown). However, both miR-16 and miR-26b expression patterns showed the highest coherence among different samples, and a significant correlation ($R^2 = 0.875$, $P < 0.0001$) between each other (Fig. 1E). Because RNU6b did not show correlation with any of the previously described normalizers such as miR-16 and miR-26b (Fig. 1F), we selected these two miRNAs as normalizers for subsequent analyses.

Comparison of miRNA profiles between stool and normal colonic mucosa

Having shown that fecal miRNAs are detectable in stool samples from healthy individuals, we next sought to evaluate the feasibility of performing miRNA profiling in stool specimens and compared it with normal colonic mucosa tissues. As shown in Fig. 2A, the miRNA expression profiles from stool samples and normal colonic mucosa showed significant similarities in the expression profiles of 284 miRNAs, including miR-16 and miR-26b.

⁵ Balaguer F, Moreira L, Lozano JJ, Link A, Ramirez G, Cuatrecasas M, Arnold M, Stoffel E, Syngal S, Jover R, Llor X, Castells A, Boland C.R., Gironella M, Goel A. MiRNA expression profiles distinguish various molecular subtypes of colorectal cancer, manuscripts in preparation.

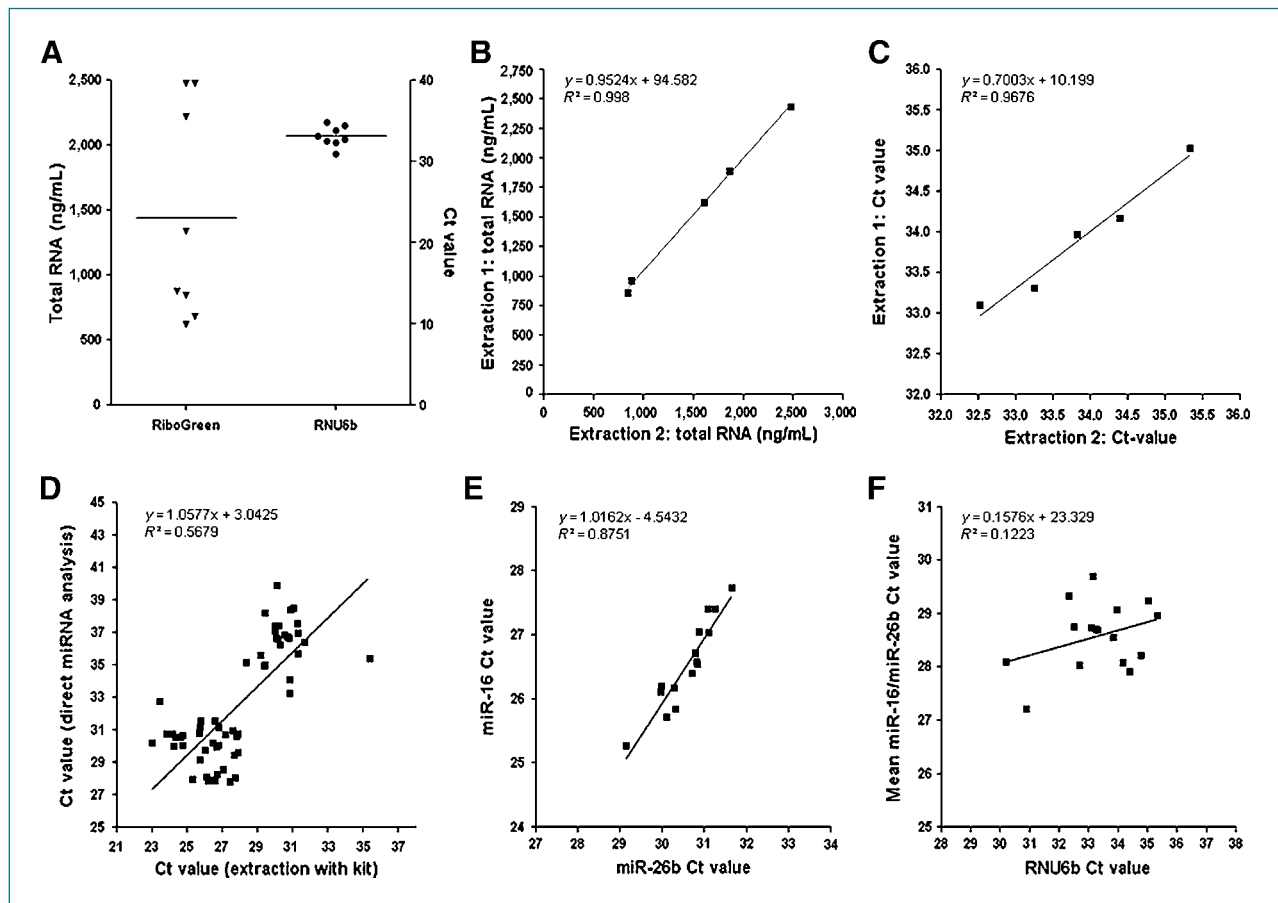


Figure 1. Total RNA and miRNA detection in the stools of healthy subjects. A, the graph shows the variation and amount of total RNA measured with RiboGreen and expression levels of RNU6b measured by TaqMan PCR. Both total RNA concentrations (B) and RNU6b expression levels (C) showed a significant correlation between independent extractions. D, comparison of Ct values detected by quantitative RT-PCR from various miRNAs showed a significant correlation between the DMA method and miRNA from Qiagen kit-extracted samples. E, significant correlation in miR-16 and miR-26b expression levels was observed among different healthy individuals. F, based on previous literature, three housekeeping RNA/miRNAs were evaluated for possible use as an internal control. RNU6b showed a high degree of variation and showed no significant correlation with miR-16 and miR-26b.

Similar miRNA expression patterns among healthy individuals

We analyzed the expression patterns of a subset of miRNAs among eight healthy individuals. To confirm the reproducibility of the analysis, we repeated the experiments in two independent RNA extractions, and showed a significant correlation ($R^2 = 0.9923$, $P < 0.0001$; Fig. 2B). Figure 2C shows the raw Ct values of the miRNAs analyzed in this study. We found that miR-21 was the most highly expressed miRNA in the stool, and the expression differences among miRNAs varied by more than 10,000-fold ($\Delta Ct \sim 14$ -15) between certain miRNAs (e.g., miR-21 versus miR-938). Analysis of the results after normalization to miR-16 and miR26b revealed that the pattern of miRNA expression was very similar among healthy subjects (Fig. 2D). Following normalization, we observed an interindividual variation in miRNA expression among healthy individuals with a SD range of $\Delta Ct \pm 0.25$ (for miR-17 and miR-21) to $\Delta Ct \pm 2.15$ (for miR-29b or miR-938).

Similarities in miRNA expression patterns at different time points

To further explore the biological relevance of a stool-based miRNA expression strategy, we investigated differences in stool miRNA expression patterns in samples collected at different time points (>2 weeks apart) from the same individuals. This analysis revealed a significant correlation ($R^2 = 0.9523$, $P < 0.0001$) in miRNA expression levels (Fig. 2E) between two different time points, suggesting that stool miRNA expression patterns remain constant over time in healthy subjects. The SD of normalized miRNA expression values among healthy individuals varied from $\Delta Ct \pm 0.2$ (for miR-17 and -21) to $\Delta Ct \pm 0.6$ (for miR-938 and miR-1238).

miRNAs could be effectively extracted and analyzed from FOBT kits

FOBT is currently the most frequently used noninvasive test for CRC screening. The feasibility of miRNA detection from FOBT collection devices would facilitate

additional possibilities for miRNA-based biomarker identification and validation as a screening tool. Following methodologic optimization, we were able to extract total RNA, including miRNA, from FOBT kits from 29 individuals. As expected, the RNA concentration was lower than in fresh stool samples (RNA concentrations varied from 9 to 87 ng/ μ L; Fig. 3A), possibly due to increased dilution of fecal specimens in the FOBT kits. However, we were able to effectively amplify all miRNAs of interest by TaqMan RT-PCR.

Differential expression of fecal miRNA in patients with colorectal neoplasia

Finally, we evaluated the potential use of fecal miRNA expression analysis to discriminate between healthy subjects and patients with colorectal neoplasia (Fig. 3A and B). Interestingly, among six tested miRNAs, we found higher expression of both miR-21 and miR-106a in stool

samples from patients with colorectal neoplasia (adenomas and CRCs) compared with subjects with normal colonoscopies ($P < 0.05$), whereas no differences were found for miR-17, miR-143, miR-622, and miR-654-3p. Separate analyses of colorectal adenoma and CRC patients showed that the mean Δ Ct \pm SD for miR-21 was 7.6 ± 1.6 and 6.9 ± 0.5 for colonic adenomas and CRC patients versus 6.1 ± 1.6 for subjects with normal colonoscopy (ANOVA, $P = 0.02$; Bonferroni's post-test normal versus adenoma, $P < 0.05$). Similarly, for miR-106a, the values were 0.5 ± 1.6 and -0.2 ± 0.5 versus 0.6 ± 1.6 (ANOVA, $P = 0.05$; post-test normal versus adenoma, $P < 0.05$) for adenoma and CRC patients versus normals (Fig. 3C and D). Surprisingly, expression of both miRNAs was higher in stool samples from patients with adenomas compared with CRCs. To further evaluate these associations, we did an analysis of miR-21 and miR-106a expression in subgroups based on the presence

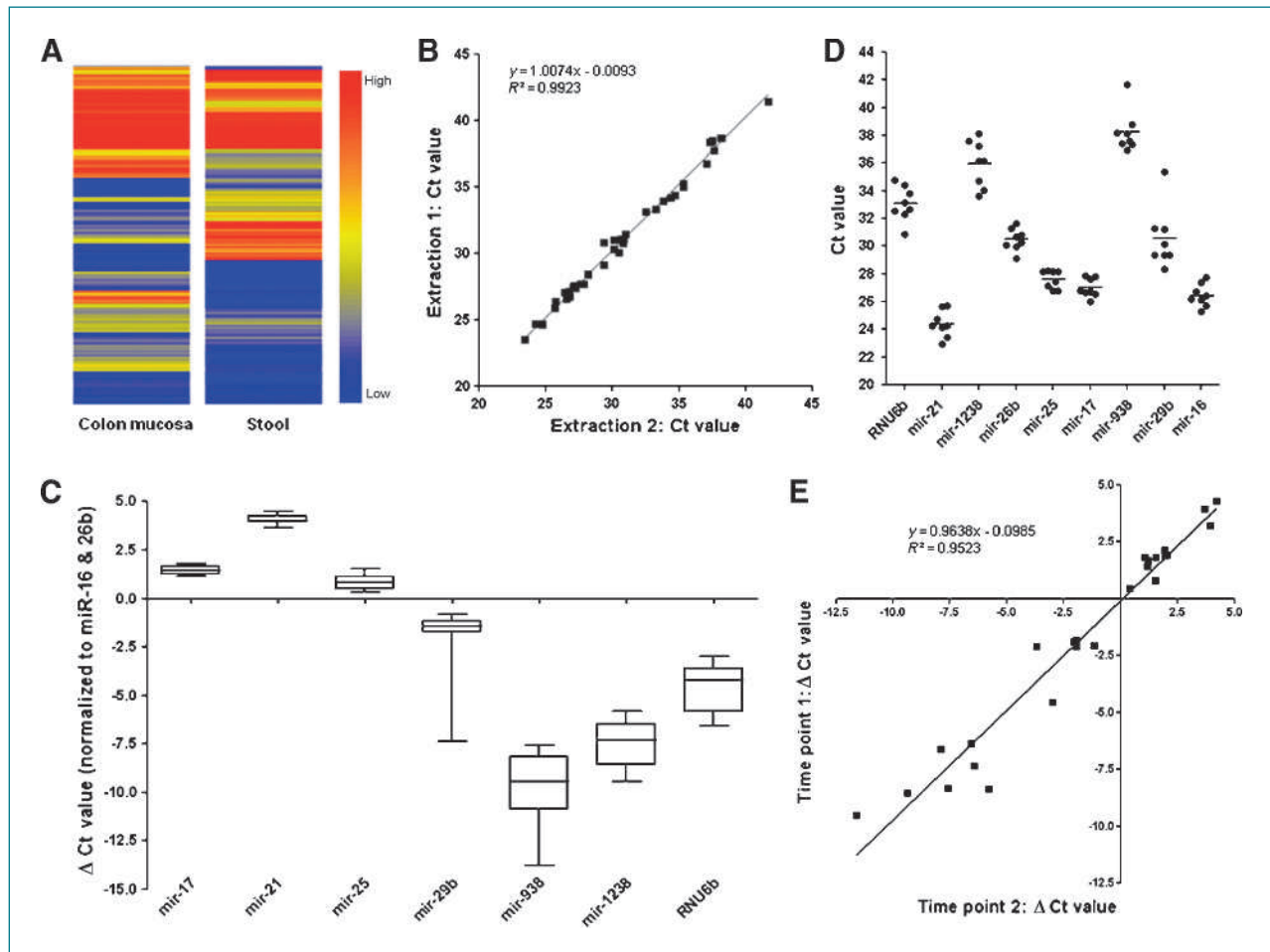


Figure 2. miRNA expression patterns in stools from healthy subjects. A, miRNA array was done on stools and normal colonic mucosa tissues. The heat map illustrates the similarities and differences in mean miRNA expression profiles. B, miRNA expression levels in stool samples showed excellent reproducibility among independent extractions. Raw (C) and normalized (D) interindividual variations of miRNA expression measured in the stool samples of eight healthy subjects (normalization was done using the standard $\Delta\Delta$ Ct method). E, stool samples collected at different time points showed significant correlation between miRNA expression patterns.

of advanced or non-advanced adenomas and tumor-node-metastasis stage. Surprisingly, the level of expression of both miRNAs decreased with higher tumor stages (Fig. 3E and F). It is important to mention that non-normalized data for both miR-21 and miR-106a showed similar results (data not shown).

Discussion

In this study, we evaluated the feasibility of fecal miRNAs as potential biomarkers for detecting colorectal neoplasia. An ideal biomarker must fulfill several criteria including the potential to be measured quantitatively, a high degree of specificity that indicates aberration in a specific biological and/or pathologic process, reliability, measurability, sensitivity, and predictability. Our data, using a small subset of stool samples from healthy individuals, represents the proof-of-principle that miRNAs

are abundantly present in stool and could be easily and reproducibly detected in stool specimens. Furthermore, the observation that intraindividual miRNA expression patterns were relatively constant highlights the potential value of miRNA as a screening tool. After determining the feasibility of detecting miRNA expression in fecal materials, we next questioned whether fecal miRNA profiles from healthy subjects were similar to those present in normal colonic epithelium. Not surprisingly, we found differences in miRNA expression patterns between stool and colonic mucosa specimens. Although these results need adequate validation in a larger set of samples, they are consistent with previous reports in which similar observations were made for miRNA profiling in blood and cancer tissues (16, 23). The fact that we could easily detect miRNAs in stool using our newly developed DMA methodology suggests that miRNA might be in the stool because of cell exfoliation and by the accumulation of

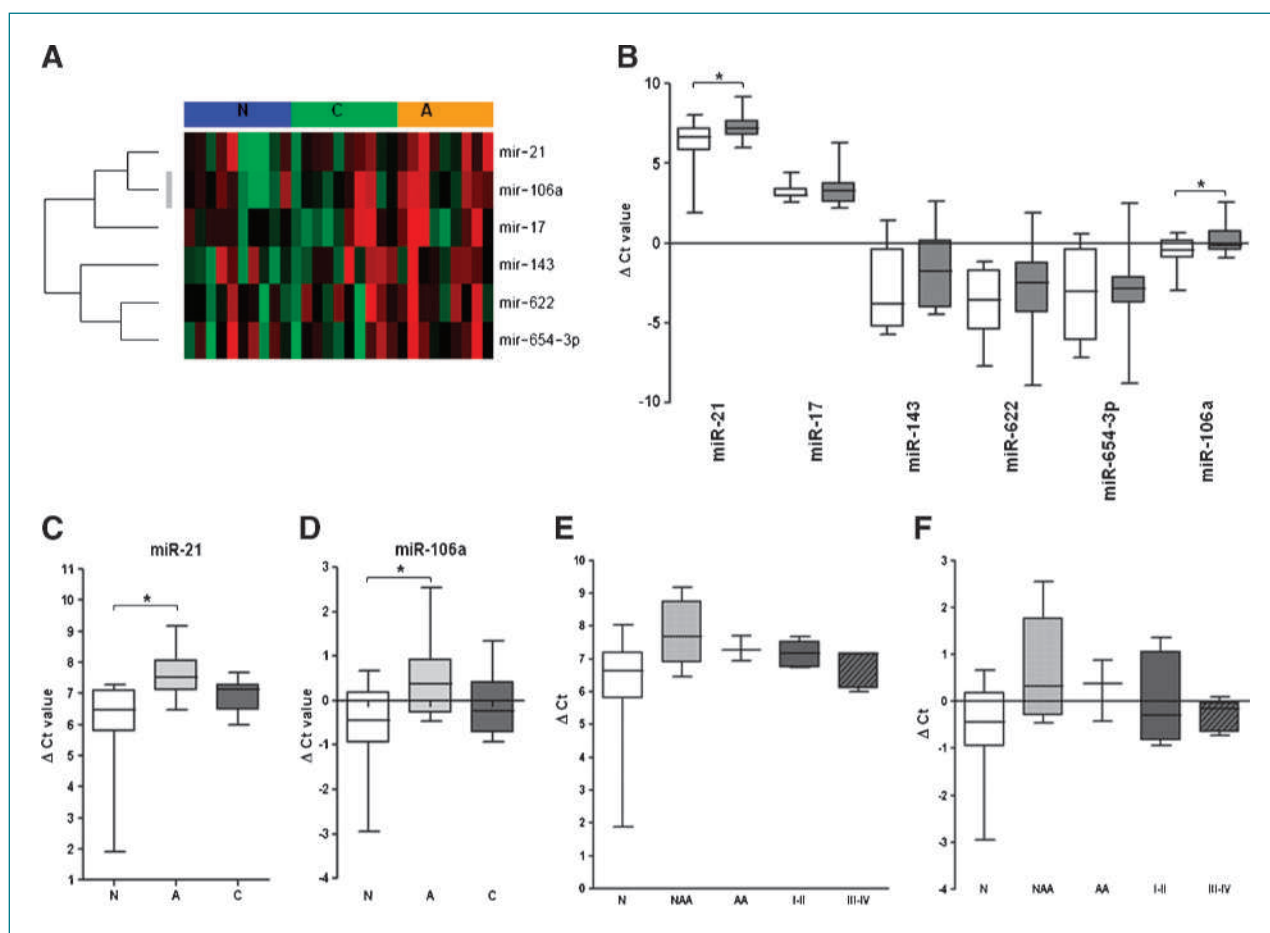


Figure 3. miRNA expression patterns in patients with adenoma and CRC. A, miRNA expression between patients with adenomatous polyps and CRC compared with subjects with no endoscopic abnormalities. Plot colors indicate low (green) and high (red) miRNA expression levels. B, quantitative RT-PCR analysis of miRNA expression between subjects with normal colons (white box plots) and patients with colorectal neoplasia (gray box plots) revealed significantly increased expression of miR-21 and miR-106a in the feces of patients with colon neoplasms. C and D, expression levels of miR-21 and miR-106a were compared among studied samples from subjects with normal endoscopy, adenomatous polyps, and CRC. E and F, subgroup analysis for miR-21 (E) and miR-106a (F) expression (*, $P < 0.05$). Abbreviations: N, subjects with normal endoscopy; A, adenomatous polyps; C, colorectal cancer; NAA, non-advanced adenoma; AA, advanced adenoma.

exosomes from cells of the gastrointestinal tract, in a similar manner as proposed for the contribution of miRNA signals by exosomes in blood (24-26).

To evaluate the potential of fecal miRNAs as biomarkers for detecting colorectal neoplasia, we did a pilot study on a small number of clinical samples. Although a blood-based test might be more practical, considering the increased number of exfoliated colonocytes shed in the colon from patients with CRC, it is highly likely that the earliest detectable neoplastic changes in the expression pattern of specific miRNAs might be in the feces rather than in the blood (10, 27). Due to the limited number of samples available to us for these experiments, we did not perform miRNA profiling on these specimens but we did the miRNA expression analysis on a subset of selected targets. The selection of miRNAs was based on the previously published role of these specific miRNAs or our own unpublished data obtained following miRNA expression profiling in CRCs and normal colonic mucosal tissues. Early premalignant adenomas, as well as early stage cancers, are ideal targets for a CRC prevention strategy. Prior studies on miRNA-based noninvasive biomarkers have mainly focused on CRC patients only, and to the best of our knowledge, no data exists on miRNA-based biomarkers for the identification of patients with colorectal adenomas. In this pilot study, we have analyzed patients from both groups—colorectal adenomas and CRC. Our observation of the higher expression of miR-21 and miR-106a in stool samples from patients with colonic neoplasia (adenomas and/or CRC) compared with subjects with normal colonoscopy is very encouraging. Ahmed et al. recently did a study in which the expression of several miRNAs was analyzed in stool samples from patients with CRC, patients with inflammatory bowel disease, and healthy subjects (18). In this study, and consistent with our results, both miR-21 and miR-106a were increased in patients with CRC compared with healthy subjects (18). Our results also support another previous study by

Schetter and colleagues in which they not only observed an increased expression of miR-21 and miR-106a in CRC and adenomas, but also the increase in the expression of these two miRNAs was also associated with poor survival and poor therapeutic outcome (28). Interestingly, the subgroup analyses of both miR-21 and miR-106a in our study revealed higher expressions of these miRNAs in patients with adenomas, supporting the rationale for developing these two miRNAs as diagnostic biomarkers for colorectal neoplasia.

In summary, we show that miRNAs could be easily, effectively, and reproducibly extracted from freshly collected stools, as well as from FOBT kits. Differential expression of miRNA in the stools of patients with colorectal neoplasia suggests that fecal miRNAs may serve as potential biomarkers. This concept requires further validation in large prospective studies. Fecal miRNAs might provide a novel, promising, and noninvasive approach for the diagnosis of early colorectal neoplasia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank participating volunteers for their effort and time. The authors would also like to thank Dr. Miriam Cuatrecasas for providing normal colonic tissue specimens.

Grant Support

R01 CA72851 and CA129286 from the National Cancer Institute, NIH to AG and CRB, funds from the Baylor Research Institute and a pilot grant from Baylor HealthCare Foundation to AG.

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Received 01/11/2010; revised 03/31/2010; accepted 04/19/2010; published OnlineFirst 06/15/2010.

References

- Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2009. *CA Cancer J Clin* 2009;59:225-49.
- Imperiale TF, Ransohoff DF, Itzkowitz SH, Turnbull BA, Ross ME. Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. *N Engl J Med* 2004;351:2704-14.
- Jankowski JA, Odze RD. Biomarkers in gastroenterology: between hope and hype comes histopathology. *Am J Gastroenterol* 2009;104:1093-6.
- Levine JS, Ahnen DJ. Clinical practice. Adenomatous polyps of the colon. *N Engl J Med* 2006;355:2551-7.
- Levin B, Lieberman DA, McFarland B, et al. Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *Gastroenterology* 2008;134:1570-95.
- Graser A, Stieber P, Nagel D, et al. Comparison of CT colonography, colonoscopy, sigmoidoscopy and faecal occult blood tests for the detection of advanced adenoma in an average risk population. *Gut* 2009;58:241-8.
- Mandel JS, Bond JH, Church TR, et al. Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. *N Engl J Med* 1993;328:1365-71.
- Lieberman DA. Clinical practice. Screening for colorectal cancer. *N Engl J Med* 2009;361:1179-87.
- Zou H, Taylor WR, Harrington JJ, et al. High detection rates of colorectal neoplasia by stool DNA testing with a novel digital melt curve assay. *Gastroenterology* 2009;136:459-70.
- Nagasaka T, Tanaka N, Cullings HM, et al. Analysis of fecal DNA methylation to detect gastrointestinal neoplasia. *J Natl Cancer Inst* 2009;101:1244-58.
- Ambros V. The functions of animal microRNAs. *Nature* 2004;431:350-5.
- Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834-8.
- Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857-66.
- Hui AB, Shi W, Boutros PC, et al. Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. *Lab Invest* 2009;89:597-606.
- Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as

- stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008;105:10513–8.
16. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 2008;18:997–1006.
 17. Cortez MA, Calin GA. MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. *Expert Opin Biol Ther* 2009;9:703–11.
 18. 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.
 19. Du P, Kibbe WA, Lin SM. lumi: a pipeline for processing Illumina microarray. *Bioinformatics* 2008;24:1547–8.
 20. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 2005;33:e179.
 21. Vandesompele J, De PK, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3, RESEARCH0034.
 22. Mestdagh P, Van VP, De WA, et al. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol* 2009;10:R64.
 23. Ng EK, Chong WW, Jin H, et al. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut* 2009;58:1375–81.
 24. Hunter MP, Ismail N, Zhang X, et al. Detection of microRNA expression in human peripheral blood microvesicles. *PLoS One* 2008;3:e3694.
 25. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* 2005;65:6029–33.
 26. Rabinowits G, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. *Clin Lung Cancer* 2009;10:42–6.
 27. Deschner EE. Early proliferative changes in gastrointestinal neoplasia. *Am J Gastroenterol* 1982;77:207–11.
 28. Schetter AJ, Leung SY, Sohn JJ, et al. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 2008;299:425–36.

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Cancer Epidemiol Biomarkers Prev 2010;19:1766-1774. Published OnlineFirst June 15, 2010.

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