Diagnostic MicroRNA Markers to Screen for Sporadic Human Colon Cancer in Stool: I. Proof of Principle

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Abstract. *To present proof-of-principle application for employing micro(mi)RNAs as diagnostic markers for colon cancer, we carried out global microarray expression studies on stool samples obtained from fifteen individuals (three controls, and three each with TNM stage 0-1, stage 2, stage 3, and stage 4 colon cancer), using Affymetrix GeneChip miRNA 3.0 Array, to select for a panel of miRNA genes for subsequent focused semi-quantitative polymerase chain reaction (PCR) analysis studies. Microarray results showed 202 preferentially expressed miRNA genes that were either increased (141 miRNAs), or reduced (61 miRNAs) in expression. We then conducted a stem-loop reverse transcriptase (RT)-TaqMan® minor groove binding (MGB) probes, followed by a modified qPCR expression study on 20 selected miRNAs. Twelve of the miRNAs exhibited increased and 8 decreased expression in stool from 60 individuals (20 controls, 20 with tumor-lymph node-metastatic (TNM) stage 0-1, 10 with stage 2, five with stage 3, and 5 with stage 4 colon cancer) to quantitatively monitor miRNA changes at various TNM stages of colon cancer progression. We also used laser-capture microdissection (LCM) of colon mucosal epithelial tissue samples (three control samples, and three samples from each of the four stages of colon cancer, for a total of 15 samples) to find concordance or lack thereof with stool findings. The reference housekeeping pseudogene-free ribosomal gene (18S rRNA), which shows little variation in expression, was employed as a normalization standard for relative PCR quantification. Results of the PCR analyses confirmed that twelve miRNAs (miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106a, miR-134, miR-183, miR-196a, miR-199a-3p and miR214) had an increased expression in the stool of patients with colon cancer, and that later TNM carcinoma stages exhibited a more pronounced expression than did adenomas. On the other hand, eight miRNAs (miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR-146a, miR-222 and miR-938) had decreased expression in the stool of patients with colon cancer, which was also more pronounced from early to later TNM stages. Results from colon mucosal tissues were similar to those from stool samples, although with more apparent changes in expression. Cytological studies on purified stool colonocytes that employed Giemsa staining showed 80% sensitivity for detecting tumor cells in stool smears. The performance characteristics of the test confirmed that stool is a medium well-suited for colon cancer screening, and that the quantitative changes in the expression of few mature miRNA molecules in stool associated with colon cancer progression provided for more sensitive and specific non-invasive diagnostic markers than tests currently available on the market. Thus, a larger prospective and properly randomized validation study of control individuals and patients exhibiting various stages of colon cancer progression (TNM stages 0-IV) is now needed in order to standardize test conditions, and provide a means for*

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determining the true sensitivity and specificity of a miRNA screening approach in stool for the non-invasive detection of colon cancer, particularly at an early stage (0-I). Eventually, we will develop a chip to enhance molecular screening for colon cancer, as has been accomplished for the detection of genetically-modified organisms (GMOs) in foods.

Mortality and morbidity from colon cancer represent a major health problem involving a malignant disease that is theoretically preventable through screening. Early detection would be greatly enhanced if accurate, practical and cost effective diagnostic biomarkers for this malignancy were available. Current screening methods (*e.g.* fecal occult blood test, FOBT) lack sensitivity (1-4), are costly, with side effects and have low compliance, or may result in mortality (*e.g.* colonoscopy) (5-7). An approach using miRNAs, which are relatively non-degradable when extracted from stool by commercially available kits and manipulated thereafter, would be preferable to a transcriptomic messenger (m)RNA-, mutation DNA-, epigenetic- or a proteomic-based test (8-17). If performance criteria are met, a non-invasive miRNA test in stool based on high throughput automated technologies and quantitative expression measurements commonly used in the diagnostic clinical laboratory should be advanced to the clinical setting and will make a significant impact on colon cancer prevention (1, 8).

A lengthy period of ~20 years is required for colon cancer to develop; therefore, an effective adenoma screening test needs to be performed less frequently than a test for early cancer. However, because only small minorities of adenomas are destined to progress to malignancy, their detection would involve gross over-treatment of patients, which would be costly and harmful to them. An optimal colon cancer screening test would be one that accurately detects advanced adenomas with a high chance of malignant progression (1, 2). Clinical management of adenomas entails removing them at the time of detection by colonoscopy. However, many eligible patients do not wish to undertake such an expensive and invasive test because of the need for bowel preparation, dietary restrictions, abdominal pain, potential perforation of the colon and even death (5-7). Given the desirability of using a non-invasive test acceptable to the target population as an initial screen, investigators have resorted to developing molecular approaches (9-17). Important considerations for developing sound tests include: nature of the specimen (invasive *versus* non-invasive), type of specimen (stool, blood, or any other body excretory/fluid), stability of specimen when handled outside the body, number of collected specimens (one sample, or multiple samples over consecutive dates), and how the specimens are stored and delivered (room temperature or frozen, posted or collected, *etc.*). Processing of samples must also be accepted by the laboratory staff. Any test that can be automated and eventually placed on a chip would be easier, more acceptable to those performing the assay, and cheaper to apply on a large scale (1).

In developing countries where colon cancer is on the rise due to adoption of a Western-type diet rich in energy (fats and carbohydrates) and low in essential nutrients (vitamins and minerals) (18), the problem is more severe, as the cost of colonoscopy often exceeds a person's yearly salary, and there are not enough trained gastroenterologists (GIs) or adequate centers to perform these tests. Our data have shown that quantitative changes in the expression of a few miRNA genes in stool that are associated with colon cancer permit for development of more sensitive and specific molecular markers than those currently available for a type of cancer that is deadly if not diagnosed before metastasis. A miRNA approach in stool could meet the criteria for test acceptability as it is non-invasive, requires at most 1 g of stool, results for stool are comparable to those for colon tissue, sampling on consecutive dates is not required, samples can be sent by mail in cold packs, the method is able to differentiate between normal tissue and colon adenomas/carcinomas, has high sensitivity and specificity of detecting advanced polyps, and can be automated (1, 8, 19), which makes it relatively inexpensive and more suited for early detection when compared to a test such as that for mutated DNA markers.

The market availability of powerful high-throughput approaches for global miRNA characterization, such as microarrays and simpler, universally applicable quantification assays for miRNA expression such as qPCR, suggests that the validation pipeline that often encounters bottlenecks (1, 19) would be more efficient for a miRNA assay. Eventually, these validated tests can be placed on chips for a more convenient testing, as has been carried out in the detection of genetically-modified organisms (GMOs) in food (20). There is, thus, a pressing need for validating sensitive and stable molecular markers, such as miRNAs, to improve the non-invasive detection of colon cancer, particularly at early TNM stages (0-I).

Materials and Methods

Acquisition of control and experimental stool and tissue samples. We used a nested case control design for sample's collection, which involved prospective collection of specimens before any outcome ascertainment from the study was known. Stool were obtained from several samples, of which 20 healthy controls and 40 patients with various stages of colon adenocarcinoma (TNM stages 0 to 4) were selected according to an approved Institutional Review Board (IRB) protocol (# 6071) by Office for Human Research Protections, U.S. Department of Health and Human Services, Rockville, MD, USA. All laboratory work was carried out and standardized under blind conditions and followed GEM Tox Labs guidelines for the handling of biohazardous material, established by its Biological Safety & Hazardous Substance protocol.

Control stool (20 g) samples were collected from consenting healthy individuals who did not have any history of polyps or inflammatory bowel diseases (IBDs), such as colitis or diverticulitis by a kit given to each participant that contained a plastic hat (Moore Medical, New Britain, CT, USA) which can be placed in the toilet,

then using a clean plastic spoon to collect samples from mucinous layers, which are rich in colonocytes (21), and from non-mucinous parts of stool in order to have a representation of the entire colon (both right- and left-sided colon) (22, 23). The participant then places 1 g of collected stool sample in a smaller clean urine container with 5 ml of the preservative RNA*Later*® (Applied Biosystems/Ambion, Austin, TX, USA) that prevents fragmentation of the fragile mRNA. The container is collected by laboratory personnel, followed by storage at –70˚C in the laboratory freezer until further processing of the preserved stool sample.

Similarly, a 20 g sample of feces was collected from patients with various stages of colon cancer visiting our collaborating GI-Surgery Clinics the night preceding surgery or earlier, and before administering any laxative. In a few cases, tissue samples were also collected from colonic mucosa to compare with results derived from stool. All samples collected were stored at –70˚C until laboratory processing. Extraction of total RNA and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) quantification was carried out randomly and blindly on all stored samples, in a short time period in order to prevent analytical bias (24, 25).

Tissue specimens were also collected by taking a small piece of tissue (~0.5 cm^3) after colonoscopy for adenoma, or at surgery for carcinoma after the pathologist had confirmed the histopathology. Samples were processed after flash-freezing in liquid nitrogen and storage at –70˚C for subsequent microdissection. Longitudinal sectioning was performed. *In situ* hybridization analysis in tissue microarray (TMA) for miR-21 was performed after digestion in protease; the tissues and probe miRNA (1 pmol/µl, 5' digoxigenin-tagged; Exiqon A/S, Vedbaek, Denmark) were co-incubated at 60° C for 5 min, then hybridized for 15 h at 37 C. After a wash in 0.1X of standard saline citrate (SSC) solution (Sigma-Aldrich, St. Louis, MO, USA) and 2% bovine serum albumin at 50 C for 10 min, the miRNA-probe complex was visualized via nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP) [Roche Molecular Biochemicals, Indianapolis, IN, USA] due to the action of the alkaline phosphatase that is conjugated to the antidigoxigenin antibody. For miR-21 expression in a TMA sample is presented in Figure 1. The figure shows high differential expression of the miRNA in the cytoplasm of the cancer cells (right image) and low expression in the adjacent normal colonic epithelia (left image). The signal is seen in blue due to NBT/BCIP staining, and counterstaining with nuclear fast red (26). Areas of the crypt, where the adenoma or carcinoma cells were marked by the pathologist, were also captured by LCM (Figure 2) for subsequent specific total RNA extraction from carcinoma cells as detailed below.

Stool samples for the study were obtained from 20 healthy control individuals, 20 patients with adenomatous polyps ≥1 cm with high-grade dysplasia (stage 0-1), 10 patients with stage 2 carcinoma, five patients with stage 3 carcinoma, and five patients with stage 4 colon carcinoma Table I).

Isolation of homogenous cancerous cells by LCM. Studies on tumor cells *in vivo* and *in vitro* revealed significant heterogeneity in the expression of myriad phenotypic parameters (*e.g.* karyotype, antigenicity, immunogenicity, biochemical properties, growth, behavior, metastatic capability and cellular susceptibility to chemotherapeutic agents) in both primary and non-metastatic lesions, including stromal and surrounding non-cancer tissue (27). Hence, it is essential to use an enrichment method to separate tumor cells from benign colonic cells and inflammatory cells. We used the

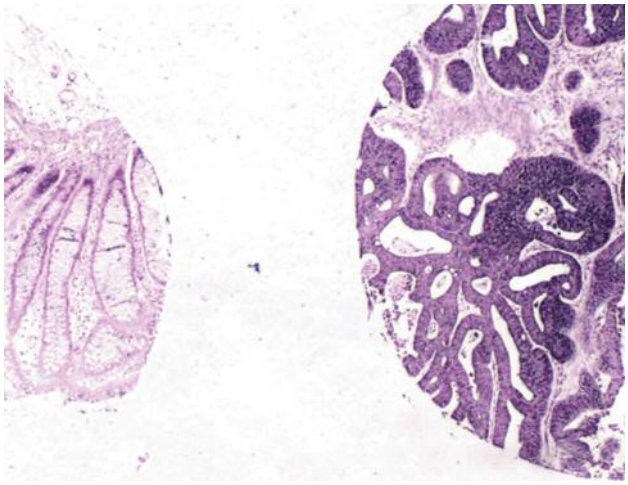

Figure 1. *Differential expression of miR-21 in colon cancer versus the adjacent normal tissue. In situ hybridization analysis for miR-21 in this core tissue microarray sample showed very high expression in the cytoplasm of the cancer cells (right part of image) and very low expression in the adjacent normal colonic epithelia (left part of image). The signal is blue due to nitroblue tetrazolium and bromochloroindolyl phosphate and counterstaining with nuclear fast red, ×10.*

PixCell II system (Arcturus Engineering, Mountain View, CA, USA), which gives a large beam of infra-red (IR) light ~7.5 µm in width (size of a single cell) (28).

LCM entails placing an ethylene vinyl acetate thermoplastic polymer film containing dyes that allow absorption of thermal energy over a tissue section, visualizing the tissue microscopically, and selectively adhering the cells of interest to the film with an infrared gallium arsenide laser diode, which produces 5-50 ms pulses, 2-30 µM in diameter. The resulting mild local heating causes the thermoplastic polymer, which is bonded to a sterilized plastic cup to flow into the interstices of the target tissue and form a bond that is stronger than the bond between the tissue section and the glass slide and selectively adheres the pulsed cells to the film (*i.e.* contact-free collection). An IR laser beam (size 5, 10, 30 or 60 µm) is selected corresponding to target morphology. The mild, brief thermal transient experienced by the tissue in the capture process is limited to 90˚C, and does not change DNA, mRNA or protein. LCM creates no chemical bonds to targeted tissue that might alter subsequent molecular architecture. Immediate cryopreservation of surgical and biopsy specimens followed by cryosectioning was found to produce better RT-PCR products than did paraffin-embedding, and fixation preparation (29). Studies have shown that a small microdissected region containing ~200 cells provides enough mRNA to make cDNA for 100 PCR reactions (30, 31).

Tissue specimens embedded in Tissue Tek® optimum cutting temperature (OCT) compound (Sakura Finetek USA, Ins., Torrance, CA, USA) before freezing were longitudinally sectioned at 7 µm in a cryostat to capture bottom mature crypt cells. Sections were picked up on a supporting polyethylene foil mounted on non-charged microscope slides (Fisher Scientific, Pittsburgh, PA, USA) coated with poly-lysine to ensure that tissue stays on during staining, and laid on the stage of the microscope. The slides were kept in a slide holder on dry ice, fixed for 1 min in 75% ethanol, dipped in nuclease free water for 30 sec,

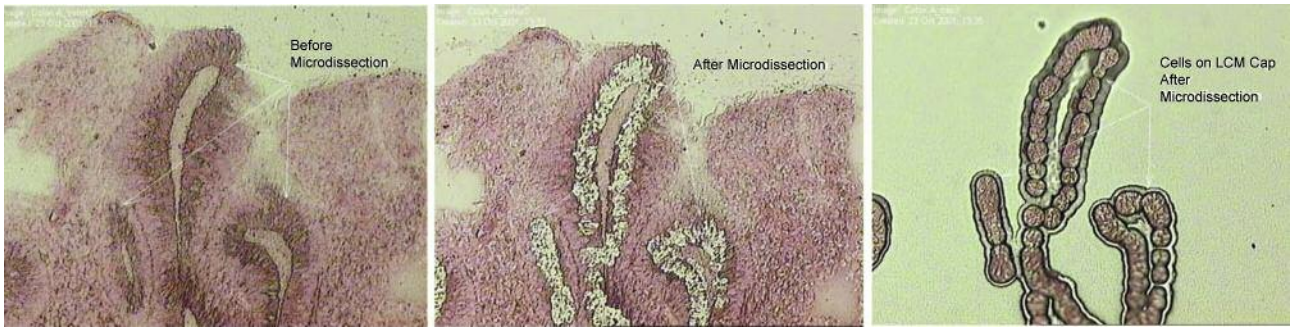

Figure 2. *A: Laser capture microdissection displaying dysplastic cells from the section shown in Figure 1 before being pulsed by an IR laser; B: The middle panel shows the same area with dysplastic cells removed. C: The right panel shows removed dysplastic regions on a film cap. ×5.*

stained with 1% Crystal Violet Acetate (Sigma-Aldrich, St. Louis, MO, USA) for 30 sec, rinsed in 75%, 95% and 100% ethanol for 30 sec each, air dried for 5 min, and stored in a slide box in a desiccator for up to 3 h before LCM. We found this procedure to result in less RNA degradation than traditional hematoxylin and eosin (H&E) staining (Sigma-Aldrich) (31).

Extraction of total RNA from stool, and ss-cDNA preparation by reverse transcription (RT). A procedure used for manual extraction of total RNA from about 0.25 g of stool was carried out using a guanidinium-based buffer, which comes with the RNeasy isolation Kit® (Qiagen, Valencia, CA, USA), as we have previously detailed (4, 17). Purity of total RNA was measured spectrophotometrically at λ 260 nm and 280 nm. The fragility of total RNA was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA, USA), utilizing the RNA 6000 Nano LabChip® (32). The Sensiscript RT Kit® from Qiagen was then employed for making a copy of ss-DNA in a 7.5 μl RT reaction containing total RNA, 50 nm stem-loop RT primer, 1× RT buffer, 0.23 nm of each of dNTP, 3.33 U/μl multiscriptase reverse transcriptase. The reaction was incubated in a 9700 thermocycler in a 96-well plate for 30 min at 16˚C, 30 min at 42˚C, 5 min at 85˚C to inactivate the Thermoscript RT, and formed ss-cDNA were held at 4˚C. A 2-3 μl of ss-cDNA was subsequently amplified by PCR (32). As detailed before, the use of non-template controls and RT-minus controls was also employed to confirm the correct performance of the RT reaction (17, 21, 24, 33).

Microarray profiling of MiRNAs. Affymetrix Gene Chip Micro 3.0 Array (Affymetrix, Inc, Santa Clara, CA, USA), which provides for 100% miRBase v17 coverage (www.mirbase.org) by a one-color approach, was employed for a universal miRNA coverage (34). The microarray contains 16,772 entries representing hairpin precursor, total probe set 19,724 for detection of 1,733 mature miRNA, 2,216 human SnoRNA and ScaRNA products in 153 species, and provides >3-log dynamic range, with >95% reproducibility and 85% transcript detection at 1.0 amol, and a dynamic range >3 logs for a total RNA input of 130-500 ng.

The enriched fraction for miRNA profiling studies was obtained by passing 10 μg of high quality, undegraded total RNA through a flashPAGE™ Fractionator apparatus (Invitrogen/Applied Biosystems), which is a specialized electrophoresis instrument for rapid and efficient PAGE purification of small, less abundant nucleic acid molecules, as compared to a traditional polyacrylamide gel electrophoresis (PAGE) purification for large, profuse macromolecules. RNA molecules were tailed and labeled using a labeling kit designed specifically for use with Affymetrix GeneChip® miRNA arrays, a 3DNA Array Detection Flash Tag Biotin HSR microarray technology (Genisphere LLC, Hatfield, PA, USA), which produces accurate, validated results from as little as 100 ng total RNA. In this method, a poly (A) tailing was first carried out at 37˚C for 15 min in a 15 μl reaction mix that contained 1× reaction buffer, 1.5 μl of 25 mM $MnCl_2$, 1 μl of 1:500 diluted ATP mix, and 1 μl phosphatidic acid phosphatase (PAP) enzyme. Flash tag ligation was then performed at room temperature for 30 min by adding 4 μl of 5× flash tag ligation mix biotin and 2 μl T4 DNA ligase into the 15 μl of reaction mix. The reaction was stopped by adding 2.5 μl of stop solution, followed by sample washing. The fluorescence on the array was scanned using an Affymetrix GCS3000 Gene Array Scanner with a high resolution 6g patch (34). Thresholding and signal scaling was generated using appropriate algorithms. The background adjusted fluorescent values generated by the scanner were normalized for each miRNA using a variation stabilization transformation method such as cyclic LOWESS, as detailed earlier (35).

Hypothetical testing with one-way analysis of variance (ANOVA) or Student's *t*-test (35) was employed for statistical analysis of miRNA arrays, as was carried out for transcriptomic mRNA arrays. *p*-Values <0.05 were considered significant. Reproducibility and linearity were evaluated using ANOVA or Pearson correlation coefficients (36, 37).

Pair-wise comparisons were carried out on differentially expressed genes identified by ANOVA (38). For each pair of treatments, a two-sample *t*-test was carried out for every gene and multiplicity correction was followed to control the false-discovery rate (FDR) using a step-up approach known as protected least significant difference (39). LSD Pairwise volcano plots were also generated. miRNAs above the horizontal lines in the plot and to the left and right of the vertical lines indicate over- or underexpression, respectively. Statistical analysis was carried out using the open source R-software (http://www.r-project.org) as we have described elsewhere (35, 40, 41).

Reverse transcription of mature miRNAs using TaqMan® minor-groove binding (MGB) probes. MiRNA stem-loop RT primers for specific miRNA species to be tested, together with probes having a MGB, containing non-fluorescence quencher at the 3' end and the fluorescence dye FAM at the 5' end, were obtained from

Table I. *Histopathological diagnoses and characteristics of patients participating in a two-year study to develop diagnostic microRNA markers for colon cancer screening.*

Number	Diagnosis (TNM stage)	Age (years)	Race	Gender	Histology location	Tumor distal	Proximal/
1	Healthy	64	W	M	--	--	--
2	Healthy	46	W	F	--	--	--
3	Healthy	49	W	M	--	--	--
4	Healthy	80	W	M	--	--	--
5	Healthy	65	W	F	--	--	--
6	Healthy	56	W	M	--	--	--
7	Healthy	53	W	M	--	--	--
8	Healthy	71	W	F	--	--	--
9	Healthy	63	W	F	--	--	--
10	Healthy	49	W	F	--	--	--
11	Healthy	61	W	M	--	--	--
12	Healthy	50	W	M	--	--	--
13	Healthy	57	B	F	--	--	--
14	Healthy	73	W	F	--	--	--
15	Healthy	51	W	M	--	--	--
16	Healthy	38	B	M	--	--	--
17	Healthy	44	W	F	--	--	--
18	Healthy	55	W	F	--	--	--
19	Healthy	47	W	M	--	--	--
20	Healthy	39	W	F	--	--	--
21	Stage 0-1, NAA	58	W	M	LGD	De	D
22	Stage 0-1, NAA	66	W	M	LGD	As	P
23	Stage 0-1, NAA	66	W	M	LGD	S	D
24	Stage 0-1, AA	61	W	F	LGD	T	P
25	Stage 0-1, NAA	60	W	M	LGD	As	P
26	Stage 0-1, AA	62	W	M	LGD	De	D
27	Stage 0-1, AA	58	B	F	LGD	S	D
28	Stage 0-1, NAA	63	W	M	LGD	T	P
29	Stage 0-1, AA	58	W	F	LGD	De	D
30	Stage 0-1, NAA	74	W	M	LGD	As	P
31	Stage 0-1, AA	76	W	F	LGD	T	P
32	Stage 0-1, NAA	77	B	M	LGD	De	D
33	Stage 0-1, AA	60	W	F	LGD	De	D
34	Stage 0-1, AA	74	W	F	LGD	S	D
35	Stage 0-1, NAA	72	W	F	LGD	As	P
36	Stage 0-1, NAA	75	W	F	LGD	S	D
37	Stage 0-1, AA	77	W	M	LGD	T	P
38	Stage 0-1, NAA	79	B	F	LGD	As	P
39	Stage 0-1, NAA	66	W	F	LGD	S	D
40	Stage 0-1, AA	83	B	F	LGD	As	P
41	Stage 2, Cancer	64	B	M	II	De	D
42	Stage 2, Cancer	63	B	M	II	De	D
43	Stage 2, Cancer	65	W	M	II	As	P
44	Stage 2, Cancer	54	W	F	II	As	P
45	Stage 2, Cancer	66	W	M	II	S	D
46	Stage 2, Cancer	83	W	M	II	S	D
47	Stage 2, Cancer	68	B	M	II	De	D
48	Stage 2, Cancer	69	W	F	II	As	P
49	Stage 2, Cancer	71	W	M	II	As	P
50	Stage 2, Cancer	62	W	F	II	De	D
51	Stage 3, Cancer	57	W	F	III	As	P
52	Stage 3, Cancer	54	W	M	III	As	P
53	Stage 3, Cancer	65	B	M	III	De	D
54	Stage 3, Cancer	69	W	M	III	S	D
55	Stage 3, Cancer	59	W	M	III	De	D
56	Stage 4, Cancer	72	W	F	IV	As	P
57	Stage 4, Cancer	59	W	F	IV	S	D
58	Stage 4, Cancer	59	B	M	IV	S	D
59	Stage 4, Cancer	67	W	M	IV	e	D
60	Stage 4, Cancer	81	W	M	IV	As	P

A, Adenoma; AA, advanced adenoma; As, ascending colon; B, black; CC, colon carcinoma; De, descending colon; D, distal; F, female; LGD, low-grade dysplasia; M, male; NAA, non-advanced adenoma; P, proximal; S, sigmoid colon; T, transverse colon; TNM, tumor node metastasis; W, white.

Invitrogen/Applied Biosystems for the following 20 miRNAs: miRNA-7, miR-9, miRNA-17, miRNA-20a, miRNA-21, miR-29b, miRNA-92a, miR-96, miR-106a, miRNA-127-5p, miRNA-134, miR-138, miRNA-143, miRNA-146a, miR-183, miR-196a, miR-199a-3p, miR214, miR-222 and miR-938.

Total RNA was briefly exposed to RNAase-free DNAase I for 5 min. RNA was then reverse-transcribed to cDNA using a long gene-specific primer (*i.e.* the antisense PCR primer) at an elevated temperature, and a Sensiscript (a thermostable reverse transcriptase from Qiagen) was employed at room temperature. Briefly, a 7.5 μl RT reaction containing an antisense primer and a primer for the *18S* rRNA internal control was heated to 80˚C for 5 minutes to denature the RNA, followed by 5-min incubation at 60˚C to anneal the primers. The reaction was cooled to room temperature and the remaining reagents (5× buffer, dNTPs, DTT, RNAse inhibitor, Sensiscript) were added according to manufacturer's protocol. The reaction proceeded for 45 min at 60˚C, followed by 5-min incubation at 85˚C to inactivate the Sensiscript (40-42)

Semi-quantitative real-time PCR of mature miRNAs. Real-time PCR analysis measures product kinetics by detecting them as they accumulate on-line at a dynamic range of target molecules at least five orders of magnitude as compared to a traditional PCR format, using Roche's PCR LightCycler's 480™ (LC). The method relies on spectral analysis to distinguish among amplicons, allowing for high-throughput detection of either 96 or 384 samples. Employing a two-step format, permits reverse transcription and amplification to be performed separately under optimal conditions, and will be used in this study because of the flexibility that this method provides for our type of multiple-parameter analysis (24, 43-45).

Although miRNAs represent a relatively abundant class of transcripts, their expression level could vary among species and tissues (46). A modified TaqMan-based quantitative real-time PCR assay has been used to quantify the RT product that includes miRNA-specific forward primer, reverse primer and a dye-labeled hydrolysis TaqMan® probes. The tailed forward primer at 5' increases its melting temperature (Tm) depending on the sequence composition of miRNA molecules (42). Better specificity and sensitivity of stem-loop primers compared to conventional linear ones occurs due to base stacking and spatial constraint on the stem-loop structure, which improves the thermal stability and extends the effective footprint of RT primer/RNA duplex that may be required for effective RT from relatively shorter RT primers, and prevent it from binding double strand (ds) genomic DNA molecules, and therefore prevent amplification of any potential genomic DNA contaminants present in the preparation (42). The TaqMan minor-groove binding (MGB) probes have also employed to increase the Tm of very small probes, and the probes have a 5' FAM and a 3' MGB (47). Components for the PCR reaction are found in the Universal Master Mix without UNG and TaqMan™ Assay, to be obtained from Applied Biosystems.

The 10 μl PCR reaction included 0.67 μl RT product (representing ~1 nm total RNA), 1X TaqMan® probe, 1 μM of forward and 0.7 μm of reverse stem-loop RT primers (~50 nM each). Reaction run conditions were as follows: 95˚C for 10 min, followed by 30 cycles of 95˚C for 15 sec and 60˚C for 1 min. All reactions were run in triplicate. Components for the assay were found in the TaqMan™ MicroRNA Reverse Reanscription Kit, Universal Master Mix without UNG and TaqMan™ Assay, all obtained from Applied Biosystems (42).

Absolute quantification of miRNA is unnecessary because constantly transcribed housekeeping genes effectively serve as internal standards for accurate quantification of miRNA genes of interest (48-52); our own experience (40, 41, 53, 54) and others' (42, 47, 52, 55, 56) support that conclusion

Our study emphasized on a commonly accepted dogma of real-time PCR analysis in which optimal data are achieved when each RT reaction is normalized to contain an equal amount of a starting total RNA (42). Normalization was carried out against an endogenous housekeeping internal standard *18S* rRNA gene, as in our hands and others, this standard seems to give consistent and reliable results for normalizing PCR expression of miRNAs (57, 58).

Comparative cross point (CP) method using Roche's 480 LightCycler (LC). The CP value (or E-method) (59) utilizing the LC Quantification Software™, Version 3-5 (60) for Roche LC PCR instruments (Mannheim, Germany). The method employs standard curves in which the relative target concentrations is a function of the difference between crossing points (or cycle numbers) that are plotted against the threshold cycle to calculate the expression of miRNA genes automatically without user's input, with a high sensitivity and specificity, using second derivative maximum calculations (61). A CP value corresponds to the cycle number at which each well has the same kinetic properties. The CP method corresponds to the $2^{-\Delta\Delta CT}$ method (62) used by other PCR instruments, although the latter method produces reliable quantitative results *only* if the efficiency [E=10-1/slope] of the PCR assay for both target and reference genes are identical and equal to 2 (*i.e.*, doubling of molecules in each amplification cycle) (63); for example if well A1 has a CP value of 15 and well A2 has a CP value of 16, we deduce that there was twice as much of the gene of interest in well A1. A 10-fold difference is shown by a difference of ~3.3 CP value. It is not possible to compare these values between different primer pairs.

It is also essential to normalize to a "*reference*" gene because the total input amount may vary from sample to sample when doing Relative Quantification. To report "Fold Change" results, the software incorporates all those factors. The CP method can normalize for run-to-run differences, as those caused by variations in reagent chemistry. For such normalization, one of the relative standards must be designated a "*calibrator*" for the target and for the reference genes, which can be any of our healthy control stool sample. These calibrator(s) can then be used repeatedly in subsequent runs to guarantee a common reference point, allowing for comparison of all experiments within the series (52, 64). If necessary, the $2^{-\Delta\Delta CT}$ can be calculated by instrument's software if samples are properly labeled; the $2^{-\Delta\Delta CT}$ calculations can also be set up manually. To determine fold change for a particular unknown cancer stool sample that has a target gene CP value of 10, one needs three additional values: a) The reference gene CP value of that same unknown stool sample/cancer stool sample, b) the target gene CP for the calibrator sample/normal stool, and c) the reference gene CP for the calibrator sample/normal stool.

Quality Control (QC) Methods. In all PCR reactions, strict attention was given to QC procedures (65, 66) because the exponential amplification of this procedure can result in major errors if contamination from any source occurs. As the field has matured, guidelines on reporting qPCR data known as minimum information for publication of quantitative real-time PCR expression (MIQUE) has also been implemented by us (67) in order to ensure the uniformity, reproducibility and reliability of the PCR reaction and data integrity.

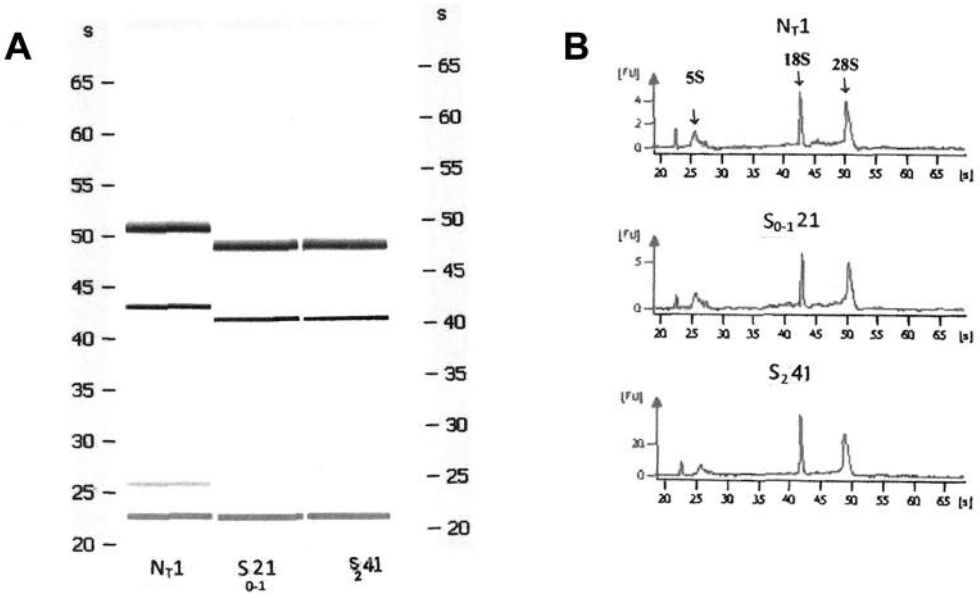

Figure 3. *A: Representative example of Agilent gels for stool and laser capture microdissected tissue samples exhibiting non-degraded RNA from normal tissue from a healthy individual [NT1], stool from a patient with adenomatous colon polyp 1 cm, villous or tubvillous, or with high-grade dysplasia (stage 0-1) [S0-121], or stool from colon carcinoma patient (stage 2) [S241]. B: Agilent electrophoretograms showing the 28S, 18S, 5S and tRNA bands for the same three total RNA stool and tissue samples as shown in A.*

Accessing test performance characteristics (TPC) of the miRNA approach. The CP values of the miRNA gene panel (or a derived preliminary microRNA index, PMI) obtained from stool colonocyte samples of cotrols and patients with colon cancer with high sensitivity and specificity were compared to the commonly used guaiac FOBT test and with colonoscopy results obtained from patients' medical records in 60 cases (20 controls, and 40 patients with colon cancer of various TNM stages) to access the TPC of the miRNA approach.

Numerical underpinning of the miRNA as a function for total RNA. Cytological methods carried out on purified colonocytes employing Papanicolaou and Giemsa staining (68) as described for colorectal cancer, showed a sensitivity for detecting tumor cells in smears comparable to that found in biopsy specimens (*i.e.* 78.1% *versus* 83.66%) (69). The numerical method was accomplished in our study by isolating a known number of colonocytes (see below) from 1 g stool (from normal and neoplastic preparations), extracting total RNA from them to determine the actual amount of total RNA per stool sample, and determining the average CP value from the panel of selected miRNA genes from qPCR measurements using Roche 480™ PCR instrument, eventually arriving at an average CP value per specific amount (pg or ng) of total RNA.

Isolation of exfoliated colonocytes from stool. Approximately 5 to 10 g of freshly-collected wet feces were homogenized with 200 ml of a buffer consisting of Hank's solution, 10% fetal bovine serum (FBS) and 25 mmol/L Hepes buffer (pH 7.35) at 200 rpm for 1 minute (70). The homogenates was filtered through a nylon filter (pore size 512 μm), followed by dividing the homogenate into five portions (~40 ml each) in 50 mL polystyrene tubes. Subsequently, 40 μl of Dynal superparamagnetic polystyrene beads (4.5 μm diameter) (Applied Biosystems/Invitrogen) coated with a mouse IgG1 monoclonal antibody (Ab) Ber-Ep4 (Dako, Glostrup, Denmark) specific for an epitope on the protein moiety of the glycopolypeptide membrane antigen Ep-CAM, which is expressed on the surface of human epithelial cells, including colonocytes and colon carcinoma cells (70), was added to each tube at a final concentration of 12 ng of Ab/mg magnetic beads (1 μg Ab/106 target cells). The mixtures were incubated for 30 minutes under gentle rolling at 15 rounds/min in a mixer at room temperature. The tubes were placed on a magnet (Dynal MPC-1) and incubated on a shaker for 15 min at room temperature. A few drops of the solution were spread on a glass slide, dried and stained with Diff-Quick stain (Fisher Scientific, Pittsburgh, PA, USA) to visualize colonocytes, and other drops counted in a hemocytometer to estimate the number of colonocytes from which total RNA was extracted. A fresh 1 mL of Hanks solution was added to each tube, the supernatant removed, and the pellet containing colonocytes stored at –70˚C until RNA extraction (71).

Statistical and bioinformatic analyses. Because the difference in gene expression between healthy individuals and patients with cancer was so large and informative for multiple miRNA genes, sophisticated classification procedures were not needed to distinguish between these two groups. Moreover, significant and informative differences in gene expression were observed among the stages of cancer so that classification procedures again were not used. The level of gene expression was displayed using parallel coordinate plots (72), produced by the lattice package in R (version 2.4.0, http://cran.r-project.org).

We have bioinformatically correlated the 2-7 or 2-8 complement nucleotide bases in the mature miRNAs with the untranslated 3' region of target mRNA (3' UTR) of a message using a basic algorithm such as Broad's Institute's TargetScan (73) http:// www.targetscan.org/archives.html, which provides a precompiled list for their prediction (8).

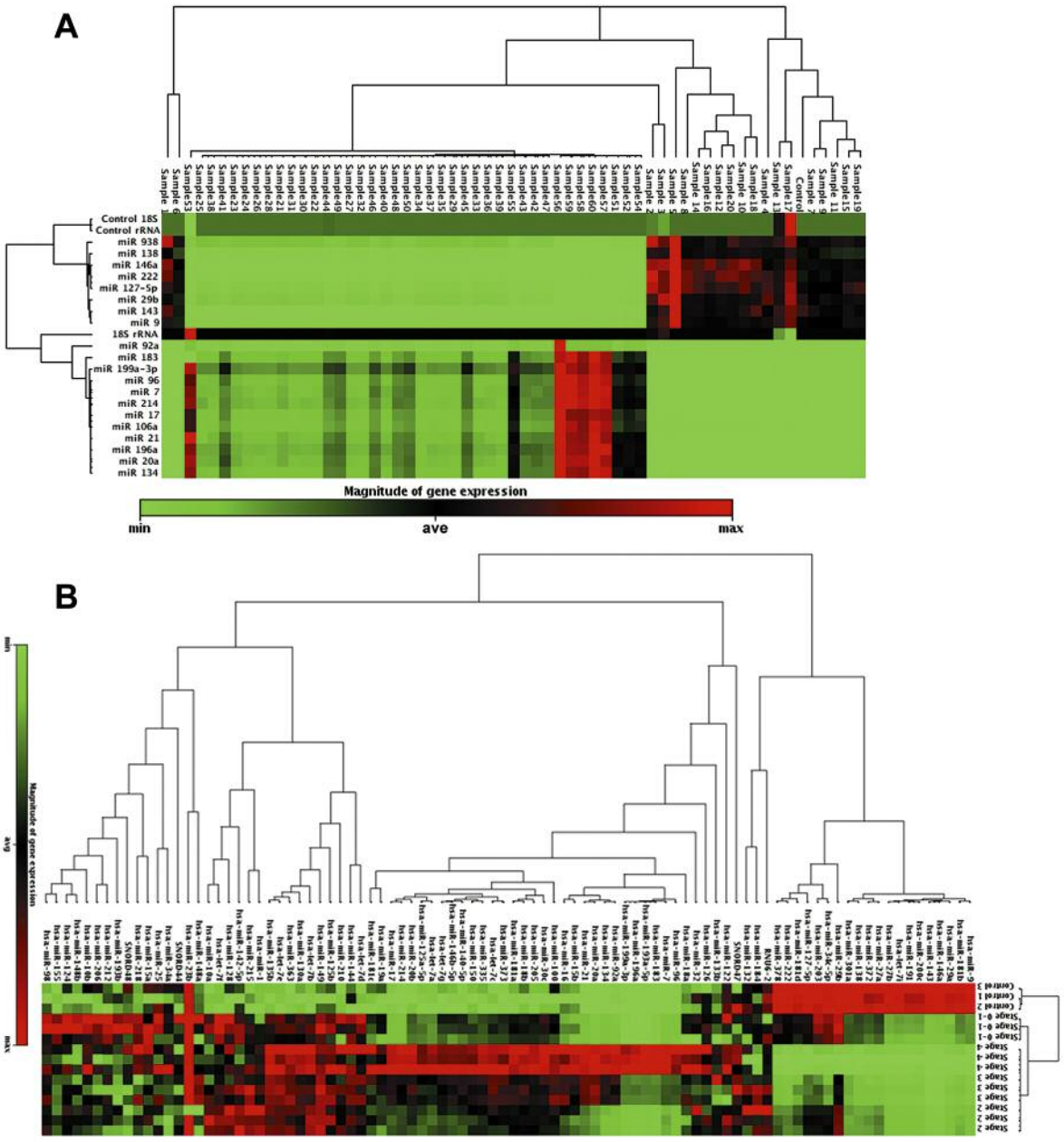

Figure 4. *A: Hierarchical clustering of an Affymetrix Gene Chip Micro 3.0 Array, depicting miRNA microarray gene expression in tissue from patients with stage 0-1 colon carcinoma. B: A hierarchical clustergram as shown in 4A, but for stool.*

Results

Tumor tissue from adenocarcinoma of the colon (containing as they generally do both normal and cancer cells) was embedded in OCT compound, flash-frozen in liquid N_2, and stored at –70˚C until ready for LCM work. Earlier on, we attempted, using the preservative RNA*Later,* to stabilize the easily-fragmented total RNA. However, we discovered that it distorted tissue morphology when used to preserve RNA, causing problems in selecting target cell populations (53, 54); thus, we stopped using this procedure, as in our hands, freezing of tissue sample at –70˚C gave satisfactory results.

The purity of total RNA from stool samples of normal tissue or tissue from various stages of colon cancer measured spectrophotometrically, gave an OD $260_{nm}/280_{nm}$ ratio from 1.9 to 2.0, indicating that it was reasonably pure. Electrophoretic runs were carried out on an Agilent 2100

Table II. *Stem-loop TaqMan® MGB probes RT-qPCR microRNA expression in human stool and tissue from healthy individuals and patients with colon cancer patients*[a,b].

Stool sample	*18S* rRNA	miR 199a-3p CP[a]	miR 196a CP[a]	miR 183 CP[a]	miR 96 CP[a]	miR 7 CP[a]	miR 214 CP[a]	miR 21 CP[a]	miR 20a CP[a]	miR 92a CP[a]	miR 134- CP[a]	miR 17 CP[a]	miR 106[a] CP[a]	miR 143 CP[a]	miR 146a CP[a]	miR 9 CP[a]	miR 222 CP[a]	miR 138 CP[a]	miR 127-5p CP[a]	miR 29b CP[a]	miR 938 CP[a]
NegCt[b]	25.87	25.68	26.26	26.04	26.04	26.14	25.88	26.44	25.90	25.78	26.04	25.82	26.12	25.66	26.08	25.92	26.42	25.98	26.10	25.78	26.04
N_s1	25.98	26.04	25.56	26.91	26.06	26.27	26.32	25.66	26.52	26.14	25.90	26.15	25.77	25.68	25.65	25.80	25.78	26.02	25.98	26.06	25.36
N_s2	26.34	25.90	25.46	25.74	25.82	25.64	26.46	26.10	26.32	25.92	25.68	26.16	25.98	26.64	25.88	26.12	25.68	25.98	25.78	26.04	25.66
N_s3	26.46	26.04	25.84	26.22	26.34	26.18	26.44	26.52	26.22	26.16	25.98	25.88	26.10	26.14	25.88	25.98	26.12	26.06	26.08	25.68	26.04
N_s4	25.88	25.64	25.68	25.11	25.96	25.98	25.62	25.84	25.92	25.96	26.08	26.16	25.96	26.02	26.16	25.80	25.86	25.96	25.78	25.56	25.86
N_s5	26.78	25.69	26.24	25.78	25.94	25.86	25.48	25.86	25.94	26.22	25.88	25.92	26.12	25.98	26.12	25.88	26.16	25.78	26.12	25-98	26.04
N_s6	25.68	25.88	25.98	26.02	26.18	26.10	25.88	25.78	25.86	26.78	26.04	26.08	25.68	26.06	25.98	26.02	25.88	26.16	25.86	26.12	25.68
N_s7	25.66	26.02	26.10	25.98	25.84	25.88	26.14	25.68	26.06	25.68	25.98	25.78	26.10	25.66	26.08	25.76	26.06	25.68	25.98	25.86	25.76
N_s8	26.04	25.78	25.88	25.76	26.08	26.18	25.68	26.14	25.84	26.06	26.12	25.94	25.68	26.10	25.84	26.02	25.78	26.08	25.76	26.06	26.14
N_s9	25.68	26.04	26.08	25.88	25.92	25.86	26.06	25.76	26.10	25.66	25.82	26.06	26.02	25.82	26.08	25.78	26.14	25.86	26.02	25.98	25.80
10	26.12	25.80	25.92	26.06	25.74	26.08	25.90	25.84	25.94	26.08	26.04	25.90	25.80	26.06	25.72	26.06	25.96	25.88	25.90	26.12	26.06
11	25.78	26.10	26.04	25.88	26.08	25.84	26.02	26.06	25.72	25.90	25.68	26.08	26.14	25.94	26.06	25.88	26.16	26.06	26.10	25.90	25.80
12	26.02	25.96	25.78	26.08	25.96	26.12	25.78	25.88	26.06	26.08	26.12	25.76	25.96	26.08	25.90	26.08	25.76	25.88	25.96	26.04	26.12
13	25.68	26.02	26.10	25.82	26.06	25.76	26.10	26.06	25.90	25.76	25.96	26.06	26.10	25.84	26.16	25.76	26.14	26.08	26.08	25.96	25.88
14	26.02	25.96	25.76	26.00	25.96	26.08	25.80	25.96	26.06	25.80	26.08	25.76	25.74	26.06	25.88	26.16	25.92	25.88	25.90	26.06	26.16
15	25.86	26.08	26.14	25.98	26.02	25.80	26.12	25.98	25.86	26.02	25.90	26.08	26.12	25.88	26.04	25.78	26.06	26.10	25.86	25.90	25.84
16	26.00	25.92	25.86	26.08	25.96	26.08	25.90	26.16	26.08	25.78	26.06	25.78	25.66	26.12	25.70	26.06	25.96	25.80	26.02	26.12	26.02
17	25.70	26.04	26.08	25.96	26.14	25.78	26.10	25.84	25.90	26.04	25.96	26.10	26.06	25.94	26.16	25.86	26.06	26.02	25.92	25.76	25.92
18	26.06	25.76	25.94	26.06	25.82	26.14	25.88	26.06	26.02	25.86	26.12	25.86	25.96	25.78	25.84	26.06	25.96	25.86	26.10	26.04	26.00
19	25.86	26.06	26.16	25.86	26.08	25.94	26.02	25.82	25.78	26.08	25.90	26.08	26.12	25.98	26.10	25.80	26.06	26.14	25.76	25.86	25.94
20	26.04	25.96	25.84	26.06	25.86	26.08	25.86	26.04	25.92	25.88	26.08	25.86	25.96	26.12	25.78	26.04	25.76	25.86	26.02	26.10	25.84
N_T	26.24	25.68	26.04	25.82	26.86	25.68	25.82	26.06	25.82	25.64	26.06	25.88	26.02	26.06	26.06	26.06	26.18	25.90	26.08	25.96	26.06
N_T	25.99	26.02	25.68	26.06	26.02	26.06	26.14	25.90	26.02	26.82	25.88	25.96	25.88	25.84	26.18	25.92	26.02	26.08	26.04	25.94	26.02
N_T3	25.86	25.82	26.12	25.92	25.66	25.88	25.74	25.62	25.92	25.86	26.06	26.08	25.90	25.66	25.78	26.08	25.66	26.02	25.88	26.06	25.88
$S_{0-1}4$	25.96	12.14	13.16	14.26	15.26	16.16	17.10	18.20	19.22	20.16	21.22	22.22	23.60	35.54	34.38	33.44	32.20	31.18	30.26	29.20	28.26
$S_{0-1}5$	26.08	12.16	13.10	14.30	15.24	16.26	17.06	18.18	19.21	20.18	21.20	22.20	23.58	35.58	34.42	33.42	32.24	31.22	30.24	29.14	28.10
$S_{0-1}6$	25.90	12.12	13.08	14.16	15.14	16.24	17.16	18.14	19.24	20.22	21.24	22.14	23.52	35.58	34.40	33.52	32.30	31.26	30.28	29.16	28.14
$S_{0-1}7$	25.86	12.16	13.02	14.20	15.28	16.14	17.08	18.12	19.16	20.24	21.26	22.16	23.44	35.62	34.50	33.54	32.36	31.28	30.14	29.22	28.18
$S_{0-1}8$	25.92	12.18	13.16	14.24	15.32	16.18	17.18	18.16	19.18	20.20	21.28	22.26	23.38	35.66	34.46	33.46	32.38	31.30	30.16	29.12	28.16
$S_{0-1}9$	25.86	12.06	13.14	14.28	15.22	16.20	17.22	18.20	19.28	20.22	21.30	22.32	23.36	35.34	34.48	33.48	32.34	31.34	30.20	29.24	28.20
$S_{0-1}10$	26.02	12.22	13.18	14.20	15.36	16.42	17.20	18.22	19.30	20.28	21.36	22.36	23.46	35.36	34.44	33.50	32.30	31.32	30.28	29.26	28.24
$S_{0-1}11$	25.94	12.16	13.08	14.18	15.24	16.38	17.24	18.10	19.24	20.26	21.34	22.34	23.48	35.42	34.52	33.38	32.32	31.36	30.32	29.30	28.28
$S_{0-1}12$	25.88	12.20	13.10	14.32	15.30	16.34	17.18	18.16	19.28	20.18	21.24	22.38	23.50	35.46	34.56	33.34	32.42	31.44	30.36	29.28	28.22
$S_{0-1}13$	26.08	12.04	13.14	14.30	15.38	16.44	17.22	18.12	19.26	20.20	21.28	22.26	23.54	35.48	34.54	33.32	32.48	31.40	30.34	29.34	28.30
$S_{0-1}14$	25.78	12.14	13.20	14.28	15.30	16.38	17.26	18.14	19.16	20.22	21.26	22.30	23.46	35.52	34.46	33.36	32.46	31.42	30.30	29.36	28.36
$S_{0-1}15$	25.92	12.12	13.18	14.36	15.18	16.26	17.22	18.18	19.24	20.18	21.32	22.32	23.38	35.58	34.36	33.40	32.44	31.48	30.38	29.38	28.44
$S_{0-1}16$	26.04	12.08	13.12	14.40	15.32	16.24	17.26	18.24	19.28	20.14	21.34	22.40	23.40	35.60	34.50	33.42	32.40	31.50	30.42	29.40	28.32
$S_{0-1}17$	25.96	12.10	13.14	14.22	15.26	16.36	17.32	18.26	19.22	20.26	21.36	22.38	23.24	35.44	34.52	33.44	32.50	31.36	30.40	29.32	28.42
$S_{0-1}18$	26.04	12.14	13.08	14.34	15.14	16.24	17.38	18.16	19.14	20.28	21.30	22.40	23.28	35.50	34.60	33.46	32.56	31.38	30.36	29.44	28.40
$S_{0-1}19$	25.88	12.06	13.10	14.38	15.18	16.12	17.30	18.24	19.26	20.24	21.38	22.36	23.42	35.56	34.38	33.32	32.52	31.44	30.34	29.42	28.38
$S_{0-1}20$	26-06	12.10	13.06	14.44	15.28	16.10	17.26	18.22	19.24	20.12	21.20	22.24	23.50	35.64	34.32	33.36	32.54	31.46	30.44	29.46	28.36
$T_{0-1}1$	25.68	10.22	11.10	12.36	13.62	14.26	15.10	16.08	17.52	18.26	19.36	20.34	21.12	37.52	36.18	35.18	34.16	33.12	32.18	31.16	30.12
$T_{0-1}2$	26.02	10.08	11.18	12.18	13.42	14.32	15.36	16.18	17.22	18.12	19.22	20.12	21.28	37.66	36.46	35.42	34.08	33.26	32.34	31.08	30.26
$T_{0-1}3$	25.88	10.18	11.02	12.22	13.56	14.46	15.66	16.24	17.36	18.08	19.14	20.28	21.08	37.72	36.38	35.20	34.36	33.18	32.08	31.02	30.36
$S_2$1	26.12	11.22	12.20	13.34	14.24	15.66	16.62	17.40	18.08	19.14	20.16	21.26	22.56	36.12	35.12	34.16	33.20	32.18	31.22	30.18	29.16
$S_2$2	25.94	11.34	12.16	13.44	14.30	15.54	16.44	17.32	18.24	19.18	20.22	21.28	22.50	36.16	35.18	34.20	33.26	32.26	31.26	30.24	29.22
$S_2$3	25.88	11.28	12.08	13.22	14.26	15.36	16.52	17.38	18.36	19.24	20.18	21.34	22.54	36.20	35.26	34.28	33.28	32.28	31.28	30.22	29.26
$S_2$4	25.96	11.30	12.24	13.28	14.38	15.42	16.56	17.40	18.34	19.26	20.24	21.36	22.48	36.26	35.28	34.36	33.24	32.34	31.24	30.20	29.18
$S_2$5	26.08	11.16	12.26	13.34	14.28	15.34	16.38	17.36	18.38	19.20	20.26	21.32	22.44	36.24	35.30	34.38	33.22	32.20	31.20	30.30	29.20
$S_2$6	25.90	11.32	12.22	13.42	14.34	15.20	16.46	17.34	18.40	19.22	20.12	21.30	22.32	36.30	35.16	34.44	33.30	32.22	31.30	30.22	29.24
$S_2$7	25.88	11.42	12.26	13.46	14.32	15.28	16.36	17.28	18.36	19.28	20.20	21.20	22.42	36.36	35.20	34.42	33.36	32.30	31.26	30.26	29.28
$S_2$8	25.86	11.36	12.40	13.44	14.36	15.32	16.24	17.32	18.38	19.30	20.30	21.18	22.38	36.38	35.24	34.48	33.32	32.40	31.30	30.24	29.30
$S_2$9	26.04	11.38	12.28	13.38	14.28	15.38	16.36	17.28	18.40	19.22	20.32	21.22	22.42	36.28	35.30	34.46	33.34	32.24	31.34	30.28	29.34
$S_2$10	25.98	11.28	12.34	13.32	14.30	15.30	16.42	17.34	18.26	19.28	20.16	21.24	22.32	36.32	35.32	34.40	33.38	32.36	31.36	30.32	29.32
$T_2$1	25.88	9.26	10.22	11.16	12.24	13.22	14.30	15.44	16.20	17.22	18.24	19.22	20.26	38.24	37.10	36.28	35.14	34.14	31.26	30.24	29.22

Table II. *continued*

Table II. *continued*

Stool sample	*18S* rRNA	miR 199a-3p CP[a]	miR 196a CP[a]	miR 183 CP[a]	miR 96 CP[a]	miR 7 CP[a]	miR 214 CP[a]	miR 21 CP[a]	miR 20a CP[a]	miR 92a CP[a]	miR 134-CP[a]	miR 17 CP[a]	miR 106[a] CP[a]	miR 143 CP[a]	miR 146a CP[a]	miR 9 CP[a]	miR 222 CP[a]	miR 138 CP[a]	miR 127-5p CP[a]	miR 29b CP[a]	miR 938 CP[a]
$T_2$2	25.76	9.18	10.12	11.32	12.14	13.14	14.26	15.32	16.34	17.30	18.36	19.28	20.18	38.38	37.24	36.12	35.24	34.22	31.28	30.22	29.26
$T_2$3	26.06	9.22	10.18	11.20	12.26	13.20	14.42	15.20	16.28	17.28	18.14	19.34	20.34	38.32	37.38	36.40	35.42	34.16	31.24	30.20	29.18
$S_3$1	25.78	10.80	11.20	12.44	13.36	14.28	15.32	16.24	17.30	18.24	19.18	20.22	21.36	37.40	36.38	35.28	34.20	33.18	32.22	31.26	30.18
$S_3$2	26.02	10.84	11.36	12.36	13.44	14.34	15.42	16.30	17.36	18.30	19.26	20.26	21.38	37.46	36.42	35.34	34.26	33.24	32.26	31.28	30.22
$S_3$3	25.96	10.72	11.42	12.30	13.40	14.36	15.36	16.32	17.32	18.26	19.20	20.28	21.34	37.48	36.48	35.30	34.28	33.26	32.24	31.30	30.20
$S_3$4	25.88	10.78	11.28	12.34	13.48	14.42	15.38	16.28	17.38	18.28	19.22	20.32	21.30	37.42	36.44	35.32	34.24	33.28	32.20	31.24	30.24
$S_3$5	26.06	10.66	11.34	12.40	13.52	14.44	15.44	16.36	17.40	18.30	19.24	20.20	21.40	37.44	36.40	35.36	34.22	33.32	32.28	31.26	30.26
$T_3$1	25.88	8.12	9.22	10.12	11.16	12.18	13.22	14.32	15.24	16.38	17.08	18.14	19.12	39.18	38.16	37.36	36.22	35.08	34.46	33.18	32.16
$T_3$2	25.76	8.08	9.12	10.02	11.04	12.10	13.18	14.16	15.38	16.24	17.24	18.24	19.10	39.26	38.36	37.82	36.12	35.36	34.36	33.26	32.28
$T_3$3	26.06	8.02	9.18	10.08	11.08	12.14	13.34	14.28	15.16	16.12	17.10	18.22	19.22	39.38	38.30	37.66	36.28	35.12	34.20	33.34	32.22
$S_4$1	26.08	10.02	10.60	11.38	12.42	13.50	14.56	15.48	16.56	17.44	18.38	19.14	20.22	38.28	37.32	36.28	35.32	34.28	33.28	32.32	31.30
$S_4$2	25.94	10.08	10.42	11.32	12.36	13.42	14.48	15.56	16.62	17.48	18.32	19.24	20.26	38.34	37.34	36.30	35.26	34.24	33.26	32.36	31.26
$S_4$3	25.84	10.10	10.56	11.28	12.30	13.38	14.40	15.50	16.48	17.52	18.40	19.22	20.28	38.36	37.36	36.36	35.36	34.22	33.34	32.34	31.24
$S_4$4	25.90	10.04	10.60	11.12	12.22	13.30	14.34	15.52	16.54	17.46	18.42	19.26	20.20	38.30	37.42	36.42	35.34	34.26	33.30	32.38	31.28
$S_4$5	26.02	10.06	10.52	11.18	12.32	13.42	14.48	15.44	16.50	17.42	18.30	19.28	20.24	38.32	37.46	36.38	35.28	34.30	33.32	32.30	31.22
$T_4$1	26.04	7.54	8.34	9.26	10.32	11.42	12.42	13.26	14.24	15.36	16.32	17.18	18.32	40.12	39.18	38.12	37.18	36.18	35.18	34.16	33.16
$T_4$2	26.12	7.26	8.42	9.34	10.18	11.26	12.38	13.22	14.42	15.22	16.42	17.12	18.12	40.26	39.22	38.26	37.46	36.28	35.26	34.08	33.24
$T_4$3	25.96	7.28	8.28	9.18	10.22	11.18	12.32	13.12	14.36	15.14	16.12	17.26	18.20	40.24	39.18	38.34	37.32	36.08	35.12	34.12	33.14

[a]Comparative crossing point or (E-value): a value of test miRNA equal to the normalization standard (*18S* rRNA) indicates similar expression, a value lower than the standard indicates increased expression, and a value greater than the standard indicates reduced expression. [b]No DNA added to reaction (negative control). All reactions were run in triplicates and then averaged. *18S* rRNA was the normalization standard. The table shows expression values obtained from stool of 60 individuals, as well as of tissues from 12 individuals who also donated stool samples: 20 non-cancerous controls (N1 to N20); 20 patients with adenomatous polyp ≥1 cm (TNM stage $S_{0\text{-}1}$1-$S_{0\text{-}1}$20); 10 patients with TNM stage 2 (S21-S210) colon cancer; 5 patients with TNM stage 3 colon cancer (S31-S35); and 5 patients with TNM stage 4 colon cancer ($S_4$1-$S_4$5). Data were also obtained from tissue of 12 individuals listed above for comparison with stool: 3 non-cancerous tissue controls (N_T1 to N_T3); tissue from 3 patients with adenomatous polyps ≥1cm (stage 0-1), 3 patients with stage 2 ($T_2$1 to $T_2$3), 3 patients with stage 3 ($T_3$1 to $T_3$3), and 3 patients with stage 4 ($T_4$1 to $T_4$3) cancer.

BioAnalyzer (74). They showed an RNA integrity number (RIN) >8.0, indicating high-quality total RNA that was intact and of human origin, making it suitable for high quality miRNA determinations (75). Although there may be daily variations between runs, the overall electrophoretogram patterns show that the *18S* and *28S* bands migrate to the same extent in all total RNA samples taken from stool (Figure 3) (17).

We estimate that by using the method outlined herein, we are able to retrieve between 75-250 μg of human total RNA per gram of stool from patients with cancer, depending on stage (the higher the stage, the greater the amount of RNA extracted), and a lesser amount (~25 μg) of human RNA per gram of stool from control individuals. Because our method employed an RLT lysis buffer (Qiagen), which was reported to lyse bacterial RNA on treatment for 5-10 min (76), our samples were free from bacterial RNA. As we reported earlier (4, 17, 40, 41, 77), no difference in the profile between RNA extracted from stool or from earlier LCM of colon mucosal samples was observed, indicating that total RNA extracted from stool of normal individuals or of patients with various stages of colon cancer is of high quality, of human origin and is intact.

Global gene expression using microarrays showed that similarity in expression between stool and tissue, as shown in the heat maps (Figure 4A and B). There were 202 preferentially expressed miRNA genes that were either increased (141 miRNAs), or reduced (61 miRNAs) in expression in in stool and in tissue samples from patients with colon cancer (Table III). A representative volcano plot of microarray data from stool samples taken from TNM stage 0-1 from colon cancer patients, for [-log10 (*p*-value for colon cancer, stage 0-1)] *versus* [mean control –mean colon cancer], is shown in Figure 5. The *p*-value for one third of the miRNAs was <0.05, and some of these small molecules were further validated by qPCR.

Our stem-loop RT-MGB qPCR of stool samples from healthy individuals and patients with colon cancer presented in Table III shows that of the 20 selected miRNAs exhibiting altered expression, and which have been shown to be related to colon carcinogenesis, 12 (miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106a, miR-134, miR-183, miR-196a, miR-199a-3p and miR214), had an increased expression in the stool of patients with colon cancer, and that of later TNM carcinoma stages

Table III. *Preferentially-expressed Homo sapiens (has)-miRNAs in stool*.

Overexpressed	Underexpressed
miR-7	miR-1
Let 7a	miR-7i
Let7b	Let-7i
Let 7c	miR-9
Let 7d	miR-10a
Let-7e	miR-15a
Let-7f	miR-18b
Let-7g	miR-23b
Let-7i	miR-24a
miR-10b	miR-27a
miR-15	miR-27b
miR-15b	miR-29a
miR-16	miR-39a
miR-17	miR-29b
miR-17-3p	miR-30-3p
miR-18a	miR-30c
miR-18b	miR-3 4-5p
miR-19-a	miR-37
miR-19b	m iR-92a
miR-20	miR-93
miR-20a	miR-95
miR-20b	miR-100
miR-18-a	miR-103a
miR-18b	miR-125b
miR-19a-b	miR-126
miR-20a	miR-127-5p
miR-20b	miR-128b
miR-21	miR-133a
miR-22	miR-138
miR-23a	miR-142-5p
miR-24	miR-143
miR-25	miR-145
miR-26a	miR-146a
miR-26b	miR-148b
miR-27a	miR-150
miR-29a	miR-191
let-30c	miR-192
miR-31	miR-193b
miR-32	miR195
miR-40-5p	miR-199-3p
miR-29b	miR-200b
miR-30-a	miR-200c
miR-30c	miR-212
miR-31	miR-215
miR-32	miR-222
miR-33a	miR-223
miR-34a	miR-301
miR-91	miR-342
miR-92a	miR-363
miR-93	miR-387
miR-95	miR-407
miR-96	miR-423-5p
miR-98	miR-424
miR-99b	miR-455
miR-100	miR-485
miR-101	miR-486
miR-103	miR-650
miR-103a	miR-522
miR-106a	miR-650
miR-106b	miR-661
miR-107	miR-938
miR-122	
miR-124	
miR-125a	
miR-125b	
miR-126	
miR-127	
miR-130a	
miR-130b	
miR-132	
miR-134	
miR-133a	
miR-133b	
miR-135a	
miR-135-b	
miR-137	
miR-139-3p	
miR-135a	
miR-135-b	
miR-140-5p	
miR-141-b	
miR-142-5p	
miR-144	
miR-146a	
miR-146b-5p	
miR-148a	
miR-149	
miR-150	
miR-153	
miR-181a	
miR-181b	
miR-181-c	
miR-181-d	
miR-182	
miR-183	
miR-184	
miR-185	
mIR-191	
miR-192	
miR-196a	
miR-196b	
miR-199a-3p	
miR-200a	
miR-200b	
miR-200c	
miR-202	
miR-203	
miR-204	
miR-205	
miR-206	
miR-210	
miR-211	
m iR-212	
miR-214	
miR-215	
miR-219	

Table III. *continued*

Table III. *continued*

Table III. *continued*.

Overexpressed	Underexpressed
miR-220	
miR-221	
miR-222	
miR-223	
miR-224	
miR-225	
miR-301a	
miR-302a	
miR-302b	
miR-320	
miR-335	
miR-338	
miR-346	
miR-355	
miR-370	
miR-372	
miR-373	
miR-387	
miR-432	
miR-484	
miR-492	
miR-493-3p	
miR-497	
miR-550	
miR-570	

exhibited a more increased expression than adenomas. On the other hand, 8 miRNAs (miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR-146a, miR-222 and miR-938) exhibited decreased expression in the stool of patients with colon cancer, which was also more pronounced from early to later TNM stages. Results from tissues were similar to those from stool samples, although with more apparent changes in expression. The data in Table II are presented graphically in Figure 7 and Figure 8 for the 20 differentially expressed miRNAs in stool samples obtained from the 20 healthy controls and 40 patients with colon cancer at TNM stages 0-IV.

We calculated the standard deviations (SDs) of individual CPs for all the miRNAs. Examples of some SD values are shown in Table IV in order of decreasing values: Numerical underpinning of the miRNAs as a function of total RNA was carried out on colonocytes isolated from stool before any preservative was added to five healthy control samples, and five stage-IV colon cancer samples, extracting total RNA from them and determining the actual amount of total RNA per stool sample, and from the average CP values. It is evident that an average CP value for stage IV colon carcinoma of 21.90, is invariably different from a CP value of 26.05 for healthy controls (Table VI).

Discussion

Colorectal cancer (CRC) is the second most common malignancy worldwide, with an estimated one million new cases and half a million deaths each year (78). Screening for CRC allows for early stage diagnosis of the malignancy and potentially reduces disease mortality (1). The convenient and inexpensive FOBT screening test has low sensitivity and requires dietary restriction, which impedes its compliance and use (4). CRC is the only type of cancer for which colonoscopy is recommended as a screening test (5). Although colonoscopy is a reliable screening tool, its invasive nature, abdominal pain and high cost have hampered worldwide application of this procedure (6, 7). In comparison to the commonly employed FOBT tests, a non-invasive sensitive screen for which there would be no requirement for dietary restriction would be a more convenient test.

Epidemiological evidence suggests that colon cancer (CC) and rectal cancer (RC) differ in their morbidities and etiologies. RC is more common in China, where it accounts for over 50% of all CRC, compared with <30% in Western countries. Data from Peking Union Medical College Hospital, China indicated that incidence of colon and rectal cancers accounted for 55.6% and 44.4% of CRCs, respectively, during the years 1989 through 2008, and RCs are more prevalent in younger Asian individuals (79). In contrast, CC was shown to account for over 60% of CRC cases in the USA and Europe, and is related to fatty foods, less exercise and a Caucasian ethnic origin (80-83), which suggests differences in carcinogenesis between CC and RC. Several structural and molecular studies have indicated differences in etiology, clinical manifestation, pathological features, genetic and molecular abnormalities between CC and RC (84-92).

The discovery of miRNAs), has opened new opportunities for a non-invasive test for early diagnosis of many types of cancer (8). There are 21,264 entries representing hairpin precursor miRNAs, expressing 25,141 mature miRNA products, in 193 species in *miRBase*, release 19 (August 2012, http://mirbase.org) (93). miRNA functions seem to regulate development (94) and apoptosis (95), and specific miRNAs are critical in oncogenesis (8, 96), effective in classifying solid (96-101) and liquid tumors (102, 103), and serve as oncogenes or suppressor genes (104). miRNA genes are frequently located at fragile sites, as well as minimal regions of loss of heterozygosity, or amplification of common breakpoint regions, suggesting their involvement in carcinogenesis (105). miRNAs have promise as biomarkers for cancer diagnosis, prognosis and for response to therapy (8, 106, 107). Profiles of miRNA expression differ between normal tissues and tumor types, and evidence suggests that miRNA expression profiles can cluster similar tumor types together more accurately than expression profiles of protein-coding mRNA genes (108, 109).

Table IV. *Standard deviations (SDs) for some miRNAs in order of decreasing values.*

miR-96	miR-143	miR-146a	miR-214	miR-21	miR-9	miR-7	miR-92a	miR-20a	miR-134	miR-938
7.898177	7.295028	6.593613	6.550193	6.356752	6.042022	5.793815	5.623533	5.450223	5.288764	5.204872
miR-222	miR-138	miR-127-5p	miR-29b	miR-17	miR-183	miR-196a	18SrRNA	miR-199a-3p	miR-106a	
5.193460	4.789436	4.139903	3.804948	3.796239	0.612726	0.5513392	0.531256	0.379780	0.144222	

Table V. *Up-regulated and down-regulated target mRNA genes detected by a DAVID Bioinformatics algorithm.*

Up-regulated target mRNA genes
BCL11B, CUGBP2, EGR3, DLHAP2, NUFIP2, KLF3, MECP2, ZNF532, APPLI1, NFIB, SMAD7, SNF1LK, ANKRD52, C17orf39, FAM13A1, GLT8D3, KIAA0240, PCT, SOCS6, TNRC6B and UHRF1BP1.
Down-regulated target mRNA genes
TGFB1, CKS2, IGF2, KLK10, FLNA, CSE1L, CXCL3, DPEP1 AND GUCA2B.

Unlike screening for large numbers of messenger (m)RNA, a modest number of miRNAs is used to differentiate cancer from normal tissue (8), and unlike mRNA (17), miRNAs in stool remain largely intact and stable for detection (40). Therefore, miRNAs are better molecules to use for developing a reliable non-invasive diagnostic screening for colon cancer, since we have shown that the presence of *Escherichia coli* does not hinder detection of miRNA by a sensitive technique such as qPCR, as the primers employed are selected to amplify human and not bacterial miRNA genes; and the miRNA expression patterns are the same in primary tumors, or diseased tissues, as in stool samples (8, 40).

The RNA isolation procedures employed herein, compared to those used for isolation of DNA from stool samples (11, 110), were standardized and simplified by us, using improved commercially available kits to extract high-quality total RNA from an environment as hostile as stool (11, 111, 112), which dispels the notion that it is difficult to employ a molecule in the stool such as miRNA as a screening substrate. Our ability to stabilize total RNA shortly after obtaining fresh stool by fixing samples in a commercially available chaotropic agent and observing that RNA does not ever fragment thereafter (17, 40) has been innovative, as fragmented RNA results in poor cDNA synthesis and ultimately in less than optimal PCR amplification.

We used Roche's LightCycler (LC) 480™ for real-time PCR analysis of product as they accumulate (*i.e.* on-line) over a large dynamic range of target molecule (*i.e.* at least five orders of magnitude as compared to traditional end-point PCR) (24, 52), which allowed for a higher sample throughput using 96 plates, precise product determination during the log/linear amplification phase, reduced labor and increased fluorescence. Moreover, employing a two-step RT-PCR format permitting reverse transcription and amplification to be performed separately under optimal conditions, is particularly attractive for experiments that require the same RT product to be used for analysis of multiple transcripts (113, 114), as compared to a one-step format that allows both cDNA synthesis and PCR amplification to be performed in a single tube, because of the flexibility provided for our particular type of multiple-parameter analysis.

Table VI. *Numerical Underpinning of miRNA markers as a function of total RNA.*

Patient #	Diagnosis	Total RNA (μg/g of stool)	CP*	
			Sample	Group
2	Healthy control	0.28	25.99	
5	Healthy control	0.30	27.21	
8	Healthy control	0.29	25.96	26.05
14	Healthy control	0.31	25.09	
17	Healthy control	0.32	26.01	
56	Stage IV carcinoma	0.31	20.18	
57	Stage IV carcinoma	0.32	22.03	
58	Stage IV carcinoma	0.31	22.43	21.90
59	Stage IV carcinoma	0.30	21.37	
60	Stage IV carcinoma	0.31	23.49	

*CP: Crossing point value calculated by an algorithm in a Roche LC 480 polymerase chain reaction instrument.

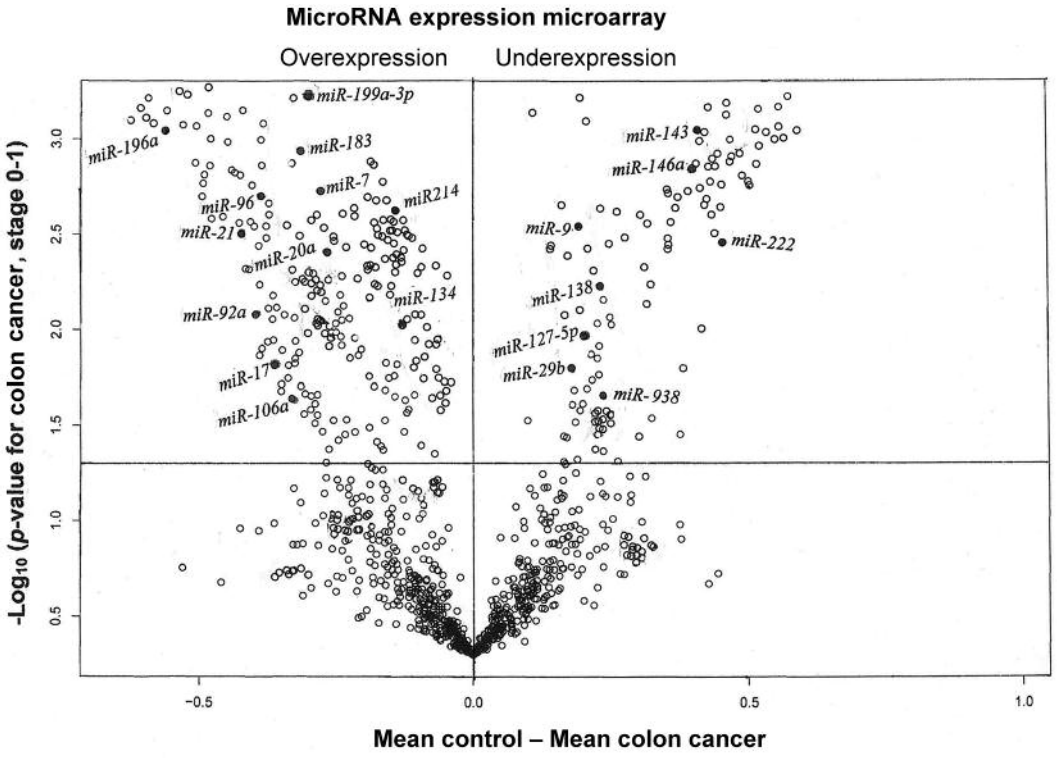

Figure 5. *A pair-wise volcano plot for miRNA gene expression in stool samples taken from twenty patients with colon cancer (TNM stage 0-1), using Affymetrix Gene Chip Micro 3.0 Array for [-log10 (p-value for colon cancer, stage 0-1)] versus [mean control–mean colon cancer] in stool. miRNAs above the horizontal line and to the left and right of the vertical line exhibited over- and underexpression, respectively. The 20 miRNAs that exhibited differential expression (12 increased and 8 decreased) are indicated on the plot.*

We used a modified TaqMan-based quantitative real-time PCR assay that incorporates two steps: a) stem-loop RT, and b) real-time PCR. Stem-loop RT primers bind to at the 3' portion of miRNA molecules and are reverse transcribed with reverse transcriptase. The RT product was then quantified using conventional TaqMan PCR that includes miRNA-specific forward primer, reverse primer and a dye-labeled hydrolysis TaqMan probes. The use of tailed forward primer at 5' increased the melting temperature (T_m) depending on the sequence composition of miRNA molecules (115) Moreover, base stacking and spatial constraint on the stem-loop structure resulted in better specificity and sensitivity compared to conventional linear ones. Additionally, base stacking improves the thermal stability and extends the effective footprint of RT primer/RNA duplex are required for effective RT from relatively shorter RT primers. The spatial constraint of the stem-loop structure may prevent it from binding double-strand (ds) genomic DNA molecules, and therefore prevent amplification of any potential genomic DNA contaminants present in the preparation (115). The TaqMan MGB probes were employed to increase the T_m of very small probes; they are designed to have a T_m that is 10˚C higher than primers, and probes were designed to have a 5' carboxyfluorescein, single isomer (FAM) and a 3' MGB (42).

To be able to screen several miRNA genes in a systematic manner using the proposed LightCycler technology in a sequence-specific RT, in which a cDNA preparation can assay for a specific miRNA, we employed a relative quantitative method involving amplification of the gene of interest (target) and a second control sequence (reference) also called an external standard, which amplified with equal efficacy as the target gene, in the same capillary, a procedure known as multiplex PCR. Quantification of the target was made by comparison of the intensity of the products (116). A suitable reference gene was the housekeeping gene 18S ribosomal (r)RNA used as a normalization standard because of the absence of pseudogenes and the weak variation in its expression (117, 118).

It should be emphasized that the Roche's LC-480 employs a non user-influenced method for highthroughput measurements, using second derivative calculations and double corrections. One correction utilizes the expression levels of a housekeeping gene of an experiment as an internal standard, which results in reduced error due to sample preparation and handling, and the second correction uses reference expression level of the same housekeeping gene for

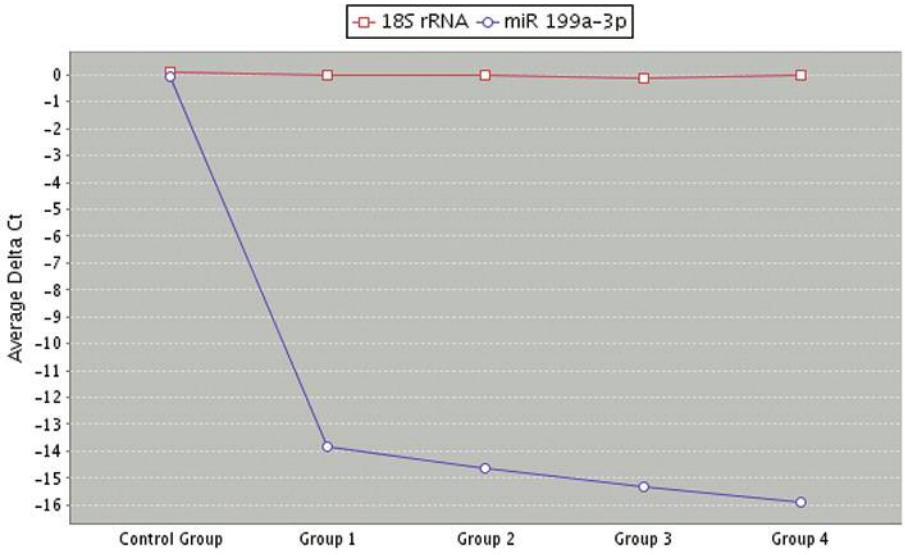

Figure 6. *A multi-group plot comparing miRNA-199a-3p to normalization standard 18S rRNA in healthy controls and the four studied colon cancer groups: Group 1. representing colon cancer TNM stage 0-1; Group 2, representing colon cancer TNM stage 2; Group 3, representing colon cancer TNM stage 3; and Group 4, representing colon cancer TNM stage 4.*

the analyzed expression in colonocytes, which avoids the variation of the results due to the variability of the housekeeping gene in each sample, especially in experiments that employ different treatments (46, 52).

Results for the expression of the 20 microRNA genes in stool are in general agreement with what has been reported in the literature for the expression of these miRNAs in tissue, blood and cells in culture (119-128). Thus, these data indicate that the choice of carefully selected miRNAs can distinguish between non-colon from colon cancer, and can even separate different TNM stages. A miRNA expression index similar to that developed for mRNA (129), or a complicate multivariate statistical analysis (130) was, therefore, not necessary in order to reach conclusions from these data.

The data presented in Figure 6 using a multigroup plot, and Figure 7 employing volcano plots exhibits minimal variance within groups resulting in low *p*-values calculated using $2^{(-dCT)}$ (SD of 0.015275 or 0.025166 is minimal, or raw CT values is only ~0.03 for three replicates). The 95% CT for group 4 was between 134.39 and 135.63, indication a slight variation between groups. However, because the raw CT variations are low, even the slightest changes resulted in significant *p*-values; for example, miR-193a-5p was induced in different groups by between two to 134-fold.

PCR data are presented in Table II and for stool are shown graphically in a scatter plot in Figure 8, as there was been no need to use receiver operating characteristic (ROC) curves because the difference in miRNA expression between healthy individuals and patients with colon cancer, and among stages of cancer was large and informative. The data show that the expression of 12 miRNAs (miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106a, miR-134, miR-183, miR-196a, miR-199a-3p and miR214) increased in stool of patients with colon cancer, and that later TNM stages exhibited a greater increase than did adenomas. On the other hand, expression of eight miRNAs (miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR-146a, miR-222 and miR-938) was decreased in stool of patients with colon cancer that became more pronounced from early to later TNM stages. Naturally this initial number of miRNA genes (20) can be refined by a larger validation study.

It should be emphasized from our data that the difference between healthy and cancer groups is several times the variability, and several times the range of the observations found within groups. When the measurements are that different between groups, when compared with the variability within groups, then sensitivity, specificity, receiver operating characteristic (ROC) analysis, and power sample/size calculations, are therefore not necessary or meaningful. As an example, the data presented can be compared to those which would be obtained from a group of students where half are first graders and the other half are high school students (although we have considered more groups, the idea can still be considered for just two groups). To separate these groups, we would use height as a measurement (in our case we used gene expression). It turns out that the shortest high school student is much more taller than the tallest first grader and all those taller are high school students. Specificity, sensitivity and area under the curve are all 100%. When we use weight (in our case, different expression) we get the same results: the lightest high school

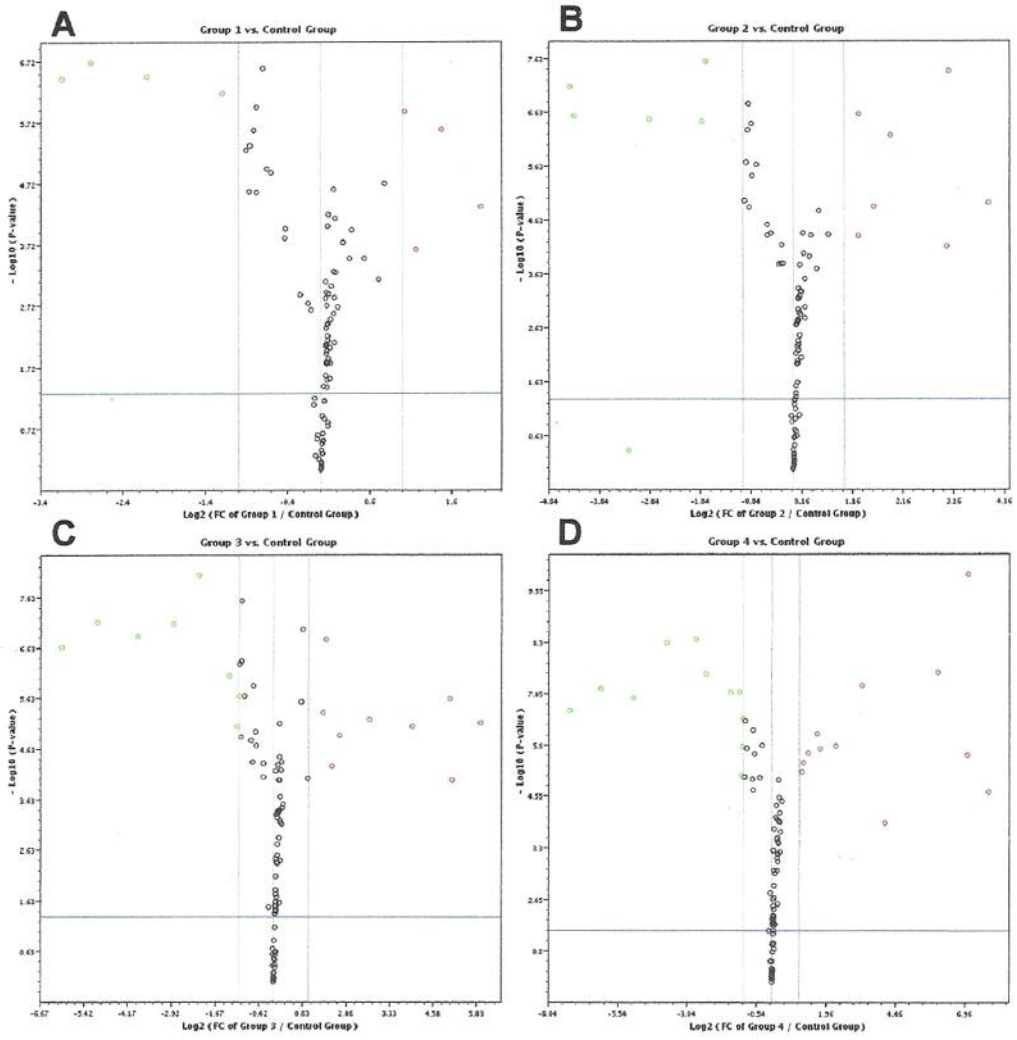

Figure 7. *Volcano plot depiction of quantification of mature microRNAs by a Stem-loop RT, TaqMan® MGB probes qPCR miRNA expression analysis of 60 human stool samples using a Qiagen Corporation program [http://pcrdataanalysis.sabiosciences/com] for colon cancer TNM stages 0-I, II, III and IV versus control in plots A-D, respectively.*

student is much heavier than the heaviest first-grader. We can use other measures, such as shoe size or reading level, and again we would obtain the same result.

Bioinformatics analysis using the TargetScan algorithm (73) for up-regulated and down-regulated mRNAs genes is shown in Table V. The program yielded 21 mRNA genes encoding different cell regulatory functions. The first 12 of these mRNAs were found with the DAVID program (131) to be active in the nucleus and related to transcriptional control of gene regulation. For down-regulated miRNAs, the DAVID algorithm found the first four of these mRNAs to be clustered in cell cycle regulation categories (Table V).

Test performance characteristics of the miRNA approach obtained by the CP values of the miRNA genes calculated from stool samples of healthy individuals and patients with colon cancer showed high correlation with colonoscopy results obtained from patients' medical records for the 20 controls, 40 patients studied.

Cytological methods carried out on purified colonocytes employing Giemsa staining (68) as described for CRC, showed a sensitivity for detecting tumor cells in smears comparable to that found in biopsy specimens (*i.e.* 78.1% *versus* 83.66%) (69), which is comparable to our method on purified colonocytes that employed Giemsa staining. A sensitivity for detecting tumor cells in stool smears of 80% was obtained by us, which is slightly better than that reported earlier (*i.e.* about 78%) (69). Isolation of colonocytes from a known amount of stool, then comparing the expression profile to that obtained from total RNA extracted from whole stool could be construed as a validation that the pattern observed in stool is truly due to the presence of tumor cells therein. We

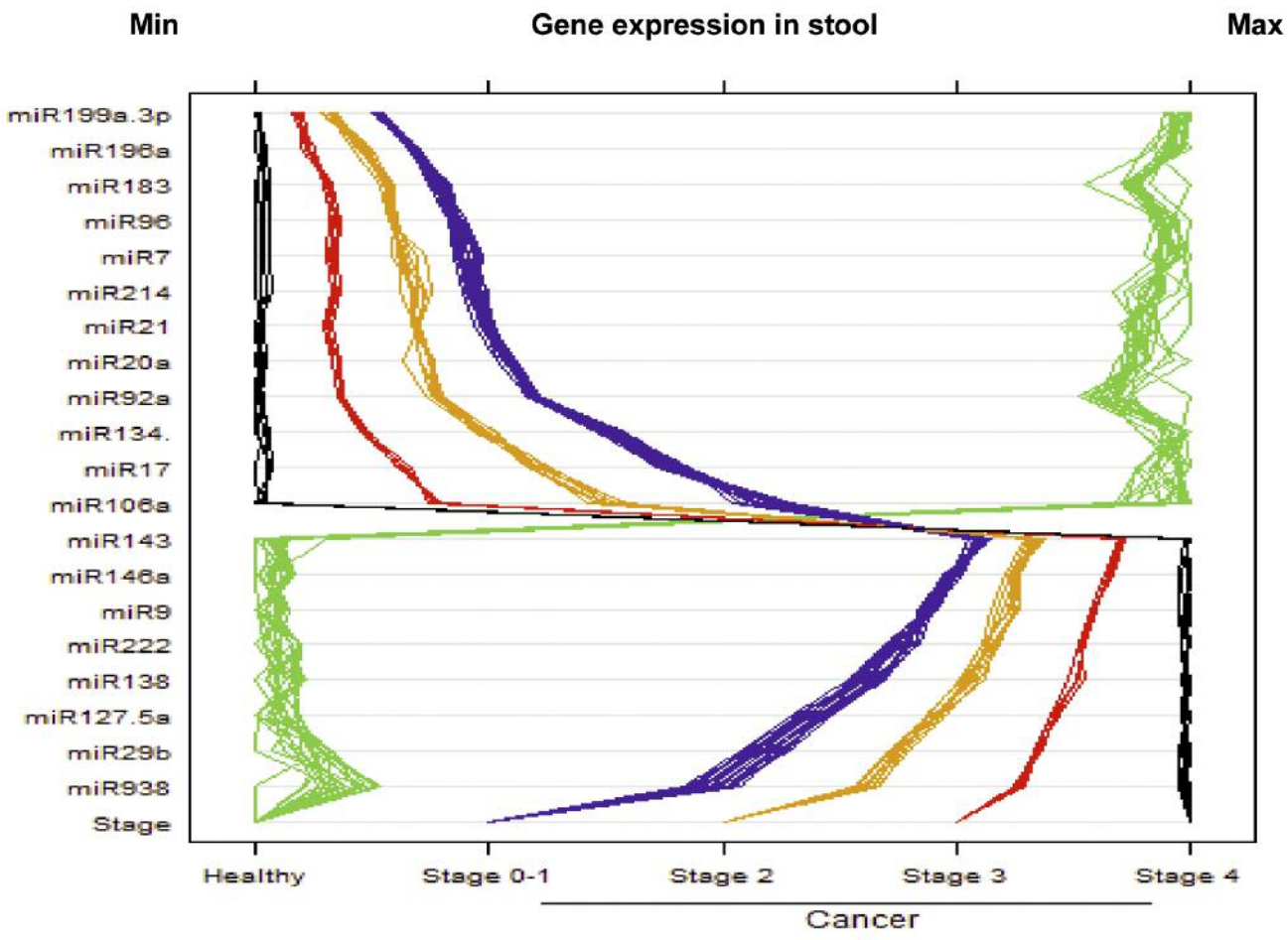

Figure 8. *MicroRNA expression in stool samples obtained from 60 individuals. The stage of cancer is indicated by the bottom row of the panel. There were 20 healthy individuals (green), and 40 with colon cancer (TNM stages 0 to 4) represented by stage 0-1 (purple), 2 (gold), 3 (red) and 4 (black) lines, respectively. Instances of high expression appear on the right (green) and those with low expression on the left (black). Expression by stem-loop RT-minor grove binding qPCR was measured by the comparative cross point (CP) or the E-method on a Roche LightCycler® 480 PCR instrument. Scales were chosen so the minimum values line up on the Min mark labeled at top left of the panel. The same is true for the maximum values, which line up under the mark labeled Max at top right of the panel.*

have also take into account that some exsosomal RNA (132) will not be released from purified colonocytes into stool, and arbitrarily corrected for that effect.

In conclusion, our results show that several miRNA genes can be used to discriminate non-invasive healthy individuals from patients with colon cancer. It would, however, be necessary to conduct a prospective randomized validation study using the methods that we have outlined herein, but on larger number of individuals to have a statistical confidence in data outcome.

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