Diagnostic MicroRNA Markers to Screen for Sporadic Human Colon Cancer in Blood

FARID E. AHMED[1,2], NANCY C. AMED[2], PAUL W. VOS[3], CHRIS BONNERUP[4], JAMES N. ATKINS[5], MICHELLE CASEY[5], GERARD J. NUOVO[6], WADE NAZIRI[7], JOHN E. WILEY[8] and RON R. ALLISON[1#]

Departments of [1]Radiation Oncology, Leo W. Jenkins Cancer Center and [8]Pediatrics-Genetics, The Brody School of Medicine at East Carolina University (ECU), Greenville, NC, U.S.A.; [2]GEM Tox Labs, Greenville, NC, U.S.A.; Departments of [3]Biostatistics, School of Allied Health Sciences, ECU and [4]Physics, School of Arts and Sciences, ECU, Greenville, NC, U.S.A.; [5]Southestern Medical Oncology Center, Goldsboro, NC, U.S.A.; [6]Department of Pathology, The Ohio State University Medical Center, Columbus, OH, U.S.A.; [7]Southern Surgical, Greenville, NC, U.S.A.

Abstract. *We carried out this study to present proof-of-principal application, showing that by using a global microarray expression analysis, followed by quantitative stem-loop reverse transcriptase in conjunction with TaqMan® polymerase chain reaction (PCR) analysis of micro(mi)RNA genes, on limited number of plasma and tissue samples obtained from 20 individuals (five healthy, five TNM stage 0-1 colon cancer, five stage 2 and five stage 3), we were able to quantitatively monitor miRNA changes at the various TNM stages of colon cancer progression, particularly at the early, pre-malignant adenoma stage (e.g. polyps ≥1 cm with high grade dysplasia). The expression of some of the tested miRNAs showed less variability in tissue than in plasma. Nevertheless, our limited preliminary data on the plasma by itself show that plasma is well-suited for screening, and that the quantitative changes in the expression of a few cell-free circulatory mature miRNA molecules in plasma, that are associated with colon cancer progression, would provide for more sensitive and specific markers than those tests currently available on the market. In addition, analysis of miRNA molecules offers a quantitative and cost-effective non-invasive diagnostic approach for screening, than currently employed methods in a prevalent cancer that can be cured if it is detected at the early TNM stages, and that becomes deadly if not diagnosed before metastasis. Thus, a larger prospective and properly randomized clinical study using plasma derived from many control individuals and at various stages of colon cancer (TNM stages 0-IV) from patients, in order to corroborate the initial results, is now urgently needed in order to allow for a statistically valid analysis, standardizing test conditions which will provide a means for determining the true sensitivity and specificity of a miRNA-screening approach. This approach, when combined with bioinformatics analysis to correlate miRNA seed data with mRNA target data, would allow for a mechanistic understanding of how miRNAs regulate mRNA gene expression, and would offer a better comprehensive diagnostic screening test for early-detection of colon cancer non-invasively.*

Abbreviations: AJCC, American Joint Committee on Cancer; cDNA, copy deoxyribonucleic acid; CRC, colorectal cancer; E-method, also referred to as second derivative maximum or CP method; FOBT, fecal occult blood test, GI, gastrointestinal; LCM, laser capture microdissection; LSD, least significant difference; MGM, minor groove binding; mRNA, messenger ribonucleic acid; QC, quality control; rRNA, ribosomal ribonucleic acid; 3'UTR, untranslated 3' region of target mRNA; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction, ss, single-stranded; TNM, tumor-lymph node metastasis; UICC, International Union Against Cancer.

[#]Current Address: Carolina Radiation Medicine 21st Century Oncology, 801 W.H. Smith Blvd., Greenville, NC 27834, U.S.A.

Correspondence to: Dr. Farid E. Ahmed, Director, Institute for Research in Biotechnology, GEM Tox Labs, 2607 Calvin Way, Greenville, NC 27834, U.S.A. (Current Address). Tel: +1 2523217261, Fax: +1 2527561656, e-mail: gemtoxconsultants@yahoo.com

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Mortality and morbidity from colorectal (CRC) represent a major health problem involving a malignant disease that is theoretically preventable through screening and early detection. Screening for CRC allows early-stage diagnosis of the malignancy and potentially reduces disease mortality (1). The convenient and inexpensive fecal occult blood (FOBT) screening test has low sensitivity and requires dietary restriction, which impedes compliance and use (2). CRC is

Table I. *Comparison of tests to be employed for pre-malignant† and malignant colon cancer screening.*

Test specification	FOBT[a] Guaiac Immunol.		Methylated genes[b] & chromosomal loci	Promoter[c] methylation	Mutated DNA[d] markers	Colonoscopy examination[e]	Proteomic-based[f]	mRNA-based[g]	MiRNA-based[h]
Non-invasive	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
Sensitivity	10.8%†	16.3%†	87.5%	31%†	18.2%	87%	75%	>80%	>90%
Specificity	95%	94.5%	82%	95%	94.4	100%	>95%	>95%	>95%
Automation	No	No	No	No	No	No	Yes	Yes	Yes
Cost[i]	$15♪	$25	$400	$150	695	$900	$650	$250	$200

†For polyps ≥1 cm in diameter. FOBT, fetal occult blood test. [a]From reference 30; [b]Only for advanced cancer, but not adenoma, based on vimentin gene and DY loci 5p21 & LOC91199, from references (31,32); [c]Based on one gene, from reference (33). From references [d](34-36); [e](10,11); [f](37); [g](66). [h]Based on our data, as presented herein; [i]Cost estimates are based on contacts with other test developers, and our experience with assay developments and requirements. Results based on a limited number of genes and a small sample size.

the only cancer for which colonoscopy is recommended as a screening test (3). Although colonoscopy is a reliable screening tool, its invasive nature, resulting in abdominal pain and high cost have hampered worldwide application of this procedure (4). Early detection would be greatly enhanced if accurate, practical and cost-effective diagnostic biomarkers for this malignancy were available. In comparison to the commonly employed FOBT stool tests, a non-invasive plasma test –as proposed herein- would be more convenient as there would be no requirement for dietary restriction, or meticulous collection of stool samples (5), and thus this proposed new screening test would be more acceptable to a broader population.

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 a small minority of adenomas are destined to progress to malignancy, their detection would involve gross overtreatment of patients, which would be both costly and harmful. An optimal CRC screening test would be one that accurately detects advanced adenomas carrying a high chance of malignant progression (6-8). 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, 9, 10). Given the desirability of using a non-invasive test acceptable to the target population as an initial screen, investigators have resorted to developing molecular diagnostic approaches.

The discovery of small noncoding protein sequences, 17-27 nucleotide-long microRNAs (miRNAs), has opened new opportunities for a non-invasive test for the early diagnosis of many types of cancer. miRNAs regulate cell processes in ~30% of mammalian genes by imperfectly binding to the 3' untranslated region (UTR) of target mRNAs, resulting in prevention of protein accumulation by either transcriptional repression, or by inducing mRNA degradation (11). Many miRNAs are thought to target hundreds of conserved mRNAs and several hundreds of nonconserved targets. Thus, miRNAs operate in a complex regulatory network, and it is predicted that miRNAs together regulate thousands of human genes (12).

miRNA functions seem to regulate development (13) and apoptosis (14), and specific miRNAs are critical in cancer initiation and metastasis (15), effective in classifying solid (16-20) and liquid tumors (21,22), and serve as oncogenes or suppressor genes (23). Genes for miRNAs 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 (24). miRNAs have promise as biomarkers for cancer diagnosis, prognosis and even response to therapy (11, 21, 25-27). 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 (28). There are ~1,898 human, 1,141 mouse and 679 rat miRNAs in Sanger *miRBase*, release 18, Nov 2011, http://microrna.sanger.ac.uk/sequences (29).

Table I presents a comparison of tests that are currently in use (30-37), or those under development for CRC screening, with respect to their invasiveness, sensitivity, specificity, ease of automation and use, as well as cost.

The aims of this study were to show that miRNA molecules can serve as screening markers for diagnosing colon cancer in patients' blood at an early pre-neoplastic stage before the disease metastasizes and the cancer becomes incurable.

Materials and Methods

Acquisition of clinical specimens. Blood and tissue samples were obtained from five consenting control individuals and 15 patients with various stages of colon adenocarcinoma [tumor-lymph node metastasis (TNM) stages 0 to 3] (38, 39), according to an

Institutional Review Board (IRB) protocol approved by the U.S. Department of Health and Human Services (DHHS). All laboratory work was carried out and standardized under blind conditions, and followed the guidelines for handling biohazardous material established by the GEM Tox Labs Biological Safety and Hazardous Substances' standard operating procedures (SOPs).

Tissue and blood samples from controls and patients with colon cancer. (i) Tissue specimens: Normal tissues were either obtained from a small piece of colon tissue (about 0.5 cm^3) removed >10 cm away from diseased tissue at surgery (40), or from biopsies taken during colonoscopy from non-diseased areas of consenting individuals. Tissues were flash-frozen in liquid nitrogen and stored at –80˚C for subsequent laser capture microdissection (LCM) work, as detailed earlier (41). Longitudinal sectioning of the tissue before LCM use, was employed to pick up epithelial cells that would eventually be shed as colonocytes into the *lamina propria* from the bottom epithelial cells among the proliferative enterocyte crypt lineage (42).

For CRC tissue, the areas of the crypt that indicated where the transformed cells are dysplastic (*i.e*. adenoma, carcinoma), which were to be captured by an Arcturus II LCM instrument (see Figure 1), were picked-up on a nylon membrane for subsequent RNA extraction, as detailed elsewhere (41-44).

(ii) Blood samples. Blood was collected in 4.5-ml vacutainers containing buffered sodium citrate, 0.105 mol/l (Becton Dickinson, Franklin Lakes, NJ, USA) from control or CRC, affected individuals visiting our participating gastroenterology (GI) offices/surgery clinics/endoscopy labs, who did not have any polyps or inflammatory bowel diseases, such as colitis or diverticulitis, according to rigid exclusion criteria (Table II). Blood was transferred to a polypropylene tube, centrifuged at 16,000 ×*g* for 10 min at 4˚C, and the top plasma layer was removed and stored at –80˚C until needed for total RNA extraction.

Manual extraction of total RNA from LCM cells and blood, and single-stranded (ss)-cDNA preparation. High quality undegraded total RNA was extracted from 500 µl of plasma, or 10,000 LCM colon cells from the same individuals, using Trizol LS® reagent (Invitrogen, Life Technologies, Grand Island, NY, USA) and the RNeasy Isolation kit® (Qiagen Corporation, Valencia, CA, USA), as we reported elsewhere (41, 43, 44). The purity of total RNA was measured spectrophotometrically at λ 260 nm and 280 nm. The intensity of RNA was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Palo Alto, CA, USA), utilizing the RNA 6000 Nano LabChip®. The Sensiscript RT Kit® from Qiagen was then employed for making a copy of ss-DNA, resulting in 40 µl of ss-cDNA, of which 2-3 µl were subsequently amplified by polymerase chain reaction (PCR). Concentrations of RNA samples quantified spectrophotometrically averaged from 15 to 20 ng/µl for plasma, and 30 to 40 ng/µl for LCM colon mucosa.

We used 50 nM sequence-specific stem-loop reverse transcriptase (RT) primers designed to anneal to the 3'-end of a mature miRNA, 1× RT buffer, 0.25 mM of dNTPs and 3.33 U/µl MultiScribe™ reverse transcriptase (all from Applied Biosystems, Foster City, CA, USA) to convert total RNA into cDNA suitable for miRNA analysis, as described earlier (44, 45).

Microarray profiling of MicroRNAs. 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 universal miRNA coverage. The microarray contains 16,772 entries representing hairpin precursor, expressing 19,724 mature miRNA products in 153 species, and provides >3 *log* dynamic range, with 95% reproducibility and 85% transcript detection at 1.0 amol for a total RNA input of 100 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), 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 1x 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 (46). Thresholding and signal scaling was generated using appropriate algorithms (47). The background adjusted fluorescent values generated by the scanner were normalized for each miRNA

Table II. *Study inclusion/exclusion criteria. In this study, which aimed to develop microRNA markers in plasma to screen for colon cancer in humans, enrolled individuals were from both genders, ages 18-80 years, who were either patients with colon cancer at any TNM stage of the disease (0-IV) or controls without disease, without signs of diarrhea, inflammatory bowel diseases (IBDs) or hepatitis, and who were willing to donate 4 cc of blood.*

Parameter	Inclusion	Exclusion
Gender	Male and female	None
Age	18-80 years	None
National origin	All nationalities	None
Race	All races	None
Organ	Colon	Rectum
Disease	Any stage of colon cancer	Diarrhea
	Hypertension	HIV
	Chronic heart diseases	IBD, Crohn's disease
	Sexually-transmitted diseases	Hepatitis
	Obesity	
	Diabetes	
	Arthritis	
	Diverticulitis	
	Tuberculosis	
	Common cold	
	Atherosclerosis	
	Alzheimer disease	
	Chronic renal disease	
	Chronic obstructive	
	Pulmonary disease	

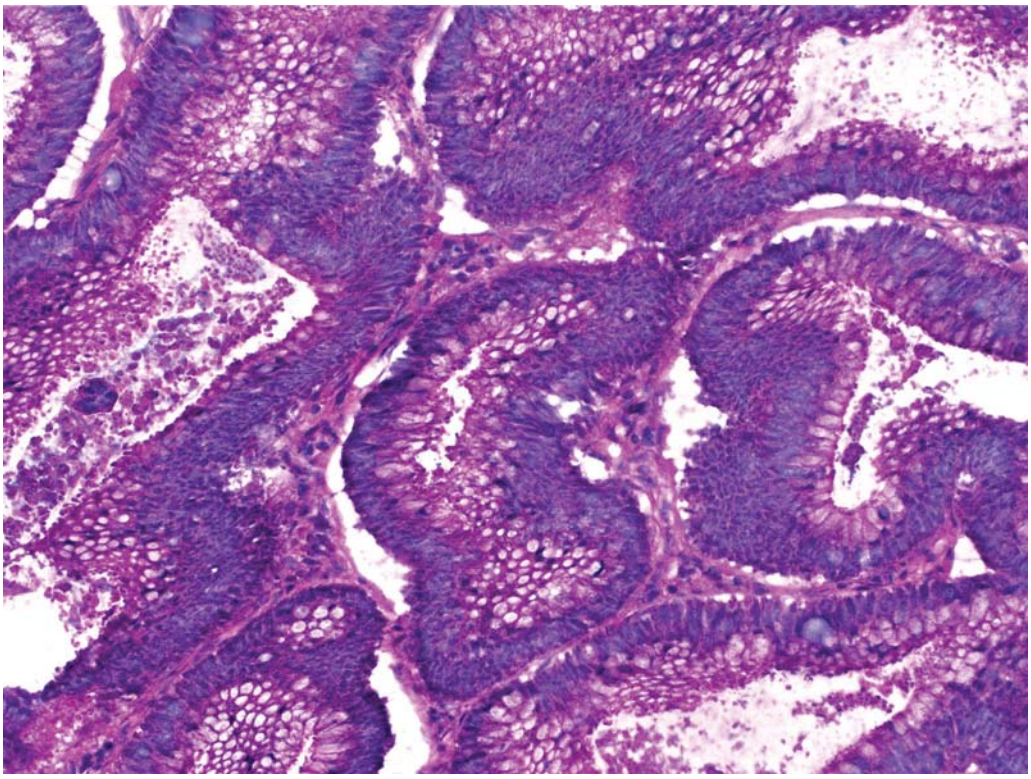

Figure 1. *Longitudinal hematoxylin and eosin (H&E) cryostat section of colon adenoma exhibiting high-grade dysplasia (i.e. carcinoma in situ, stage 0), ×40 magnification.*

using a variation stabilization transformation method such as cyclic LOWESS (48), as detailed before in reference (47).

Hypothetical testing with one-way analysis of variance (ANOVA) or Student's *t*-test (48) was employed for statistical analysis of miRNA array. *p*-Values <0.05 were considered significant. Reproducibility and linearity were evaluated using ANOVA or Pearson correlation coefficients (49, 50).

Pair-wise comparisons were carried out on differentially expressed genes identified by ANOVA. 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, referred to as protected least significant difference, LSD (51). 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 described elsewhere (44, 47, 52).

Real-time PCR and relative quantification of mature miRNAs employing the TaqMan® minor groove binding (MGB) probes. Although miRNAs represent a relatively abundant class of transcripts, their expression levels vary greatly among species and tissues (11). Less abundant miRNAs routinely escape detection with technologies such as cloning, northern hybridization and microarray analysis. Low sensitivity becomes a problem for miRNA quantification because it is difficult to amplify these short RNA targets. Furthermore, low specificity may lead to false-positive signals from closely related miRNAs, precursors and genomic sequences (53). A modified TaqMan®-based quantitative real-time PCR assay was designed, incorporating 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. Then the RT product is quantified using conventional TaqMan PCR that includes miRNA-specific forward primer, reverse primer and a dye-labeled hydrolysis TaqMan probes. The purpose of the tailed forward primer at 5' is to increase its melting temperature (T_m) depending on the sequence composition of miRNA molecules (45). 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. Moreover, base stacking 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. 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 (45). The TaqMan minor groove binding (MGB) probes are 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 are designed to have a 5' 6-carboxyfluorescein (FAM) probe and a 3' MGB probe (53).

This assay is specific for mature miRNAs and discriminates among related miRNAs that differ by as little as one nucleotide, and is not affected by genomic DNA contamination (45). miRNA stem-

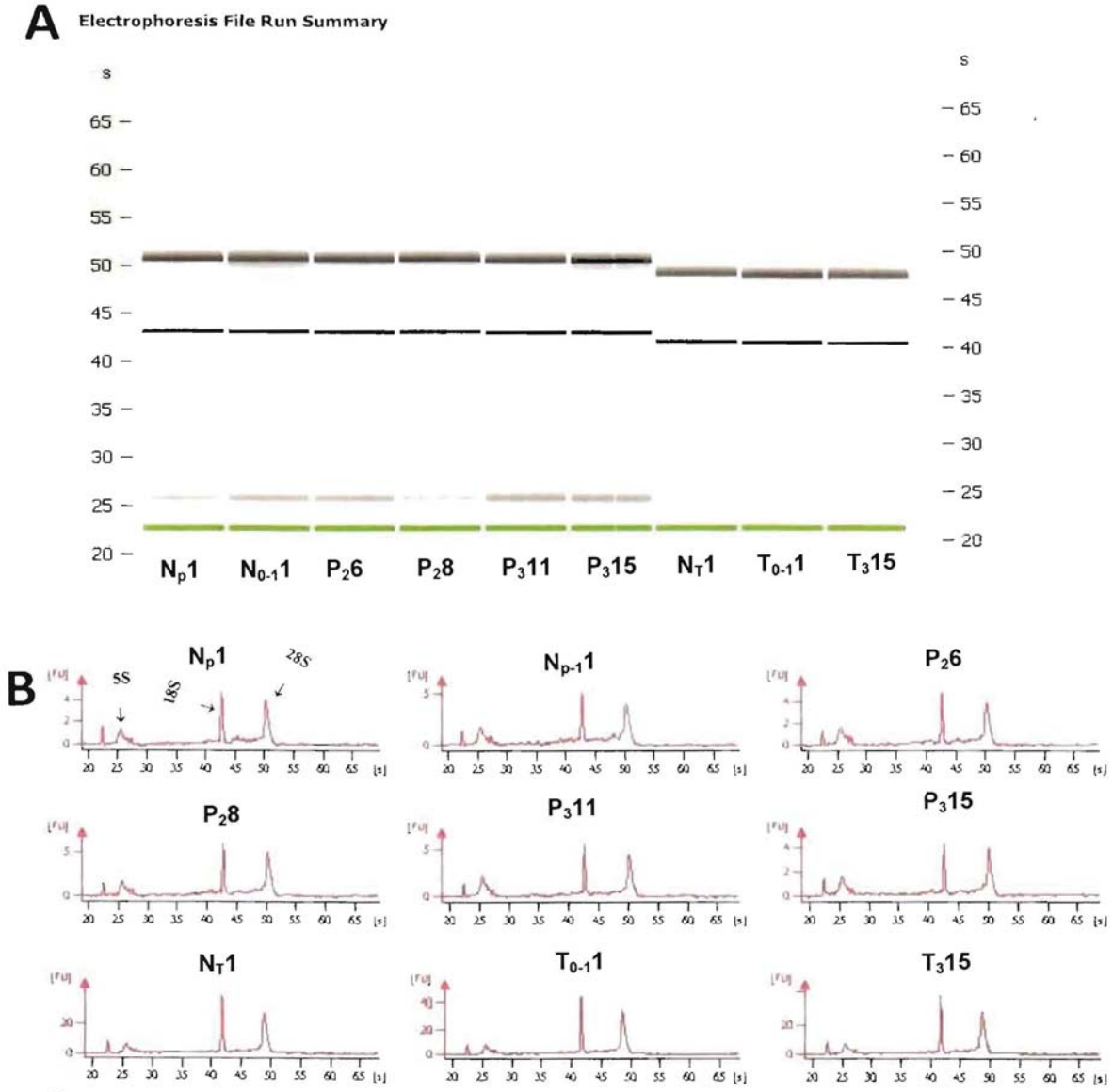

Figure 2. *A: Agilent 2100 gel showing representative plasma and laser capture microdissection (LCM) tissue samples showing non-degraded RNA from nine individuals who are either healthy, or with various TNM stages of colon cancer. Np1, Plasma from a healthy individual; N_T1, tissue from a healthy individual; $P_{0-1}1$, plasma from a patient with adenomatous colon polyp 1 cm (stage 0-1); P_22, plasma from a patient with colon carcinoma (stage 2); P_33, plasma from a patient with colon carcinoma patient (stage 3); $T_{0-1}1$, tissue from a patient with adenomatous colon polyp of 1 cm (stage 0-1); T_22, tissue from a patient with colon carcinoma (stage 2); T_33a, tissue from a patient with colon carcinoma (stage 3); and T_33b, tissue from another patient with colon carcinoma (stage 3). B: Agilent 2100 electrophoretograms showing the 28S, 18S and tRNA, 5.8S and 5S bands from the same samples, as shown in panel A.*

loop RT primers for specific miRNA species to be tested, together with probes having an MGB with non-fluorescence quencher at the 3' -end and the fluorescence dye FAM at the 5' -end, were obtained from Applied Biosystems (Foster City, CA, USA) for the following fifteen miRNAs: miRNA-7, miRNA-17-3p, miRNA-20a, miRNA-21, miRNA-92a, miRNA-96, miRNA-124, miRNA-127-3p, miRNA-138, miRNA-143, miRNA-146a, miRNA-183, miRNA-196a, miRNA-214, and miRNA-222 (Table III). The reference housekeeping pseudogene-free ribosomal gene (*18S* rRNA) that showed weak expression variations (54) was employed as a normalization standard for relative PCR quantification in plasma and LCM tissue obtained from the tested 20 control individuals and colon cancer subjects, at various TNM stages (38, 39).

The 10 μl PCR reaction included 0.67 μl RT product (representing ~1 nm total RNA), 1× 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 s and 60˚C for 1 min. All reactions were run in

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

Overexpressed	Underexpressed	Overexpressed	Underexpressed
miR-7	miR-1	miR-103	miR-342
Let 7a	miR-7i	miR-106a	miR-363
let7b	let-7i	miR-106b	miR-387
let 7c	miR-9	miR-107	miR-407
let 7e	miR-15a	miR-124	miR-424
let-7g	miR-18b	miR-125a	miR-455
let-7f	miR-24a	miR-125b	miR-484
milet7d	miR-27a	miR-126	miR-522
miR-10b	miR-27b	miR-127	miR-650
miR-15b	miR-29a	miR-130a	miR-661
miR-16	miR-29b	miR-130b	miR-132
miR-17-3p	miR-30e-3p	miR-134	miR-133a
miR-18a	miR-30c	miR-133b	miR-135a
miR-18b	miR-27a	miR-135-b	miR-137
miR-19-a	miR-34-5p	miR-139-3p	miR-135a
miR-19b	m iR-37	miR-135-b	miR-141b-
miR-20	miR-92a	miR-146-5p	miR-148a
miR-20a	miR-93	miR-150	miR-153
miR-20b	miR-95	miR-181a	miR-181b
miR-17	miR-125a	miR-181-c	miR-181-d
miR-18-a	miR-126	miR-182	miR-183-
miR-19a-b	miR-127-5p	miR-184	miR-185
miR-20a	miR-128b	mIR-191	miR-192
miR-21	miR-133a	miR-196a	miR-196b
miR-24	miR-142-5p	miR-199a-3p	miR-200a
miR-25	miR-133a	miR-200b	miR-200c
miR-26a	miR-138	miR-202	miR-203
miR-31	miR-142-5p	miR-204	miR-205
miR-40-5p	miR-143	miR-206	miR-210
miR-29b	miR-145	miR-211	m iR-212
miR-30-a	miR-146a	miR-214	miR-219
miR-30c	miR-148b	miR-220	miR-221
miR-31	miR-150	miR-222	miR-223
miR-32	miR-191	miR-224	miR-210
miR-33a	miR-192	miR-301a	miR-302a
miR-34a	miR-193b	miR-302b	miR-320
miR-91	miR-195	miR-335	miR-338
miR-92a	miR-200b	miR-346	miR-355
miR-93	miR-200c	miR-370	miR-372
miR-95	miR-212	miR-373	miR-378
miR-96	miR-215	miR-387	miR-432
miR-99b	miR-222	miR-484	miR-492
miR-100	miR-223	miR-493-3p	miR-497
miR-101	miR-301	miR-550	miR-570

triplicate. Components for the assay were included in the TaqMan™ MicroRNA Reverse Transcription Kit, Universal Master Mix without UNG and TaqMan™ Assay, all obtained from Applied Biosystems.

The comparative cross point (CP) method, also called the E-method (55), was used for semi-quantitative PCR analysis on a Roche LightCycler (LC™), model 480 PCR instrument, utilizing the LC Relative Quantification Software™ (56). The method employs standard curves in which the relative target concentration is a function of the difference between crossing points (or cycle numbers) as calculated by the second derivative maximum, in which the Cycler's software algorithm (v4.0 software for the LC™) identifies the first turning point of the fluorescence curve in the graph showing fluorescence *versus* cycle number (56), which corresponds to the second derivative curve (57). The CP method was shown to produce more accurate results than the 2-ΔΔCt method (58), as it compensates for differences in target and reference gene amplification efficiency [$E=10^{-1/slope}$] (59), whether within an experiment, or between experiments (51). Normalization against a housekeeping internal standard or in some cases against several standards is customarily employed in this method (53, 54, 60).

In all PCR reactions, strict attention to quality control (QC) procedures was adhered to in order to ensure the uniformity, reproducibility and reliability of the PCR reaction (61, 24). The level of gene expression was displayed using parallel coordinate plots (65), produced by the lattice package in R (version 2.4.0, http://cran.r-project.org).

Statistical analyses. Because the difference in gene expression between healthy and cancer patients 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 were again not used.

Results

The purity of total RNA measured spectrophotometrically showed that the OD $260_{nm}/280_{nm}$ ratio was from 1.9 to 2.0, indicating that it was reasonably pure. Electrophoretic runs carried out on the Agilent Bioanalyzer showed that all total RNAs extracted from LCM and blood samples of normal or of various stages of colon cancer were intact and of human origin, making them suitable for high-quality miRNA determinations. Although there may be daily variations between runs, as shown by comparing the first six gels that were processed on one day with three other gels that were processed on another day (Figure 2A and B), the overall electrophoretogram patterns show that the 18S and 28S bands migrated to the same extent in all nine total RNA samples taken from blood or tissue. Moreover, the RNA integrity assessed on the Agilent 2100 BioAnalyzer had an RNA integrity number (RIN) >7.0, indicating high quality total RNA. We have shown previously that high-quality total RNA is needed to improve miRNA expression results (44, 52).

Global gene expression using microarrays showed that there were 176 preferentially expressed miRNA genes that were either increased (122 miRNAs), or reduced (54 miRNAs) in expression in colon cancer in plasma and in tissue samples (Table III).

A volcano plot of microarray data from plasma samples taken from five cancer patients (TNM stage 0-1), for [–log10 (*p*-value for colon cancer, stage 0-1)] *versus* [mean control –mean colon cancer] is shown in Figure 3. One third of the miRNAs had a *p*-value <0.05, and some of these molecules were further validated by qPCR.

Our stem-loop RT-MGB qPCR in plasma and tissue samples from healthy individuals and patients with colon cancer presented in Table IV show that out of the 15 selected miRNAs exhibiting preferential expression and which have been shown to be related to colon carcinogenesis; nine of them (miR-7, miR-17-3p, miR-20a, miR-21, miR-92a, miR-96, miR-183, miR196a and miR-214) exhibited increased expression in plasma (and also in tissues) of patients with CRC, and that later TNM carcinoma stages had a more increased expression than did adenomas. On the other hand, six of the selected miRNAs (miR-124, miR-127-5p, miR-138, miR-143, miR-146a and miR-222) were reduced in expression in plasma (and also in tissues) of patients with CRC, the reduction becoming more pronounced during progression from early to later TNM carcinoma stages. The data in Table IV are presented graphically in Figure 4 for 15 preferentially expressed miRNAs in plasma (Figure 4A) and tissue (Figure 4B) samples, obtained from 15 patients with cancer at TNM stages 0-3.

Discussion

Using stable molecules, such as miRNAs, which are not easily degradable when extracted from blood and manipulated thereafter, a miRNA approach is preferable to a transcriptomic mRNA-, mutation DNA-, epigenetic- or a proteomic-based test (7, 31-37, 44-46). If performance criteria are met, as proposed in this undertaking, the non-invasive miRNA-based test in plasma based on high-throughput automated technologies and quantitative expression measurements, commonly used nowadays in diagnostic clinical laboratories, would offer an advancement to the clinical setting and would have a significant impact on the prevention of CRC.

In developing countries where CRC is on the rise due to the adoption of Western diet (63), the problem is more severe, as the cost of colonoscopy often exceeds a person's yearly salary, and there are not enough trained personnel or adequate centers to perform these tests (6). We are of the opinion that an approach utilizing miRNA in plasma could meet the criteria for test acceptability as it is non-invasive, requires at most <2 ml of blood (60% of which is plasma), does not need sampling on consecutive dates, can be sent by mail in cold packs, is able to differentiate between healthy individuals and those with colon adenoma/carcinoma as shown in our studies, has high sensitivity and specificity for detecting advanced polyps, and can be automated, which makes it relatively inexpensive and more suited for early interfering clotting products present in serum. miRNAs are stable in plasma, and only 500 μl of plasma are required to perform the assay using commercially available kits. The availability of powerful approaches for global miRNA characterization such as microarrays and simple, universally applicable assays for quantification of miRNA expression such as qPCR suggests that the validation pipeline, which often encounters bottlenecks (64), will be more efficient for this assay. There is a pressing need for discovering non-invasive sensitive and stable molecular markers, such as miRNAs, to improve the detection of CRC, particularly at early disease stages (0-I), before the cancer metastasizes and becomes incurable.

A recent study examined the global expression of 735 miRNAs in 315 samples of normal colonic mucosa, tubulovillus adenomas, adenocarcinomas proficient in DNA

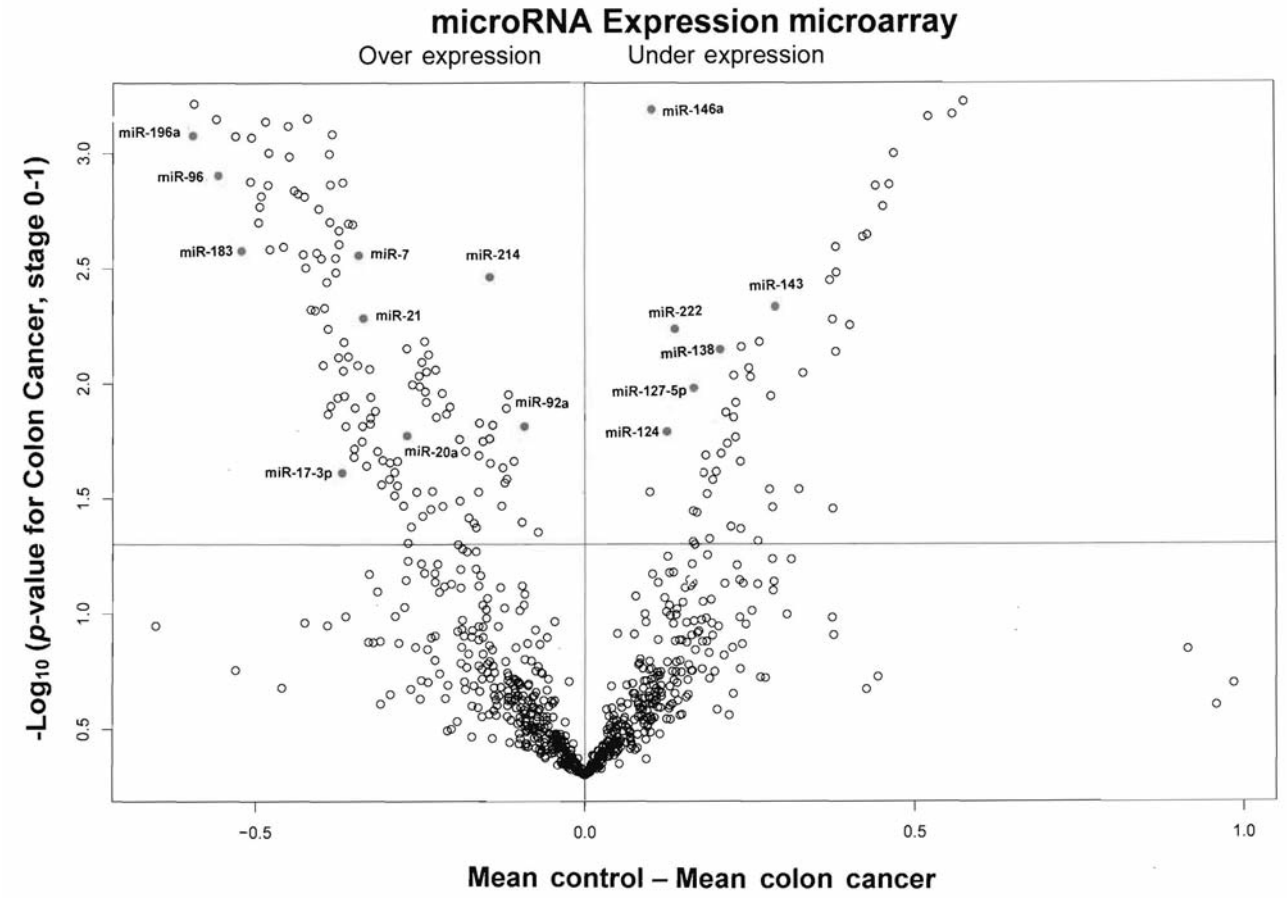

Figure 3. *A: A pairwise volcano plot for miRNA gene expression in plasma samples taken from five patients with colon cancer (TNM stage 0-1), using Affymetrix Gene Chip Micro 3.0 Array for [–log$_{10}$ (p-value for colon cancer, stage 0-1)] versus [mean control –mean colon cancer] in plasma. miRNAs above the horizontal line and to the left and right of the vertical line exhibited over- and underexpression, respectively. The 15 miRNAs that showed differential expression (9 increased and 6 reduced) are indicated on the plot.*

mismatch repair (pMMR), and defective in DNA mismatch repair (dMMR) representing sporadic and inherited CRC stages I-IV (26). Results showed that: a) the majority of miRNAs that were differentially expressed in normal tissue and polyps (miR-1, miR-9, miR-31, miR-99a, miR-135b and miR-137) were also differentially expressed at a similar magnitude in normal *versus* both the pMMR and dMMR tumors; b) all but one miRNA (miR-99a) demonstrated similar expression differences in normal tissue *versus* carcinoma, suggesting a stepwise progression from normal colon to carcinoma, and that early tumoral changes were important in both the pMMR- and dMMR-derived carcinomas, c) several of these miRNAs were linked to pathways identified for colon cancer, including adenomatous polyposis coli *(APC)*/pathway for embryogenesis and cancer (*WNT*) signaling and an oncogene known to be translocated in Burkitt's lymphoma (*cMYC*), and d) four miRNAs (miR-31, miR-224, miR-552 and miR-592) showed significant expression differences (≥2 fold changes) between pMMR and dMMR tumors. The data suggest involvement of common biological pathways in pMMR and dMMR tumors in spite of the presence of numerous molecular differences between them, including differences at the miRNA level (65).

Another study that looked at the expression of a panel of 95 cancer-related miRNAs by a real-time PCR-based array, reported that five miRNAs (miR-17-3p, miR-92, miR-95, miR-135n and miR-222) were up-regulated in plasma, as well as in tissue of all CRC Dukes' stages. Validation of the miRNA data indicated that miR-17-3p and miR-92 molecules were particularly elevated in the plasma obtained from 45 patients with CRC (5).

Since 1999, tumor-associated RNAs, and more recently, freely-circulating miRNAs, have been detected in serum or plasma of patients with CRC (43, 66-70). There are, however, discrepancies among the different studies, partially due to the heterogeneity between the different series

Table IV. *Stem-loop RT-TaqMan® MGB qPCR miRNA CP expression in plasma and in tissue of healthy and colon cancer patients*

Stool sample	18S rRNA stnd	miR 21	miR 214	miR 96	miR 196a	miR 20a	miR 183	miR 7	miR 17-3p	miR 92a	miR 146a	miR 222	miR 138	miR 143	miR 127-5p	miR 124
Neg Ct	26.28	25.92	25.86	25.66	26.04	26.14	25.56	26.08	26.08	26.04	25.78	25.68	26.12	26.04	25.94	26.02
N_p1	26.44	26.08	25.66	25.90	26.06	26.27	26.02	25.86	26.02	25.94	25.90	26.02	25.88	25.98	25.88	25.88
N_p2	26.34	25.96	25.78	25.54	25.88	25.94	25.86	26.06	26.10	25.92	25.88	26.06	26.06	25.88	25.80	26.08
N_p3	26.16	26.04	25.80	26.48	26.04	26.02	26.08	26.56	26.02	26.02	25.92	25.96	26.16	26.08	25.68	25.94
N_p4	25.98	25.84	25.94	25.90	25.96	25.88	25.92	25.80	25.88	25.88	26.06	26.02	25.86	26.02	26.08	25.78
N_p5	26.28	26.16	26.08	25.94	25.72	25.78	26.06	25.78	26.04	25.88	25.94	25.88	26.04	25.86	25.92	26.02
$P_{0-1}1$	26.02	16.52	15.66	12.44	11.80	18.06	12.70	14.56	20.02	17.02	32.90	31.88	31.98	32.20	29.04	27.62
$P_{0-1}2$	25.88	16.72	15.52	12.52	11.64	17.98	12.54	14.64	20.98	16.88	32.88	31.64	31.99	32.34	28.98	27.55
$P_{0-1}3$	25.36	16.88	15.18	12.32	11.72	18.08	12.82	14.42	20.08	16.94	32.97	31.73	32.17	32.46	29.14	27.46
$P_{0-1}4$	26.16	16.66	15.34	12.46	11.76	17.88	12.64	14.26	20.22	16.76	33.02	31.88	32.10	32.29	28.88	27.39
$P_{0-1}5$	26.33	16.74	15.22	12.32	11.68	17.79	12.54	14.38	20.11	16.89	33.06	32.02	32.18	32.63	28.79	27.58
P_26	25.44	15.66	14.46	11.20	10.80	16.24	11.60	13.44	19.88	15.96	33.96	32.74	32.86	33.26	31.24	29.66
P_27	25.74	15.48	14.62	11.46	10.82	16.44	11.76	13.22	19.96	16.04	33.93	32.55	33.07	33.40	31.16	29,55
P_28	26.02	15.26	14.70	11.32	10.78	16.50	11.56	13.36	19.90	15.88	33.88	32.46	33.14	33.39	31.20	29.60
P_29	26.36	15.36	14.58	11.26	10.62	16.40	11.70	13.32	19.98	16.02	33.98	32.22	33.25	33.43	31.34	29.57
P_210	25.31	15.12	14.62	11.36	10.66	16.36	11.62	13.16	19.76	15.98	33.92	32.46	33.10	33.59	31.18	29.70
P_311	25.64	13.46	13.16	10.44	9.66	15.08	10.72	12.46	18.80	14.74	34.96	33.90	34.06	34.36	32.50	31.31
P_312	26.12	13.50	13.33	10.12	9.40	14.96	10.62	12.30	18.72	14.66	34.96	33.86	34.02	34.42	32.33	31.20
P_313	26.42	13.62	13.52	10.26	9.50	15.04	10.58	12.56	18.84	14.88	35.02	33.70	33.98	34.56	32.29	31.16
P_314	26.08	13.84	13.44	10.36	9.12	15.10	10.70	12.44	18.68	14.46	34.98	33.76	33.96	34.39	32.38	31.22
P_315	26.12	13.70	13.10	10.04	9.36	14.89	10.56	12.36	18.78	14.36	35.04	33.84	34.04	34.46	32.41	31.33
N_T1	26.06	25.56	25.14	25.69	26.14	26.04	26.18	25.79	25.69	25.73	26.04	25.94	26.04	25.83	26.04	26.03
N_T2	26.11	26.16	25.28	26.77	26.02	25.79	25.77	25.91	25.90	25.77	26.11	26.03	25.91	26.05	25.77	25.92
N_T3	25.76	26.03	25.84	26.06	25.80	26.01	26.04	25.78	26.14	26.11	25.79	25.76	25.69	25.80	26.11	26.03
N_T4	26.03	25.76	26.16	25.88	26.11	26.16	25.81	26.11	26.08	25.66	25.88	26.14	26.09	25.66	26.01	26.10
N_T5	25.76	25.94	25.46	25.71	25.90	25.89	26.07	25.80	25.69	25.89	26.08	25.92	25.77	26.12	25.86	25.88
$T_{0-1}1$c	25.98	14.36	13.12	10.44	9.36	16.23	10.59	10.70	16.12	13.00	34.62	34.00	34.10	35.10	33.22	29.71
$T_{0-1}2$	26.04	14.22	13.08	10.38	9.30	16.21	10.64	10.74	16.23	13.06	34.73	33.98	34.18	35.08	32.16	29.59
$T_{0-1}3$	25.78	14.16	13.16	10.50	9.42	16.13	10.59	10.66	16.29	13.08	34.88	33.89	34.22	34.90	32.30	29.66
$T_{0-1}4$	25.88	14.02	13.22	10.40	9.34	16.10	10.63	10.69	16.22	13.02	34.80	33.90	34.33	35.02	32.12	29.73
$T_{0-1}5$	25.90	14.07	13.09	10.32	9.44	16.08	10.71	10.62	16.10	13.04	34.75	33.99	33.39	35.04	32.15	29.77
T_26[c]	26.04	13.18	11.66	9.36	8.55	15.30	9.52	9.66	15.04	11.62	35.84	34.59	35.92	36.55	34.00	31.50
T_27	25.88	13.92	11.54	9.44	8.41	15.21	9.61	9.72	15.00	11.66	35.91	34.65	35.96	36.64	33.90	31.56
T_28	25.82	13.86	11.40	9.52	8.39	15.19	9.66	9.76	15.06	11.53	35.96	34.71	35.88	36.59	33.94	31.67
T_29	25.88	13.62	11.45	9.44	8.50	15.17	9.71	9.69	15.12	11.29	35.80	34.75	35.90	36.70	33.89	31.69
T_210	26.02	13.78	11.58	9.38	8.40	15.25	9.55	9.65	15.08	11.20	35.89	34.92	35.92	36.66	35.15	31.71
T_311[c]	26.08	11.08	10.66	8.18	7.21	13.22	8.48	8.61	13.06	9.44	36.44	35.78	36.99	38.00	35.22	33.15
T_312	25.90	11.16	10.46	8.12	7.36	13.28	8.58	8.73	13.00	9.58	36.53	35.85	36.97	37.94	35.34	33.29
T_313	26.04	11.20	10.62	8.14	7.30	13.32	8.62	8.77	13.10	9.61	36.61	35.90	37.04	37.87	35.30	33.26
T_314	25.69	11.14	10.40	8.10	7.33	13.40	8.44	8.66	13.04	9.48	36.59	35.93	37.11	37.80	35.39	33.34
T_315	26.08	11.22	10.38	8.13	7.41	13.36	8.51	8.69	13.02	9.54	36.59	35.88	37.08	37.89	35.40	33.38

CP: Comparative crossing point or (E-value), a value of test miRNA equal to the normalization standard indicates similar expression, a value lower than the standard indicates increased expression, and a value greater than the standard indicates reduced expression. Neg Ct: No DNA was added to the PCR reaction (negative control). All PCR reactions were run in triplicates and then averaged. The upper table was obtained from plasma of 20 individuals: 5 non-cancerous controls (Np1 to NS5); 5 patients with adenomatous polyp ≥1cm (stage 0-1), 5 patients with stage 2 (P26-P310) and 5 patients with stage 3 colon cancer (P311-P315). The lower table represents tissue of the same 20 individuals listed in the upper table, processed after flash-freezing in liquid nitrogen and storage at –80˚C for subsequent laser capture microdissection (LCM): 5 non-cancerous tissue controls (NT1 to NT5); 5 tissues with adenomatous polyp ≥1cm (stage 0-1), 5 patients' tissue with stage 2 (T26-T310), and 5 patients' tissue with stage 3 colon cancer (T311-T315). No RNALater® (Invitrogen, Carlsbad, CA) was used for preserving tissue samples because it often distorted tissue morphology causing artifacts and visualization problems when selecting target-cell populations in LCM processed tissue samples (43, 44, 66).

associated with tumor stage, tumor location, genetic background of the tumors, technical issues and different types of microarrays employed to study miRNA gene expressions.

A recent study confirmed that cell-free circulating mature miRNAs are stable in plasma and in cell culture media, and that extracellular miRNAs are predominantly exosome/microvessicle-free, and are associated with part of the RNA-

Figure 4. *MicroRNA expression in plasma (A), or tissue (B) samples taken from 20 individuals. The condition of the patient is indicated by the bottom row of the panel. There were 5 healthy patients and 15 with cancer (TNM stages 0 to 3). Instances of high expression appear on the right and those with low expression on the left. Expression by stem-loop RT-minor grove binding qPCR was measured by comparative cross point (CP) or E-method, and scales were chosen so that minimum values line up on the "Min" mark labeled at the top of the panel. The same is true for the maximum values which line up under the mark labeled "Max".*

induced silencing complex, (Ago2) protein (71). Most circulating miRNAs are found in lipid or lipoprotein complexes, such as apoptotic bodies, microvesicles or exosomes, and are therefore highly stable (72), permitting the use of stable miRNAs in the circulation as non-invasive reliable markers for CRC detection (5).

Unlike screening for large numbers of mRNA, a more modest number of miRNAs can be used to differentiate cancer from normal tissue, and unlike the fragile plasma mRNA, miRNAs in blood remain largely intact and stable for detection when handled with methods which do not lead to its contamination by fragmenting enzymes as those present in the hand, and in a standardized fashion (5, 71, 73). Therefore, we are of the opinion that miRNAs are better molecules to use for developing a reliable non-invasive screening for diagnostics, prognostic outcome, or response to therapy modality for CRC.

Out of the selected 15 miRNAs that exhibited quantifiable preferential expression by qPCR, and have also been shown to be related to colon cancer carcinogenesis (74-94), nine of them (miR-7, miR-17-3p, miR-20a, miR-21, miR-92a, miR-96, miR-183, miR196a and miR-214) exhibited increased expression in plasma (and also in tissues) of patients with CRC, and later TNM carcinoma stages exhibited a more increased expression than did adenomas. On the other hand, six of the selected miRNAs (miR-124, miR-127-3p, miR-138, miR-143, miR-146a and miR-222) exhibited reduced expression in plasma (and also in tissues) of patients with CCR, with the reduction becoming more pronounced during progression from early to later TNM carcinoma stages (38, 39).

We propose to follow this preliminary work with a prospective randomized design (95) study using a larger number of individuals (*e.g.* 100 controls and 200 CRC cases at various TNM stages), in order to have statistical confidence in the study outcome, by using methods such as those that we have outlined herein.

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