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Chip-Based 3D dPCR for Absolute Quantification of Colon Cancer Micronics

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Abstract

There is currently no validated micro(mi)RNA diagnostic stool test to screen for Colon Cancer (CC) on the market because of the complexity of fecal density, vulnerability of stool to daily changes, and the presence of three sources of miRNAs in stool (cell-free from fecal homogenates, exsosomal miRNAs from fecal exosomes, and fecal colonocytes). To address these complexities, we have earlier on carried out a microarray miRNA experiment, using Affymetrix GeneChip miRNA 2.0 Arrays, on immunocaptured and enriched stool colonocytes of 15 subjects [three healthy controls and twelve colon cancer patients [three TNM stage 0-1 (e.g., polyps ≥ 1 cm, villous or tubvillous, or with high grade dysplasia), three stage 2, three stage 3, and three stage 4] in triplicates to select a smaller panel of 14 preferentially expressed mature miRNAs associated with colon cancer (12 Up-Regulated, miR-19a, miR-20a, miR-21, miR-31, miR-34a, miR-96, miR-106a, miR-133a, miR-135b, miR-206, miR-224 and miR-302; and 2 Down-Regulated, miR-143 and miR-145). In a subsequent preliminary study, absolute quantitative digital PCR on these 15 stool samples from stages 0-4 was subsequently carried out on total small RNA extracted by immunocapture, followed by RT that employed TaqMan® miRNA Reverse Transcription (RT) Kit and a Custom TaqMan RT Primer Pool, and absolute quantification of miRNAs, in copies/μl, was measured using a chip-based Absolute Quant Studio 3D Digital PCR analysis, to validate microarray results. To ensure that we have chosen human and not bacterial small total RNA, we have carried out co extraction protocols with *E. coli* K1 strain RS18, compare Agilent electrophoretic patterns, and also sequenced random samples throughout this research using mRNA/miRNA sequencing.

Our initial quantitative dPCR miRNA data presented in this article, showed that the quantitative changes in the expression of a few mature miRNA genes in stool, which are associated with right and left colon cancer, would provide for a more convenient, sensitive and specific diagnostic screening markers. More useful than those test markers currently available on the market, such as the low-sensitivity (<15%) Fecal Occult Blood Test (FOBT); result in better compliance; and is more economical than the invasive and expensive colonoscopy exam in colon cancer, which can be cured if that cancer is detected at the early TNM stages, and that becomes incurable and deadly if not diagnosed before metastasis. Initial test performance characteristics of the miRNA approach showed that the test has a high numerical predictive value in colon cancer. Moreover, underpinning of the miRNA markers as a function of total RNA showed that the test can numerically differentiate between control subjects and colon cancer patients, particularly at the early stages of that curable cancer.

We propose to extend our initial research results to a larger prospective and randomized five-years nested case-control study, to validate the expression of the above 14 miRNAs, in stool of 180 individuals in an epidemiologically designed study, using [30 controls and 150 colon cancer patients [thirty precancerous polyps (stage 0-1), forty five stage 2, and seventy-five colon cancer stages 3 or 4] chosen randomly by an epidemiological method from 900 control and CC subjects to allow for an adequate time to collect the required 900 stool samples, as well as allowing for statistically valid analysis, standardized test conditions, and to provide a mean for determining the true sensitivity and specificity of a miRNA-screening approach in noninvasive human stool. Power-analysis has indicated that a total of 180 individuals, which will take us 5 years to enroll in testing, is an appropriate number of subjects to standardize and validate our proposed miRNA screening test. We may find out at the end of the proposed validation study in stool that fewer miRNAs, or even one miRNA, may suffice to serve as an efficient and a quantitative marker for the non-invasive diagnostic screening of colon cancer in human stool.

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The above approach when combined with bioinformatics analysis, to correlate miRNA seed data with our previously published messenger (m)RNA target data in stool, allows for a thorough mechanistic understanding of how miRNA genes regulate mRNA expression, and would offer a better comprehensive diagnostic screening test for the non-invasive early detection stage (0-1) of colon cancer.

In order to show the clinical sensitivity and specificity of the proposed miRNA test, the absolute miRNA PCR values, in copies/µl, will be correlated with FOBT, colonoscopy, and pathology data. Standardization will establish test's performance characteristics (sample selection, optimal sample running conditions, preservation and storage) to ensure that the assay will perform the same way in any laboratory, by any trained personnel, anywhere in the World. Ultimately, a smaller number of selected validated miRNAs (<10) showing increased and reduced expression could suffice to give quantitative miRNAs colon cancer expression values, useful for the early diagnostic screening of that curable cancer.

Keywords: Chips; Diagnosis; Epidemiology; Immunobeads

Introduction

The discovery of small non coding protein sequences, 17-27 nucleotides long RNAs (microRNAs), has opened new opportunities for a non-invasive test for early diagnosis of many cancers [1]. The latest miRBase release 22 on, March 12, 2018 [http://ww.mirbase.org] indicates the total number of miRNAs labeled "high confidence" has increased by 168, to 1996, than in the previous release. That increase is partly due to incorporation of more deep sequencing datasets, and also because of relaxation of one criterion: A few sequences labeled as high confidence in miRBase 20 have disappeared in the miRBase 21 set, because high confidence sequences must either: (a) have at 10 reads mapping to each arm, as before, or (b) have at least 5 reads mapping to each arm and at least 100 reads mapping in total. The latter case helps to catch some of the well-established, highly expressed miRNAs that have very high arm expression bias that is, a large number of reads mapping to one arm, and a small number to the other [2].

MiRNA functions seem to regulate development and apoptosis [3,4], and specific miRNAs are critical in oncogenesis [1,5], effective in classifying solid & liquid tumors [6,10-12], and serve as oncogenes or suppressor genes [13]. MiRNA genes are frequently located at fragile sites, as well as minimal regions of loss of heterozygosity, or amplification of common break-point regions, suggesting their involvement in carcinogenesis [14]. MiRNAs have promise to serve as biomarkers for cancer diagnosis, prognosis and/or response to therapy [1,15-17]. Profiles of miRNA expression differ between normal tissues and tumor types, and evidence suggests that myna expression profiles cluster similar tumor types together more accurately than expression profiles of protein-coding mRNA genes [1,18,19].

A recent study examined global expression of 735 miRNAs in 315 samples of normal colonic mucosa, tubulovillus adenomas, adenocarcinomas proficient in DNA mismatch repair (pMMR), and defective in DNA mismatch repair (dMMR) representing sporadic and inherited CRC stages I-IV [20]. Results showed that: a) the majority of miRNAs that were differentially expressed in normal and polyps (miR-1, miR-9, miR-31, miR-99a, miR-135b and miR-137) were also differentially expressed with 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 versus carcinoma, suggesting a stepwise progression from normal colon to carcinoma, and that early tumor changes were important in both the pMMR- and dMMR-derived cancers, c) several of these miRNAs were linked to pathways identified for colon cancer, including APC/WNT signaling and 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 biologic pathways in pMMR and dMMR tumors in spite of the presence of numerous molecular differences between them, including differences at the miRNA level [20,21].

Unlike screening for large numbers of messenger (m) RNA, a modest number of miRNAs is used to differentiate cancer from normal [1,10,16,18-20], and unlike mRNA [22], miRNAs in stool remain largely intact and stable for detection [23]. Therefore, miRNAs are better molecules to use for developing a reliable noninvasive diagnostic screen for colon cancer, since we have found out during preliminary studies that: a) the presence of *Escherichia Coli* does not hinder detection of miRNA by a sensitive technique such as dPCR, as the primers employed are selected to amplify human and not bacterial miRNA genes [24], and b) the miRNA expression patterns are the same in primary tumor, or diseased tissue, as in stool samples [1,21,23]. The gold standard to which the miRNA test will be compared is colonoscopy, which will be obtained from patients' medical records. However, because the low sensitivity guaiac FOBT is still the most commonly used screen in annual checkups [25-29], we will also include this test for comparison with our proposed molecular diagnostic screening miRNA approach in human stool.

Isolation of colonocytes from stool samples is needed to perform an acceptable cytology, and will be used to provide a quantitative estimate of how our miRNA method performs. Although we may miss exosomal RNA, a parallel test could also be carried out on miRNAs obtained from stool samples to compare the extent of loss when colonocytes are only used, and an appropriate corrections for exsosomal loss can then be made [30].

The biomarker validation approach outlined in this proposal has been designed to test the hypothesis that "quantitative measurement of the expression of a carefully-selected panel of miRNAs in stool by dPCR is a reliable, sensitive and specific diagnostic indicator, for early non-invasive screening of colon cancer". To prove this hypothesis, it must first be validated in a study, as proposed herein, using a nested case control epidemiology design and employing a prospective specimen collection, retrospective blind evaluation (PRoBE) of control subjects and test colon cancer patients [31], as specifically delineated by the National Cancer Institute's (NCI's)

Table 1: Characteristics of Fourteen Up- or Down-Regulated MicroRNAs in Human Stool.

MiRNA	Up-Regulated	Down-Regulated	Chromosome Location	Known Putative Cancer Target Gene(s)
MiR-19a	Yes	No	13q31.3	Undetermined
MiR-20a	Yes	No	13q31.3	PTEN, TMP1
MiR-21	Yes	No	17q23.1	PTEN,BCL2,PDCD4,TIMP3,SPRY2,REC,T1AM1
MiR-31	Yes	No	9p21.3	T1AM1,AX1N1,FOXC2,FOXP3,H1F1AN
MiR-34a	Yes	No	1p36.22	BCL2,TP53, E2F3, NOTCH1, E2F1, S1RT
MiR-96	Yes	No	17q32.2	KRAS
MiR-106a	Yes	No	Xq26.2	PTEN,E2F1,RB1
MiR-133a	Yes	No	18q11.2/20q13.33	BAX,KRAS
MiR-135b	Yes	No	1q32.1	MSH2
MiR-200c	Yes	No	12p13.31	ZEB1
MiR-224	Yes	No	Xp23	Undetermined
MiR-30a	No	Yes	6q13	RASA1, ERG, SEMA6D, SEMA3A
MiR-143	No	Yes	5q32	KRAS, MAPK7.DNMT3A
MiR-145	No	Yes	5q32	TGFBRE, APC, IRS1, STAT1,YES1, FLI1

Early Detection Research Network (EDRN) http://edrn.nci.nih.gov for cancer biomarker discovery studies.

Innovation of the dPCR-miRNA stool screening approach lies in the collective use of many methods in the proposed research, such as: immunoparamagnetic beads [25,26] to capture colonocytes from the harsh, but noninvasive stool environment, whose extracted fragile total small RNA is stabilized shortly after stool excretion by commercial kits so it does not ever fragment, followed by standardized analytical quantitative miRNA dPCR-chip profiling in noninvasive stool samples, which are neither labor intensive, nor require extensive sample preparation, to develop a panel of few stable miRNAs for absolute quantitative diagnostic screening of early sporadic colon cancer (stage 0-1), more economically and with higher sensitivity and specificity than any other colon cancer screening test on the market today [1,21-29].

Epidemiology of colon cancer

Colorectal Cancer (CRC) is the third most common malignancy worldwide, with an estimated one million new cases and a half million deaths each year [1,23]. Screening for CRC allows early stage diagnosis of the malignancy and potentially reduces disease mortality [28,29]. The convenient and inexpensive Fecal Occult Blood Test (FOBT) screening test has low sensitivity and requires dietary restriction, which impedes compliance and use [29]. CRC is the only cancer for which colonoscopy is recommended as a screening test [21-23]. Although colonoscopy is a reliable screening tool, the invasive nature, abdominal pain and high cost have hampered worldwide application of this procedure [25]. In comparison to the commonly employed low sensitivity FOBT tests, a noninvasive sensitive screen for which there would be no requirement for dietary restriction would be a more convenient test. Epidemiological evidence suggests that Colon Cancers (CCs) and Rectal Cancers (RCs) differ in their morbidities and etiologies [32-38]. RC is more common in China where it accounts for over 50% of CRC, compared with <30% in western countries. Data from Peking Union Medical College Hospital, China, indicates that colon & rectal cancers accounted for 55.6% and 44.4% of CRC, respectively, during the years 1989 through 2008, and are more prevalent in younger Asian individuals [39]. In contrast, colon cancer 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 [27-29], which suggest differences in carcinogenesis between CC and RC. Several structural and molecular studies have indicated differences in etiology, clinical manifestation, pathological features and genetic abnormalities between CC and RC [31-33]. The proximal colon, distal colon and rectum have different embryological origin. Molecular studies found that tumor suppressor genes, point mutations and genetic instability differ according to the subsite colorectum. CC has been reported to more likely have CpG island methylator phenotype and k-ras mutations, whereas rectal and distal colon tumors are more likely to have p53 and APC mutations [34-37]. Gene hybridization techniques have shown amplification of 20q in CC, compared with amplification of 12p in RC [38].

A study indicated significant differences between rectal and colon cancer in the amplification of genes for cell cycle as cyclin-A2, -B1, -D1 and –E [40]. An omic study using Illumina HT-12 V4.0 Expression Bead chip oligonucleotide microarrays, found RC to be more complex than CC as 676 genes related to 11 pathways in CC and 1,789 genes related to 30 signal pathways altered in RC, with 824 common differentially expressed genes up- or down-regulated in both Cancers [40], leading to the conclusion that colon and rectal cancers represent two distinct types of tumors. We have focused in our research on colon cancer as it is more abundant in the USA, and more CC patients report to our Collaborating Clinics, compared to RC.

Materials and Methods

We have first carried out a global microarray expression analysis study [41-43] using an exfoliated colonocytes enrichment strategy [44-47], which employed 15 subjects (three controls, three TNM stage 0-1, three stage 2, two stage 3, and three stage 4 colon cancer) in triplicates, using Affymetrix Gene Chip miRNA 2.0 Array, containing 15,644 probe sets that provides 100% miRBase v15 coverage, to select a panel of miRNAs for subsequent dPCR studies, as we have detailed before [41-43]. Microarray results showed 180 preferentially expressed miRNA genes that were either increased (124 miRNAs), or reduced (56 miRNAs) in expression in stool samples from colon cancer patients. We then carefully selected 14 miRNAs, 12 of them showed increased expression and 2 showed decreased expression, as

Table 2: Absolute Quantification of Up-/Down- Regulated miRNAs in Stool by QuantStudio™ 3D Chip-Based Digital PCR.

Type	miR-19a	miR-20a	miR-21	miR-31	miR-34a	miR-96	miR-106a	miR-133a	miR-135b	miR-200c	miR-224	miR-30a	miR-143	miR-145
control	9964.23	9724.14	9699.68	9591.16	9580.92	9590.59	9464.64	9574.13	9568.15	9556.85	9631.73	9401.81	9585.54	9683.18
control	9984.55	9890.38	9795.44	9588.24	9602.9	9587.82	9592.68	9680.24	9515.46	9511.29	9592.62	9580.92	9504.61	9506.12
control	9950.19	9898.88	9938.74	9791.83	9894.82	9862.24	9875.88	9800.08	9824.18	9843.18	9810.2	9780.74	9699.52	9823.54
stage01	7998.16	8011.92	7949.68	7864.18	7880.18	7790.44	7682.74	7687.88	7561.64	7402.8	6994.24	6892.54	1995.92	1884.54
stage01	7814.22	7901.24	7890.32	7798.92	7780.28	6849.68	6999.68	6742.6	6640.16	6616.1	6872.54	6640.24	1879.04	1764.92
stage01	7764.5	7745.38	7690.32	7549.28	7610.32	6787.62	6870.96	6739.42	6690.82	6584.74	6477.52	6454.44	1799.92	1668.19
stage2	7414.42	7569.16	7529.9	7492.68	7384.82	7189.64	6794.88	6690.98	6504.2	5702.16	5464.16	4870.22	1346.48	1040.26
stage2	7390.84	7490.96	7501.62	7379.04	7202.28	7102.28	6472.48	6598.24	6242.82	4387.76	5414.08	4189.42	988.14	862.08
stage2	7208.16	7378.74	7402.68	7299.76	7124.56	7098.04	6402.18	6401.16	6218.92	4123.18	4098.78	3894.9	872.4	763.14
stage3	6850.14	6936.16	6902.04	6890.14	7092.18	6586.18	6319.08	5898.36	5386.66	3821.22	3679.62	3601.4	365.42	256.28
stage3	6792.75	6790.29	6776.26	6658.78	6674.54	6560.68	6116.84	5602.16	4999.16	3715.22	3686.92	3570.92	260.14	154.02
stage3	6622.84	6662.9	6694.28	6558.84	6554.28	6510.27	6039.84	5404.68	5498.82	3421.22	3614.62	3120.18	194.84	133.37
stage4	6506.92	6538.8	6419.02	6227.54	5978.48	5766.32	5686.36	5256.81	4973.28	3327.28	3479.52	2052.38	92.45	88.49
stage4	6468.22	6384.12	6397.92	6117.12	5856.66	5681.82	5259.84	4905.76	3840.86	3244.16	3276.42	1096.44	76.88	67.42
stage4	6488.38	6434.48	6346.06	5898.78	5466.16	5372.56	4896.36	4812.44	3784.56	3164.8	3186.14	678.56	56.82	49.26

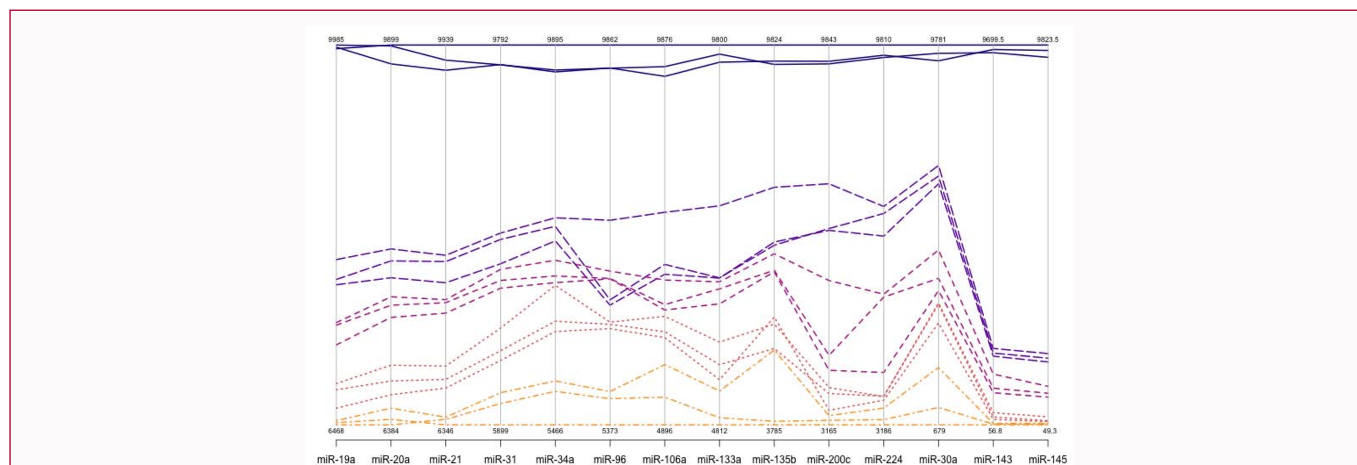

Figure 1: Absolute Quantification of Up- or Down-Regulated miRNAs in Human Stool by QuantStudio™ 3D Digital PCR Chip System.

presented in Tables 1 & 2, and Figure 1 below, for further analysis of absolute miRNAs expression by a chip-based digital (d) PCR test in a proposed validation study [48-50].

Our absolute dPCR data tabulated in Table 2, and presented graphically in Figure 1 below, show 14 preferentially expressed mature miRNAs associated with colon cancer (12 Up-Regulated, miR-19a, miR-20a, miR-21, miR-31, miR-34a, miR-96, miR-106a, miR-133a, miR-135b, miR-206, miR-224 and miR-302; and 2 Down-Regulated, miR-143 and miR-145) in stool samples from healthy controls, and stages 0-1 to 4 individuals with colon cancer. We further calculated Standard Deviations (SD) obtained from the one way ANOVA, using the 5 level factors Type (normal, stage 01, stage 02, stage 03, stage 04). The adjusted R-squared values representing the proportion of variation explained by Type are also reported. Type was statistically significant for every gene; all p-values were less than 0.000001 (no adjustments for multiple comparisons). These data are tabulated in Table 3, and Shown graphically in Figure 1.

For each gene on the graph in Figure 1, we have shown the min and max in order to make the presentation clearer. At top left is high expression Value of 9985, which is the maximum value for that gene, at the bottom one finds the value for the minimum The colors range from dark blue (control) to orange (stage 4). The groups are also distinguished by line type: control (solid), stage 0-1 (long dash), stage 2 (dash), stage 3 (dot), stage 4 (dash and dot). The figure is a parallel coordinate plot made in R [51], using the package MASS [52].

Stool collection and storage

Stool was obtained from 15 participating subjects {three healthy controls and twelve colon cancer patients of all the colon cancer stages [three TNM stage 0-1 (e.g., polyps ≥ 1 cm, villous or tubvillous, or with high grade dysplasia), three stage 2, three stage 3, and three stage]} [23]. All stools were collected with sterile, disposable wood spatulas in clean containers, after stools were freshly passed; it was then placed for storage into Nalgene screw top vials (Thermo Fisher Scientific, Inc., Palo Alto, CA, USA), each containing 2 ml of the preservative RNA later (Applied Biosystems/Ambion, Austin, TX, USA), which prevents the fragmentation of the fragile mRNA molecule [22], and vials were stored at 80°C until samples were ready for further analysis. Total small RNA, containing miRNAs, was extracted from all frozen samples at once, when ready, and there was no need to separate mRNA containing small miRNAs from total RNA, as small total RNA

Table 3: Representation of SDs and R^2 for miRNAs tested by absolute digital PCR.

Type	miR-19a	miR-20a	miR-21	miR-31	miR-34a	miR-96	miR-106a
sd	92.2390	111.10331	99.76355	146.64101	209.04905	278.47558	301.87638
r2	99.4831	99.18486	99.34603	98.65141	97.63002	96.13899	96.19772
Type	**miR-133a**	**miR-135b**	**miR-200c**	**miR-224**	**miR-30a**	**miR-143**	**miR-145**
Sd	300.06189	409.67168	449.86741	376.84372	424.99723	132.76331	110.89266
r2	96.85741	95.49454	96.70427	97.61795	97.95389	99.87075	99.91289

Table 3: Timeline for accomplishing research aims during the proposed 5 years study.

	Aim 1	Aim 2	Aim 3	Aim 4	Aim 5	Aim 6
Method-Aim/ Months	Standardize sample acquisition, handling & epidemiol- ogically select population OR Collect samples in yrs 2-5	Standardize total small RNA extraction; use dPCR to study miRNAs gene expression	Use statistics for data analysis &_ bioinformatics to identify control elements	Finalize accessing test performance characteristics of the dPCR-miRNA approach	Provide numerical under pinning of miRNA as a function of total RNA	Provide alternate standardized methods to achieve aims
1-4	..........[a]					
1-8	....	.				
1-12		....	...	.	.	
13-16	....					
17-20	....	.				
21-24		....	...	.	.	.
25-28	....	.				
29-32	....	.				
33-36		....	...	.	.	.
37-40	....	.				
41-44	....	.				
45-48		....	...	.	.	.
49-52	....					
53-56	....	.				
57-60		.	....	..	..	.

.[a] Refers to potential frequency and/or level of effort needed to accomplish/complete project aim.

was suitable to make ss c-DNAs.

Extraction of total small RNA

A procedure used for extracting small total RNA from 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 [22]. DNase digestion was not carried out, as our earlier work had demonstrated no difference in RNA yield or effect on RT or PCR after DNase digestion [23,41-43,53-57]. The time taken to purify aqueous RNA from the entire 15 A frozen stool sample was ~ two hrs. Small RNA concentrations were measured spectrophotometrically at λ 260 nm, 280 nm and 230 nm, using a Nano-Drop spectrophotometer (Themo-Fischer Scientific). The integrity of total RNA was determined by an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA, USA) utilizing The RNA 6000 Nano LabChip®. RNA integrity number (RIN) was computed for each sample using instrument's software [22,23,42,43,53-57].

Preparation of ss-cDNA for molecular analysis

The RT2 miRNA First Strand Kit® from SABiosciences Corporation (Frederick, MD, USA) was employed for making a copy of ss-DNA in a 10.0 µl Reverse Transcription (RT) reaction, for each RNA samples in a sterile PCR tube, containing 100 ng total RNA, 1.0 µl miRNA RT primer & ERC mix, 2.0/µl 5X miRNA RT buffer, 1.0 µl miRNA RT enzyme mix, 1.0 µl nucleotide mix and Rnase-free H2O to a final volume of 10.0 µl. The same amount of total RNA was used for each sample. Contents were gently mixed with a pipette or, followed by brief centrifugation. All tubes were then incubated for 2 hrs at 37°C, followed by heating at 95°C for 5 mins to degrade the RNA and inactivate the RT. All tubes were chilled on ice for 5 mins, and 90 µl of Rnase-free H_2O was added to each tube. Finished miRNA First Strand cDNA synthesis reactions were then stored overnight at -20°C [22,23,42,53-57].

Experimental digital absolute quantitative PCR approach

Because the use of 96 or 384-well plates for a single sample is nether practical or affordable, nor very accurate, widespread implementation of dPCR technology has necessitated the introduction of nanofluidic techniques and/or emulsion chemistries. Three enhancements associated with dedicated instruments have helped promote the use of dPCR: (a) Partition volumes have been lowered to as little as 5 picoliter (pl); (b) The partitioning process has been automated, and (c) The number of partitions has been increased to over 100,000 for a single experiment. These innovative elements have simplified dPCR, and increased its precision, while holding down the total reaction value of a single experiment, compared to that of a conventional qPCR [48-51].

Figure 2: Diagram illustrating QuantStudio™ 3D Digital PCR System Chip; ChipCase Lid (1);Digital PCR 20K 10 mm² nanofluidic v2 chip (2), which contains 20,000 reaction wells; QuantStudio™ 3D Digital PCR Chip Case (3); Chip ID (4); Fill port (5); and Reaction wells, the 20,000 physical holes that suspend individual PCR reactions.

Digital PCR is a new approach to miRNAs quantification that offers alternate method to qPCR for absolute quantification, by partitioning a sample of DNA or cDNA into many individual, parallel PCR reactions; some of these reactions contain the target molecule (positive), while others do not (negative). A single molecule can be amplified a million-fold or more. During amplification, TaqMan chemistry with dye-labeled probes is used to detect sequence-specific targets. When no target sequence is present, no signal accumulates. Following PCR analysis, the fraction of negative reactions is used to generate an absolute count of the number of target molecules in the sample, without the need for standards or endogenous controls. In conventional qPCR, the signal from wild-type sequences dominates and obscures the signal from rare sequences [55-57]. By minimizing the effect of competition between targets, dPCR overcomes the difficulties inherent to amplifying rare sequences and allows for sensitive & precise absolute quantification of the selected miRNAs.

Applied Biosystem Quant Studio™ 3D instrument used in this research study only performs the imaging and primary analysis of the digital chips. The chips themselves must be cycled offline on a Dual Flat Block GeneAmp® 9700 PCR System. Or the ProFlex™ 2x Flat PCR System. The Quant Studio™ 3D Digital PCR System can read the digital chip in less than 1 minute, following thermal cycling [48]. It allows for one sample per chip; although, duplexing allows for analysis of two targets per chip. Sample prep for digital PCR is no different than for real-time PCR, when using the Quant Studio™ 3D Digital PCR System. To figure out the concentration of cDNA stock from results, if one includes all of the necessary dilution factors into the Analysis Suite™ software, the software will give the copies/μL in the stock.

There are 2 dilutions that one needs to take into account: (a) The first is the dilution of the sample in the reaction, and (b) the second is the dilution of the stock that one makes before adding it to the digital PCR reaction. For example, if one wants to add 1 μL of a sample that has been diluted 1:10 from the stock. Thus, if one adds 1 μL of his/her sample to a 16 μL (final volume) reaction, the dilution factor of the sample is 1:16 or 1/16=0.0625. Since the stock has also been diluted 1:10 (0.1), one also needs to factor this in. The final dilution factor to enter into the software is 0.0625 × 0.1=0.00625 (1:160). One can use either annotation to indicate the dilution factor in the Analysis Suite™ software. If one enters that value into the "Dilution" column, the software will give the copies/μL in the starting material (stock). The Poisson Plus algorithm for projects that contain Quant Studio™ 3D Chips with target, quantities >2000 copies/μL. The Poisson Plus algorithm corrects for well-to-well load volume variation, on a per Chip basis. This becomes important at higher target concentrations. There is also an option to export the Chip data as XML on the Export tab-thousands of discrete subunits prior to amplification by PCR, each ideally containing either zero or one (or at most, a few) template molecules [50].

Each partition behaves as individual PCR reactions as with real-time PCR fluorescent FAM probes [or others, as VIC fluorescence]. Samples containing amplified products are considered positive (1, fluorescent), and those without product –with little or no fluorescence (i.e., are negative, 0). The ratio of positives to negatives in each sample is the basis of amplification. Unlike real-time qPCR, dPCR does not rely on the number of amplification cycles to determine the initial amount of template nucleic acid in each sample, but it relies on Poisson Statistics to determine the absolute template quantity. The unique sample partitioning step of dPCR, coupled with Poisson Statistics allows for higher precision than both traditional and qPCR methods; thereby allowing for analysis of rare miRNA targets quantitatively and accurately [50,51].

The use of a nanofluidic chip, shown below, provides a convenient and straight forward mechanism to run thousands of PCR reactions in parallel. Each well is loaded with a mixture of sample, master mix, and Applied Biosystems TaqMan Assay reagents, and individually analyzed to detect the presence (positive) or absence (negative) of an endpoint signal. To account for wells that may have received more than one molecule of the target sequence, a correction factor is applied using the Poisson model. It features a filter set that is optimized for the FAM™, VIC®, and ROX™ dyes, available from Life Technologies [49].

A workflow of the dPCR procedure by the QuantStudio™ 3D Digital PCR System Chip is presented in Figure 3, below. Digital PCR, however, has several tips to follow: 1) A rough estimate of the concentration of miRNAs of interest has to be first carried out, in order to make appropriate dilutions, so that not too many partitions will get multiple copies that prevent accurate calculation of the copy number of miRNAs of interest; 2) Non-template controls and a

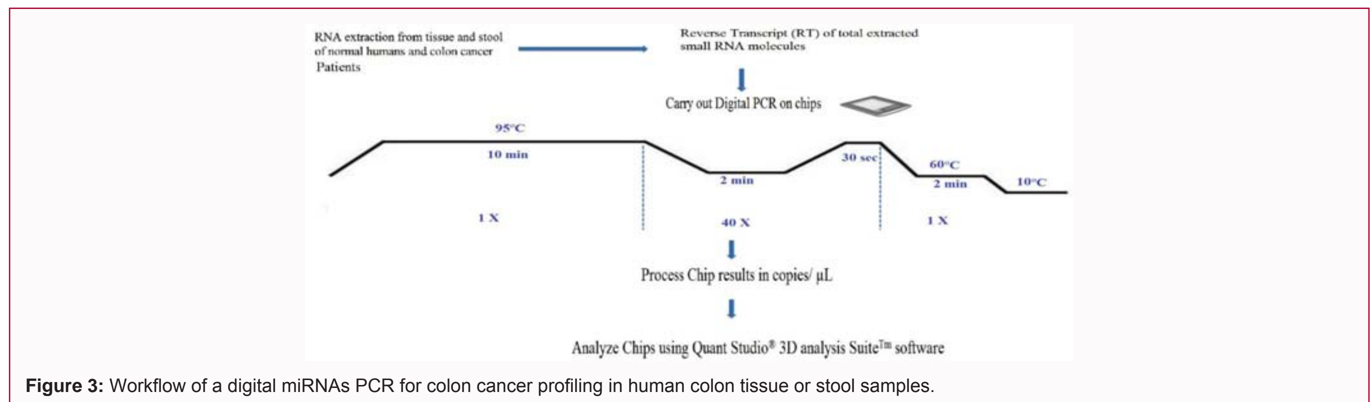

Figure 3: Workflow of a digital miRNAs PCR for colon cancer profiling in human colon tissue or stool samples.

RT negative control must be set up for each miRNA, when using a "primer pool method" for retro-transcription; 3) A chip-based dPCR method requires less pipetting steps, which reduces potential PCR contamination compared to another type of dPCR marketed by Bio-Rad Laboratories, thus called "Bio-Rad's droplet digital PCR", which requires multiple pipette transfers that potentially increase the risk of contamination [50], and 4) Quant Studio TM 3D chip has 20,000 fixed reaction wells, whereas Bio-Rad's droplet PCR relies upon the generation of droplets; a step that could be extremely variable, as reported by Miotto et al. [11,48].

Acquisition of patients and specimens for carrying out the clinical study

Our collaborating clinicians are aware of the constraints imposed by working with RNA and the need to preserve it so it does not ever fragment thereafter [22,23,41-43]. Participating clinics will consent prospective individuals when they report to the clinic for consult. Those individuals not showing any polyps, or inflammatory bowel diseases, such as colitis or diverticulitis, will be asked by their physicians if they wish to participate in the study. If they agree, they/their guardian will be consented, each given a stool collection kit and detailed collection instructions. Each study subject will collected one 10 g stool sample, in a standardized fashion, in a large 40 cc plastic jacket given to each participating, consented individual, prior to any bowel preparation. The study nurse will show and ask participants to brush both the mucinous layer, which is rich in colonocytes, and the non-mucinous parts of stool in order to have a representation of the entire colon (both right and left side colon) [1,27,54-57], to be preserved overnight at room temperature in the fixative RNA Later® (Invitrogen) added at 2.5 ml per 1 g of stool, followed by calling the laboratory personnel to pick up the sample by next morning. Samples will be stored at -80°C in small aliquots until needed. International Collaborators will also give study participants these written instructions in their native languages to ensure standardization, and will explain to them what's needed to collect samples representing both right and left side colon. When ready for analysis, samples are defrosted at room temp, filtered through a nylon mesh by laboratory personnel, in order to remove the preservative, and any debris prior to extraction of total small RNA. All laboratory work will be carried out and standardized under blind conditions and, in accordance with organization's Standard Operating Procedures (SOPs) for handling of biohazard us waste material [1,21-23,41-43,54-57].

Randomized selection of control human subjects and case patients

To avoid bias, and ensure that biomarker selection and outcome assessment will not influence each other, a prospective specimen collection retrospective blinded evaluation (PRoBE) design randomized selection of control subjects and case patients from our consented cohort population, will be employed [31], without no prior knowledge of who has what diagnosis, and stool specimens collected prior to removal of the lesion on patients undergoing colonoscopy, which will form the cohort.

By the 8th months of each year, we would have a cohort of 135 subjects, who are representative of the entire cohort, to select 6 control subjects and 30 CC patients. This will undoubtedly be the least common of the three groups (normal, adenoma & cancer) by far. We will then match 1 to 1 adenoma cases to the cancer cases for age (+/- 5 years), gender, clinic and month of diagnosis. Similarly, we will then match the normal controls from among the collected specimens to the cancer and adenoma cases. If there is no match, we will liberalize the data restriction to allow +/- 2 months. Thus, we will collect a case-case-control group nested in the overall colonoscopy cohort that is collected. The absolute quantitative dPCR miRNA expression analysis will be carried out on all coded samples at once during the last three months of each study year as shown in the time line Table 3, with the investigators blind to knowledge of the patients' diagnosis, so that no analytical bias is introduced to the study.

While we believe that the 135 stool samples collected every year are representative of the overall cohort, there may be some volunteer bias, which we will not know how it would affect the studied miRNA markers. Therefore, we will collect demographic & clinical data on both groups (those who participated & those who did not) and compare for the following factors: age, gender, race/ethnicity, reason for colonoscopy, diagnoses, so that an assessment can be made at study conclusion as to what degree selection may have affected the study results.

Enrichment & exfoliation strategy of colonocytes from stool for miRNA profiling

Approximately 1 g of thawed stool is homogenized in a Stomacher® 400 EVO Laboratory Blender (Seward, UK) at 200 rpm for 3 min, with 40 ml of a buffer of Hank's solution, containing 10% Fetal Bovine Serum (FBS) and 25 mmol/L Hepes buffer (pH 7.35). The homogenates is filtered through a nylon filter (pore size 512 µm), followed by addition of 80 µl of Dynal superparamagnetic polystyrene beads (4.5 µm diameter) (Invitrogen, Carlsbad, CA, USA) 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 (58,59), at a final concentration of 12 ng of Ab/mg magnetic beads (1 μg Ab/106 target cells). The mixture is incubated for 30 min on a shaking platform at room temperature. To visualize colonocytes. a drop of the solution is spread on a glass slide, dried and stained with Diff-Quick stain (Fisher Scientific, Pittsburgh, PA), and another drop is placed on a hemocytometer, and counted under the microscope to estimate the number of colonocytes form which total small RNA will be extracted. The supernatant is removed and the pellet containing colonocytes will be stored at –80°C until small RNA extraction [22,59,60].

By the 9th month of each study year, isolation of colonocytes from stool, and comparing the Agilent electrophoretic (18S and 28S) patterns to those obtained from total RNA extracted from whole stool, and differential lysis of colonocytes by RT lysis buffer (Quagen), could be construed as a validation that the electrophoretic pattern observed in stool (18S and 28S) is truly due to the presence of human colonocytes, and not due to stool contamination with Escherichia coli (16S and 23S) [24]. One must also take into account that some exsosomal RNA will be released from purified colonocytes into stool, and correction is made for that effect [30].

Why use a miRNA assay for colon cancer screening

The expression of individual genes may be altered by mutations in the DNA, or by a change in their regulation at the RNA or protein levels. Epigenetic silencing is an important mechanism that contributes to gene inactivation in CRC [21]. Analysis of promoter methylation of hypermethylated in cancer 1 (HIC1) gene in stool showed it to be highly specific (98%) for colon adenoma and carcinoma, but sensitivity was quite low (31% for adenoma & 42% for all cancer) [61], which suggested that an epigenetic marker only is not good enough for screening, but a combination of genetic and epigenetic markers would be required to reliably identify CRC at an early stage.

Working with the stable DNA has been relatively easy. A study by scientists affiliated with Exact Sciences Corp., Marlborough, MA, which markets a mutation-based DNA test, assessed a newer version of a fecal DNA test for CRC screening using a vimentin methylation marker and another mutation DY marker plus non degraded DNA in a limited sample of 44 CRC patients and 122 normal controls. It cited a sensitivity of 88% and a specificity of 82% only for advanced cancer, but not adenoma [62,63]. Besides, DNA mutation tests are not cost-effective, as screening for multiple mutations is expensive because these demanding mutation tests are not automated and are labor intensive. In addition, mutation detection in oncogenes and suppressor genes suffers from: a) the detection of mutations in these genes in fewer than half of large adenomas and carcinomas, b) the detection of gene mutations in non-neoplastic tissues, c) mutations found only in a portion of the tumor, and d) mutations often produce changes in the expression of many other genes [63,64].

Protein-based methods are currently not suited for screening and early diagnosis either because proteins are not specific to one tumor or tissue type (e.g., CEA), their susceptibility to proteases, current lack of means to amplify proteins, no function is known for more than 75% of predicted proteins of multicellular organisms, there is not always a direct correlation between protein abundance and activity, and most importantly because detection of these markers exfoliately often signifies the presence of an advanced tumor stage. The dynamic range of protein expression in minimally-invasive body fluids (e.g., blood) is as large as 1010 [65]. Moreover, mRNA levels do not necessarily correlate with protein expressions [66]. Protein microarray studies revealed that protein expression vastly exceeds RNA levels, and only posttranslationally modified proteins are involved in signal transduction pathways leading to tumorigenesis [67]. There is no well-documented protein test that has been shown in clinical trials to be a sensitive and a specific indicator of colon neoplasia, especially in early stages. More recently, a serum proteomic study employing Liquid Chromatography (LC)-Mass Spectrometry (MS) carried out in a nonbiased fashion failed to differentiate between individuals with large adenoma (≤ 1 cm) and normal individuals [68]. Proteomic research is a relatively new discipline, so it will take considerable time to identify and validate proteins suitable for use as clinical markers, and resolve issues of bias and validations [65,69].

On the other hand, a transcriptomic mRNA approach has shown promise to detect adenomas and colon carcinomas with high sensitivity and specificity in preliminary studies [22], but no randomized, standardized, blinded prospective clinical study has been carried out to validate the superiority of the mRNA approach. A study indicated that a combination of a transcriptomic mRNA and miRNA expression signatures improves biomolecular classification of CRC [69]. Furthermore, not only does miRNAs regulate mRNA, but they also regulate protein expression. Two studies have shown that a single miRNA act as a rheostat to fine tune the expression of hundreds of proteins [70,71]. Hence, for CRC screening, miRNA markers are much more comprehensive and preferable to a DNA-, epigenetic-, mRNA- or a protein-based marker [23]. An added advantage of the use of the stable, no degradable miRNAs by PCR expression, or chip-based methods is being automatable, which makes them much more economical and more easily acceptable by laboratory personnel performing these assays [48-51].

Suitability of stool for developing a highly sensitive diagnostic biomarker screen for colon cancer

Links between miRNAs and CRC have been reported in several studies in colon cancer cell lines, cells in culture, blood, colon tissue of CRC patients, and human stool [23,43,54-57,73-86].

Stool testing has several advantages over other colon cancer screening methods as it is truly noninvasive and requires no unpleasant cathartic preparation, formal health care visits, or time away from work or routine activities. Unlike sigmoidoscopy, it reflects the full length of the colorectum and samples can be taken in a way that represents the right and left side of the colon. It is also believed that colonocytes are released continuously and abundantly into the fecal stream [79,80], contrary to blood that is released intermittently as in guaiac FOBT [25]; therefore, this natural enrichment phenomenon partially obviates the need to use a laboratory-enrichment technique to enrich for tumorigenic colonocytes, as for example when blood is used for testing. Furthermore, because testing can be performed on mail-in-specimens, geographic access to stool screening is essentially unimpeded. The American Cancer Society (ACS) has recognized stool-based molecular testing as a promising screening technology for CRC (www.cancer.org).

Our results and others have show that even the presence of bacterial *E. coli* DNA, non-transformed RNA and other interfering substances in stool does not interfere with measuring miRNA expression [1,22,23,51-57,77-86], when an enrichment method such as the immunological paramagnetic capture method is used [25,26], when good ss-cDNA is produced [87], and when appropriate PCR primers are employed [23,55-57,77,79,81-86], as in this study. Besides, stool

colonocytes contain much more miRNA (than that available in free circulation such as in plasma [53, 57,84,88]. Considerable effort has gone into selecting a reasonable number of miRNA genes (fourteen) from among the many mature human miRNA sequences identified in a previous preliminary microarray data generation study, as a number that can be screened reliably by PCR in a subsequent quantitative dPCR study to ultimately validate smaller panel of miRNA diagnostic screening gene markers, preferably 10 or less, for routine use.

Extraction of total small RNA from stool samples

We have routinely carried out RNA isolation procedures (both manual and automatic) from colon tissue, blood and stool samples in our labs, manually, as well as by employing the Roche MagNA pure LC™ automated system, using Qiagen's RNeasy Isolation Kit® from Qiagen, Valencia, CA, containing RLT buffer (a guanidinium-based solution) and other commercial RNA extraction preparations, which provide the advantage of manufacturer's established validation and quality control standards, increasing the probability of good results [21,22,43,53-57,82-91], to extract high quality total RNA from an environment as hostile as stool; thus, shattering the myth that it is difficult to employ RNA as a screening substrate. The trick has been to stabilize total RNA shortly after obtaining fresh stool by fixing samples in a chaotropic agent [RNALater® (Invitrogen)] and observing that RNA does not ever fragment thereafter. Fragmented RNA results in poor cDNA synthesis and ultimately in less than optimal PCR amplification.

We found total small RNA isolated from stool to be suited for dPCR analysis, without further mRNA purification because: a) purified mRNA involves additional steps [87], and the increased sensitivity could be balanced by possible loss of material, b) not all mRNA molecules have poly A tail, and c) the concentration of mRNA may be insufficient to allow quality assessment using the Agilent 2100 Bioanalyzer [87]. Good human stool preparations showed two sharp ribosomal 18S and 28S rRNA (28S/18S=0.33), with a small fraction of micro RNA and 5S rRNA or tRNA molecules in the Agilent capillary electrophoresis equipment fitted with a RNA 6000 Nano LabChip [22]. However, *E. coli* shows 16S and 23S (23S/16S=1.8) [24]. RNA will be quantified spectrophotometrically. Acquiring sufficient small mRNA to analyze from stool or isolated colonocytes is feasible, as each cell contains ~ 20 pg total RNA or 0.4 pg mRNA (equivalent to 0.36 pg ss-cDNA). Only few nanograms of that DNA are needed per PCR reaction] [92].

Reverse Transcription (RT) & preparation of single stranded copy deoxy ribonucleic acid (ss-cDNA)

An Applied Biosystem kit (the TaqMan® MicroRNA Reverse Transcription Kit) that makes high quality ss-cDNA from total small RNA, and has been employed in earlier studies, will also be used in this study. It uses 50 nM RT primers that bind to the 3' portion of miRNA molecules, 1 × RT buffer, 0.25 mM each of dNTPs, 3.33 U/µl RT in a 7.5 µl reaction for 30 min at 96°C, 2 min at 56°C, 30 sec at 98°C, 2 min at 60°C and held at 10°C, the chip is then processed, and results expressed in copies µl [48,50], as shown in the workflow in Figure 3.

Quality Control (QC), and good laboratory practices (GLPs) procedures

Rigid QC considerations are necessary to ensure the uniformity, reproducibility and reliability of dPCR amplification technology. Compared to real-time quantitative PCR (qPCR), dPCR clearly offers more sensitive and considerably more reproducible clinical methods that could lend themselves to diagnostic, prognostic, and predictive tests. But for this to be realized, the technology will need to be further developed to reduce cost and simplify application. Concomitantly the preclinical research will need be reported with a comprehensive understanding of the associated errors [48,50].

The term "absolute quantification" used in dPCR refers to an estimate derived from the count of the proportion of positive partitions relative to the total number of partitions and their known volume. When the sample is sufficiently dilute, most partitions will not contain template and those that do are most likely to contain single molecules. As the sample becomes more concentrated, the chance of more than 1 molecule being present within a positive partition increases. This does not pose too great of a challenge, because the distribution of molecules throughout the partitions approximates a Poisson distribution, and a Poisson correction is applied. The dynamic range of a dPCR assay can extend beyond the number of partitions analyzed but the assay precision deteriorates at each end. In contrast, qPCR precision deteriorates only at low copy numbers [50].

dPCR benefits from a far more predictable variance than qPCR, but dPCR is susceptible to upstream errors associated with factors like sampling and extraction. dPCR can also suffer systematic bias, particularly leading to underestimation, and internal positive controls are likely to be as important for dPCR as they are for qPCR, especially when reporting the absence of a sequence. Calibration curves are frequently employed to reduce the error associated with qPCR, but they in turn are challenging to select, value assign, and apply in a manner that will be reproducible; their application also contains inherent error that is almost never considered. Arguably, a key problem with applying qPCR to areas such as the discovery of biomarkers that will eventually be translated to clinical care, is understanding whether poor reproducibility is biological, or if it is due to issues related the fact the qPCR technique is difficult to perform reproducibly. Taking all these arguments in consideration, we are therefore in the opinion that chip-based dPCR is more suited than qPCR in our proposed validation; 5-years study [50]. dMIQUE Guidelines have been implement on dPCR data [93]. Adopting these guidelines helps to standardize our experimental protocol, maximize efficient utilization of resources, and enhance the impact of this technology. Measuring miRNA by dPCR takes the last 3 months of every study year, after all stool samples have been collected.

Statistical methods for validating the microRNA approach

If the difference in gene expression dPCR value in copies/µl between healthy and cancer patients and among the stages of cancer at the end of the proposed validation study is as large and informative for multiple miRNA genes as in the limited preliminary results, suggesting that classification procedures could be based on values exceeding a threshold, then sophisticated classification procedures would not be needed to distinguish between these two groups; otherwise, we will use predictive classification, as detailed below. The goal will be to assign cases to predefined classes based on information collected from the cases. In the simplest setting, the classes (i.e., tumors) are labeled .cancerous and .non-cancerous. Statistical analyses for predictive classification of the information collected (i.e., quantitative PCR results on miRNA genes) attempt to approximate an optimal classifier. Classification can be linear, nonlinear, or nonparametric [94,95].

The miRNA expression data will be analyzed first with parametric

statistics such as Student t-test or analysis of variance (ANOVA) test if the data distribution is random, or with nonparametric Kruskall-Wallis, Mann-Whitney and Fisher exact tests if the distribution is not random [96]. If necessary, more complicated models such as multivariate analysis and logistic discrimination will be employed [97]. For the corrected index, cross-validation will be used to protect against overfitting. Efron and Tibshirani suggested dividing the data into 10 equal parts and using one part to assess the model produced by the other nine [98]; this is repeated for each of the 10 parts. Cross-validation provides a more realistic estimate of the misclassification rate.

The area under the ROC curves, [in which sensitivity is plotted as a function of (1–specificity)], will be used to describe the trade-off between sensitivity and specificity [99]. We will also employ Principal Component Analysis (PCA) method [100], which is a multivariate dimension reduction technique to simplify grouping of genes that show aberrant expression from those not showing expression, or a much reduced expression.

In cases where several genes by themselves appear to offer distinct and clear separation between control and cancer cases in either stool or tissue samples, a PMI [101,102] may not be needed. If the miRNA gene panel (or a PMI) derived by the end of the study is better than existing screening methods, all of the data generated will be used to assess the model so over-fitting is not a concern.

Cross-validation will be used to protect against over-fitting. The level of gene expression will be displayed using parallel coordinate plots produced by the lattice package in R (version 2.9.0, http://cran.r-project.org) [51,103-105]. Other packages such as GESS (Gene Expression Statistical System) published by NCSS (www.ncss.com) will also be employed in the study.

Each subject will have his or her medical record number as the key ID for merging various tables in the database. A database will be established using widely available software like MS-Access, which output spreadsheets that will be analyzed with R (version 2.9.0, The R Foundation for Statistical Computing, http://www.r-project.org/) and S-plus software (Insightful Corporation, Seattle, WA).

Results

Accessing test performance characteristics (TPC) of the MiRNA approach

The copies/μl values of the miRNA gene panel (or a derived microRNA index, PMI) obtained from stool/colonocyte samples of normal subjects and colon cancer patients with high sensitivity and specificity will be compared to the commonly used guaiac FOBT test and with colonoscopy results obtained from patients' medical records in 180 subjects (30 controls & 150 CC patients) at Study end to access TPC of the microRNA approach.

False positive discovery rates (expected proportion of incorrect assignment among the accepted assignments) will be assessed in our proposed approach by statistical methods [103-105], as it could reflect on the cost effectiveness of our test. The number of optimal miRNA genes (whether 14 or less) to achieve an optimum miRNAs' gene expression panel is established by appropriate statistics, as detailed below.

Providing numerical underpinning of the method as a function of total RNA

Cytological methods on purified colonocytes employing Papanicolaou and Giemsa staining, which showed a sensitivity for detecting tumor cells in smears comparable to that found in biopsy specimens (78.1% *vs.* 83.66%), have been employed [106]. A known number of the colonocytes isolated from 1g 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 copies/μl value from the panel of selected miRNAs from dPCR using the QuantStudio™ 3D Digital PCR Chip instrument will ultimately give an average value per a certain amount (pg or ng) of total RNA.

Determining a panel of MiRNA genes, or a predictive MicroRNA index (PMI)

If results using a nested case-control design that involves prospective collection of specimens before outcome ascertainment from the study cohort are found to provide a clear cut miRNA expression value, similar to data from the Preliminary Study, one may not need to derive a PMI. It may, however, be necessary to do so if data evaluation dictates the need for that alternative. In this case, the results of the quantitative expression of miRNA genes used to derive the index. Wiley et al. [102,103] considered 15 genes to derive a mRNA gene expression index for lung cancer, and derived an index defined as the product of two genes divided by the expression of a third gene that was 100% successful in identifying cancerous tissue. Derived research data will be used to check the sensitivity and specificity of the index. If the sensitivity falls below 90% or the specificity falls below 95%, forthcoming data using additional miRNA genes may be used with linear or logistic discriminant analysis to refine the index. To determine the usefulness of the PMI as a screening test, the clinical sensitivity (i.e., no or small number of false negatives) and specificity (i.e., no or small numbers of false positives) of the index is used, and a cutoff for a positive or negative index established. All obtained results are classified as either a true positive, false positive, true negative, or false negative by using a two by two matrix (Table 4). The cutoff is defined so that the specificity is at least 95%, and the corresponding sensitivity is expected to be better than current pne (i.e ~ 85%). The relationship between other values of sensitivity and specificity will be described using ROC curves [99]. Once results are classified, the clinical sensitivity and specificity of the PMI are calculated using conventional calculations [107].

The positive and negative predictive values are also calculated, although the population being tested will heavily influence these calculations. To measure the clinical utility of gene expression testing as a screening test, the diagnostic sensitivity and specificity of the miRNA gene panel selected, or a derived PMI, are compared to the published sensitivity and specificity of the commonly employed diagnostic screening test, guaiac FOBT, which for over 3 decades in large adenoma averaged <12%, and in carcinoma averaged ~30%, and the specificity averaged ~95% [108-111], and to the gold screening standard colonoscopy results obtained from participants' medical records that averaged 87% for sensitivity and 100% for specificity [112]. The limitations of FOBT are biologically inescapable and cannot be reversed by technological advances [113]. Based on our data, we will be able to screen colon cancer, particularly at the pre-malignant stage, with >90% sensitivity and >95% specificity, employing ≤ 10 miRNA genes in a functional assay, which is better than any available noninvasive test. Thus, a large number of patients will be spared the discomfort, risk and expense of screening colonoscopy. Only those patients truly at risk of having a colon cancer will need to undergo colonoscopy.

Table 4: Predictive MicroRNA Index (PMI).

Cancer Cases	Tue Positive (TP)	False Negative (FN)
Normal Subjects	False Positive (FP)	True Negative (TN)

%Sensitivity=TP/TP+FN*100
%Specificity=TN/FP+TN*100

Bioinformatic methods to correlate seed miRNA data with mRNA data

To provide information about complex regulatory elements, it is important to correlate miRNA resulting from this study with our mRNA data, which we produced in our earlier published research [22], as well as those data available in the open literature using several computer models [e.g., Target Scan [114]. DIANA-micro [115], miRanda [116], PicTar [117], EMBL [118], EIMMo [119], mieWIP [120] or PITA Top [121]], each algorithm having its advantages and disadvantages. The authenticity of functional miRNA/mRNA target pair, once identified, will be validated by fulfilling four basic criteria: a) miRNA/mRNA target interaction can be verified, b) the predicted miRNA and mRNA target genes are co-expressed, c) a given miRNA must have a predictable effect on target protein expression [i.e., if a gene is a true target of a given miRNA, its miRNA mimic will decrease the target gene expression level while a miRNA Antisense Ss-Oligonucleotides (ASO) inhibitor will increase the target gene expression level [122], and d) miRNA-mediated regulation of target gene expression should equate to altered biological function [123]. To examine the significance of the gene-term enrichment, a modified Fisher exact test [EASE score] is used to calculate the p-value & Bonferroni criterion employed to correct for multiple hypothesis testing (threshold 0.05), having the human genome as background. MiRNAs are annotated based on their targets identified via miRDB [124]. A thermodynamic biomarker discovery approach is to apply Shannon's mathematical theory of communication encompassing normalized Shannon entropy [125] & Jensen-Shannon divergence to trace the transcriptional changes in CC as the disease progress [125,126]. Information theory measures allow the identification of biomarkers for progressive and relatively sudden transcriptional changes leading to malignant phenotypes on omics-generated data [127].

Recommended Alternate Methods for Achieving Study Aim

We have proposed the most practical, least labor-intensive and economical approach to accomplish study aims. However, in a few samples (<5%) in control, pre- or malignant cases, it may be necessary to use methods other than automatic RNA extraction, or dPCR for sample analysis. However, because the error rate is so small and would occur in control and cases, adopting different extraction/analysis methods will not bias results.

Manual extraction of total RNA from problematic samples using the AGPC method

In very few samples, inhibitors present in stool may make it difficult to isolate RNA automatically using Qiagen kits that provide the advantage of manufacturer's validation and QC standards, increasing the probability of good results, may not be suitable. In such cases we will manually isolate RNA by a modification of the classical acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method using the chaotropic agent guanidinium thiocyanate (GSC) that inactivates ribonucleases and most microorganisms. Only total small RNA samples with an OD 260/280 nm ratio from 1.9 to 2.0, and RNA integrity (RIN) on Agilent 2100 BioAnalyzer of >7.0, may be used [128].

Use of RT2 focused PCR arrays to study miRNA expression in conjunction with qPCR system

Qiagen introduced a focused human PCR array in a 96 well plate containing 88 cancer-related miRNA genes, 4 normalization housekeeping synthetic miRNA genes, 2 RT controls and 2 controls to test the efficiency of the dPCR reaction. These focused arrays could be used to study miRNA expression by a universal multiplex qPCR assay using Roche 480 Light Cycler PCR instrument, in which a single cDNA preparation can quantitatively assay 88 miRNA genes with high specificity due to the use of universal primers containing a modified oligonucleotide [129].

Use next generation sequencing technologies (NGS) for MiRNA profiling (miRNA-seq)

miRNA-seq in more expensive than microarray or qPCR, requires larger amount of total RNA, involves extensive amplification, more time consuming, and is inaccurate estimating miRNA abundance, but it does not require a prior sequence information, allowing identification novel miRNA and miRNA isoforms (isoMirs), distinguish sequentially similar miRNAs, and identify point mutations [130,131].

Use of a plate assay to study microRNA expression

Signosis, Inc, Sunnyvale, CA (www.signosisinc.com) uses high throughput plate assay to monitor individual miRNAs, without the need to carry out a RT reaction. In that assay one of the bridge oligos is partially hybridized with the miRNA molecule and the capture oligo, and another bridge forms a hybrid between the miRNA molecule and the detection oligo. The hybrid is immobilized onto plate through hybridization with an immobilized oligo and detected by a streptavidin-Horse Radish Peroxidase (HRP) conjugate and chemiluminescent substrate using a plate reader. This hybrid structure is sensitive to the sequence of the miRNA molecule. One oligonucleotide difference will prevent the formation of the hybrid and therefore miRNA isoform could be differentiated.

MiRNA measurements from exosomes and microvessicles extracted from stool

MiRNAs are resistant to ribonucleases present in stool, probably by inclusion in lipid or lipoprotein complexes in either microvessicles (up to 1 µm), or in small membrane vesicles of endocytic origin known as exosomes (50 nm to 100 nm) [132]. The mechanism of release of miRNA from exosomes and microvesicles is unclear, although an apoptotic delivery candidate is shed from cells during apoptosis [133]. Exosomes released from human and murine mast cell lines were show to contain mRNAs and miRNAs [134]. MiRNAs in microvessicles were shown to regulate cellular differentiation of blood cells and certain metabolic pathways, and to modulate immune functions [135].

MiRNA signatures of tumor-derived exosomes were shown to function as diagnostic markers in ovarian cancer, and tumor-derived miRNA profiles and profiles of exosomal miRNAs were not significantly different [30]. If necessary, exosomal miRNAs extracted from stool colonocytes by differential centrifugation, followed by filtration through 0.22 µm filters, total RNA extracted by Trizol & concentration measured at λ 280 [134].

Use of real-time qPCR to study microRNA expression

dPCR has the edge over qPCR with the respect to technical

reproducibility, because the digital output derived from diluting the sample essentially counts the number of molecules, which is far more reproducible than the analog Cq output offered by qPCR that potentially improves both quantitative and qualitative molecular measurements.

One key advantage of qPCR, however, is it being readily scalable. Consequently, although dPCR has the potential to be more sensitive than qPCR when sample volumes are matched; qPCR will have the edge if sensitivity can be improved by performing a larger-volume reaction [136].

Conclusion

Quantitative Milestones Expected to be accomplished by the End of the Research.

The following three milestones are expected to be achieved by the end of proposed research to judge success:

Milestone 1: Derive a workable miRNA gene panel, or a PMI in stool indicative of premalignant & malignant conditions using total small RNA extracted from stool of 150 CC patients and 30 control subjects.

This milestone is achieved, if ≥ 114 (95%) of the patients with cancer have a miRNA panel that gives numerical pre- and malignant copies/µl values in stool by QuantStudio™ 3D Digital PCR System.

Milestone 2: Access TPC & Provide numerical underpinning of the method as a function of total RNA

Test Performance Characteristics (TPC) of the miRNA approach are determined by comparing copies/ul values of the miRNA gene obtained from stool samples of normal subjects and colon cancer patients with guaiac FOBT test and with colonoscopy results obtained from patients' medical records on the 150 subjects. A numerical underpinning of the method are determined by calculating the amount of total small RNA in 1 g of stool, and determining the average copy/ul value for the miRNA gene per a known amount (pg or ng) of total RNA.

Milestone 3: Establish the clinical sensitivity and specificity of the miRNA gene panel, or a PMI, using total small RNA extracted from stool of 180 subjects (30 controls and 150 with pre- and malignant CCs)

This milestone ia carried out as follows:

Guaic FOBT [Hemoccult II Sensa, Beckman Coulter, Fullerton, CA] standardized at research facility is performed in parallel with the miRNA panel for each stool sample obtained from the 30 normal & 150 colon cancer.

Colonoscopy results, which are considered as the "Gold Standard" for CRC screening, are reviewed by Gastro-enterologists, as well as blindly checking Histopathologic results of biopsies/ surgical specimens and final patients' diagnosis, including those carried out on polyp biopsies, if removed, as obtained from patients' medical records.

Using the copies/ul results from the panel of genes selected (or a PMI) obtained from stool samples of normal, and from stool samples of cancer patients, a 2 × 2 tables (see Predictive MiRNA Index, Table 4) is constructed to determine the clinical sensitivity and specificity of the microRA assay from miRNAs stool specimens' results.

The calculated sensitivity/specificity of the miRNA assay is compared to the FOBT assay in all the 180 subjects assessed in the same laboratory by the same investigators, as well as colonoscopy results obtained from patients' medical records, to establish TPCs. If the results are at least as specific as the FOBT (95%) and the sensitivity ≥ 95%, which exceeds colonoscopy, then this milestone will have been successfully achieved.

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