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MicroRNAs and Colon and Rectal Cancer: Differential Expression by Tumor Location and Subtype

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Abstract

MicroRNAs are thought to have an impact on cell proliferation, apoptosis, stress responses, maintenance of stem cell potency, and metabolism and are, therefore, important in the carcinogenic process. In this study, we examined 40 colon tumors, 30 rectal tumors, and 30 normal tissue samples (10 proximal colon, 10 distal colon, and 10 rectal paired with cancer cases) to examine miRNA expression profiles in colon and rectal tumors. MiRNA expression levels were adjusted for multiple comparisons; tumor tissue was compared with noncancerous tissue from the same site. A comparison of normal tissue showed 287 unique miRNAs that were significantly differentially expressed at the 1.5-fold level and 73 with over a two-fold difference in expression between colon and rectal tissue. Examination of miRNAs that were significantly differentially expressed at the 1.5-fold level by tumor phenotype showed 143 unique miRNAs differentially expression for microsatellite instability positive (MSI+) colon tumors; 129 unique miRNAs differentially expressed for CpG Island Methylator Phenotype positive (CIMP+) colon tumors; 135 miRNAs were differentially expressed for *KRAS2*-mutated colon tumors, and 139 miRNAs were differentially expressed for *TP53*-mutated colon tumors. Similar numbers of differentially expressed miRNAs were observed for rectal tumors, although the miRNAs differentially expressed differed. There were 129 unique miRNAs for CIMP+, 143 unique miRNAs for *KRAS2*-mutated, and 136 unique miRNAs for *TP53*-mutated rectal tumors. These results suggest the importance of miRNAs in colorectal cancer and the need for studies that can confirm these results and provide insight into the diet, lifestyle, and genetic factors that influence miRNA expression.

Introduction

MicroRNAs (miRNA) are a class of small regulatory RNAs that mediate post-transcriptional silencing of specific target mRNAs. These small non-coding RNAs are 18–24 nucleotides long (Smalheiser and Torvik, 2004). Like protein-coding genes, miRNAs show complex patterns of tissue- and disease-specific expression that merge with their ability to regulate many targets that are crucial to the carcinogenic process. They have been shown to have an impact on cell proliferation, apoptosis, stress responses, maintenance of stem cell potency, and metabolism. miRNAs are thought to have both oncogene and tumor suppressor gene regulatory roles. Although studies now show the importance of miRNAs in the etiology of colorectal cancer (CRC), few individuals and few individual miRNAs have been evaluated. Often data are from cell lines rather than population-based studies. Studies have not

evaluated colon and rectal cancer separately to determine associations with miRNAs expression.

In 2003, it was first reported that differential expression of miRNAs was associated with CRC (Michael et al., 2003). At that time, Michael and colleagues illustrated tumor suppressor-like activity for miR-143 and miR-145 in colon cancers and hypothesized that these miRNAs were targeting *ERK5* and *IRS1* (Michael et al., 2003). Over 100 miRNAs have been implicated in CRC since then (Yang et al., 2009). Generally, studies have focused on describing differential expression of miRNA and have yet to delve into the etiology of that differential expression. Akao and coworkers showed downregulation of let-7a-1 in colon tumors and in cell lines (Akao et al., 2006); miR-34a has been shown to inhibit cell proliferation and be downregulated in colon cancer cells (Yang et al., 2009), whereas miR-31, miR-96, miR-31, miR-135b, and miR-183 were upregulated in CRC cells in the same study (Yang et al., 2009).

MiRNA associations with specific tumor markers also have been reported. *TP53*, which is frequently mutated in CRC, has been a target of much of this research. Loss of miR-34 has been shown to impair *TP53*-mediated cell death while over-expressed miR-34 leads to apoptosis (He et al., 2007a; He et al., 2007b; He et al., 2007c). miRNAs also have been shown to be involved with oncogene regulation and with tumor suppressor gene regulation. MiR-143 and let-7 expression levels have been associated with *KRAS2* mutations (Schetter et al., 2010).

The ability to use paraffin-embedded tissue is essential to study tumor changes at the population level. A study of breast cancer demonstrated the ability to use paraffin-embedded tissue to examine miRNA expression (Hui et al., 2009). In that study, 365 miRNAs were examined in 40 paraffin-embedded breast tumors. Technical reproducibility was high, with intrasample correlation above 0.9 with 93% accuracy. Paraffin-embedded tissue also was compared to fresh-frozen tissue from the same breast cancer samples, and a correlation of 0.94 was found between miRNA expression levels.

In this study, we evaluate 70 colon and rectal tumor samples from a population-based sample of colon and rectal cancer and compare them with a smaller subset of 30 normal (20 colon and 10 rectal) tissue samples obtained from paraffin-embedded tissue from the colon and rectal cancer cases. We evaluate differences in expression of miRNA by colorectal site as well as by differences in specific tumor alterations, ie. CIMP+, MSI+, *KRAS2*, and *TP53*. We compare these specific tumor alterations with normal tissue expression levels as well as with expression levels observed for other tumor alterations to identify unique miRNA expression that accompanies specific disease pathways as indicated by unique molecular changes in tumors.

Material and Methods

Samples come from participants in two case–control studies, one of colon cancer and one of rectal cancer. The first study, a population-based case–control study of colon cancer, identified cases of first primary colon cancer between October 1, 1991 and September 30, 1994 (Slattery et al., 1997b). The second study, with identical data collection methods, included cases with cancer of the rectosigmoid junction or rectum identified between May 1997 and May 2001. Eligible criteria included: being between 30 and 79 years old at time of diagnosis (cases only), English speaking, mentally competent to complete the interview, having had no previous history of CRC, and having no known (as indicated on the pathology report) familial adenomatous polyposis, ulcerative colitis, or Crohn's disease. All participants included in this study resided in Utah or were members of the Kaiser Permanente Medical Care Program (KPMCP) in Northern California. Tumor tissue was

obtained for 97% of all diagnosed cases in Utah and 85% of diagnosed cases at KPMCP. Study details have been previously reported (Slattery et al., 1997a; Slattery et al., 2003b)

Tumor site within the colon was defined as proximal (cecum through transverse colon) or distal (splenic flexure, descending, and sigmoid colon) or rectal (rectosigmoid junction and rectum) as classified by the tumor registries. Samples were selected for this study based on known unique tumor characteristics so that comparison could be made for individuals with only CIMP+, MSI+, *KRAS2*, and *TP53*+ tumors. MSI+ tumors were not selected for rectal cancer given the rarity of these alternations in rectal cancer.

RNA Isolation From Paraffin-Embedded Tissue and Microarray Analysis

Formalin-fixed paraffin embedded (FFPE) tissue representing 30 matched normal tissue samples, 20 from colon cancer cases and 10 from rectal cancer cases, and 70 colon and rectal tumor samples were used. Five-micron tissue slices from these samples were mounted on glass slides and stained with either H&E for tumor reference or Aniline Blue for dissection. Depending on tumor size, we dissected one to four slides per FFPE tissue block to isolate enough RNA to generate expression data on the Agilent human miRNA microarray platform. The area on each slide dissected ranged from 2 mm × 3 mm to 10 mm × 4 mm. Total RNA containing miRNA was extracted, isolated, and purified using the RecoverAll Total Nucleic Acid isolation kit (Ambion, Austin TX). RNA yields were determined using a NanoDrop spectrophotometer. RNA yields ranged from 100 ng to 1 µg total RNA.

For all 100 tissue samples described above, total RNA preps were labeled with cy3 and hybridized to Agilent Human miRNA V3.0 Microarrays. Quality control reports from the arrays were examined, and one array was excluded due to high background noise on the array (approximately three times higher than all other arrays). Normalized intensity values from the remaining arrays were filtered to remove nonuniform outlier and population outlier features. The remaining intensity values were shifted (to eliminate negative values), log transformed (log base 2), and quantile normalized in R software using the BioConductor package (Gentleman et al., 2004). Hierarchical clustering using Ward's method in Spotfire DecisionSite software [<http://spotfire.tibco.com>] revealed a batch effect that corresponded to the date on which the microarrays were processed. The batch effect was corrected using ComBat software (Johnson et al., 2007).

We also prepared technical replicates by isolating two independent preps of RNAs from sequential sections derived from four normal colon blocks. These paired replicas were assessed for expression levels of the 866 human miRNAs present on the Agilent microarrays. All four pairs were highly reproducible with Pearson's correlations of greater than 0.95. It appears that our collections of tumor sections are able to provide high quality miRNAs, allowing us to generate global miRNA expression data.

Statistical Analysis

The log-scale normalized intensities were loaded into GeneSifter software for initial microarray analysis [<http://www.geospiza.com>]. Up- and downregulation of miRNA was determined based on site-specific, i.e., colon and rectal, expression levels in the normal tissue. For group comparisons, we determined differential expression in specific types of epigenetic and genetic tumor molecular phenotype based on site-specific normal tissue expression. We also assessed expression levels from specific tumor alterations to all other tumor alterations for colon and rectal cancer separately. We used the following criteria for all assessments: *t*-test *P* value of 0.05, adjusted *P* value by Benjamini and Hochberg (Benjamini, 1995), and a 1.5-fold difference in expression between comparison groups, i.e.,

tumor and normal and specific alterations versus other alteration. We report findings that are statistically significant using these criteria.

The Agilent human miRNA microarray V.3.0. targets 866 unique human miRNAs. The miRNA array contains 2370 unique human sequences and averages six features per probe sequence. miRNAs are referred to using standard nomenclature used in the miRBase database (Griffiths-Jones et al., 2006). Briefly, the first three letters signifies the organism, followed by a unique number. The number is followed by a dash and number (i.e., -1 or -2) if more than one loci codes for the miRNA. A lettered suffix denotes closely related miRNAs. If 2 miRNAs are coded for by the same precursor product, then the minor product is assigned the suffix (*). If predominant/minor product status is not known then the suffix -5p and -3p are used to denote 5' arm and 3' arm.

Results

We detected statistically significant 1.5-fold differences in expression for 287 miRNA probes comparing noncancerous normal tissue from the proximal and distal colon and rectal sites. Figure 1 shows the top 73 miRNAs with over a twofold difference in expression. It is of interest that differences in miRNA expression generally were not observed for distal colon and proximal colon tissue, but differences were common between rectal and colon tissue. The one exception to this was miR-150 that had more than a twofold decrease in expression for distal colon tissue, while rectal and proximal colon tissue showed similar levels of expression. Nineteen of the top 73 miRNA had multiple probes displaying similar site-specific differential expression. Because of these differences in expression levels of miRNAs between colon and rectal normal tissue, all subsequent tumor comparisons were based on-site specific comparisons.

One hundred forty-three (143) unique miRNA probes showed more than a 50% difference in expression when MSI+ colon tumors were compared with normal colon tissue. Of these, 48 miRNAs had two or more independent probes that were significantly up- or downregulated. The top 30 of these differentially expressed miRNAs are shown in Table 1; a complete listing of all statistically significant associations can be found online. Eight miRNAs appeared to be uniquely differentially expressed at a level of 1.5-fold or greater in MSI+ tumors compared to other tumor phenotypes.

Among colon tumors that were CIMP+, 230 miRNA probes, 129 of which were unique miRNAs, had greater than 50% differential expression compared to normal colon tissue. The top 30 miRNAs based on ratio of difference in expression compared with normal are shown in Table 2, and a complete listing of all significant miRNAs is included in the Supporting Information Tables. Comparison of CIMP+ colon tumors with other mutation-containing colon tumors showed eight unique miRNAs associated with CIMP+ tumors. For rectal CIMP+ tumors, there were 129 unique miRNAs (204 total probes) with a 50% or greater difference in expression compared to normal tissue. Of these, miR-135-b had the highest upregulation, with a 5.18 and 4.74 ratio (independent probes) compared with normal tissue. Comparing CIMP+ rectal tumors with all other mutated rectal tumors, there were eight unique miRNAs. MiR-146a had the greatest upregulation at 3.21 and 2.69 ratios.

Comparison of *KRAS2*-mutated colon tumors with normal colon tissue showed 135 probes differentially expressed at or above the 1.5-fold level; the top 30 of these miRNAs based on ratio of differences in expression are shown in Table 3. MiR-650 had the highest level of upregulation, being 3.02 greater than normal tissue. There were no significant differences

Additional Supporting Information may be found in the online version of this article.

between *KRAS2* mutations and other colon tumor mutations after adjusting for multiple comparisons. For rectal cancer, 87 of the 143 probes that showed statistically significant differences in miRNA expression were unique miRNAs. The top three miRNAs, all with duplicate probes, were miR-224 (upregulated ratio of 5.33 and 4.08), miR-135b (upregulated ratio of 4.42 and 4.29), and miR-451 (downregulated ratio of 4.27 and 4.15). Comparison of *KRAS2*-mutated rectal tumors with other rectal tumor mutations showed 10 unique miRNAs that were differentially expressed. MiR-146a had the largest degree of downregulation, with a ratio 2.63 and 2.43 compared to other tumor markers.

Colon *TP53*-mutated tumors compared with normal tissue demonstrated differential expression for 139 unique miRNA and 242 total probes; there were 11 that were unique to *TP53* when compared with other colon tumor phenotypes. The highest level of upregulation when compared with normal was for miR-224 with a ratio of 5.97 above normal; miR-650 was downregulated at a 4.61 ratio to normal tissue. The top probes with differential expression comparing normal tissue with tumor tissue are shown in Table 4. There were 136 unique miRNA associated with rectal *TP53*-mutated tumors compared with normal tissue; 82 of these miRNA had duplicate probes that exhibited up- or downregulation. MiR-224, miR-183, and miR-21 showed the highest degree of upregulation. A comparison of *TP53*-mutated rectal tumors with other rectal tumor markers showed five unique miRNA that were up- or downregulated. MiR-187, which was down-regulated, showed the highest degree of difference from other tumor markers.

Discussion

Numerous studies have shown that miRNAs are extensively involved in cancer pathogenesis of solid tumors and support their function as dominant or recessive genes (Volinia et al., 2006). We know that miRNAs are highly conserved across species, are tissue specific, and play a critical role in regulation of proliferation, differentiation, apoptosis, and stress response (Iorio and Croce, 2009). What is not known is the extent to which miRNAs are involved in colon and rectal cancer. Data from this study suggest that miRNA are differentially expressed in colon and rectal cancers.

It is believed that miRNAs are tissue specific (Aslam et al., 2009). Our work and that of others have shown that both disease-correlated genetic and lifestyle factors differ for colon and rectal cancers (Alexander and Cushing, 2010; Slattery et al., 2003a; Slattery et al., 2003b; Slattery et al., 2004a; Slattery et al., 2004b; Slattery et al., 2004c; Slattery et al., 2007; Iacopetta et al., 2009; Slattery et al., 2009). Additionally, other characteristics of these cancers suggest two separate diseases, such as differences in survival, younger age at diagnosis for rectal cancer, smaller risk associated with family history of CRC for rectal cancer and fewer inherited syndromes, such as Lynch syndrome, adenomatous polyposis coli (APC), and attenuated APC involving rectal cancer. The data we present add support for these disparate findings, in that over 200 miRNAs are differentially expressed in colon and rectal normal tissue. These differences in miRNA expression levels support the hypothesis that two diseases each arising from different tissues exist and that associations can be masked when studying them as one disease.

It has been suggested that regulation of miRNA expression in colon and rectal cancers may involve epigenetic mechanisms. Transfection of miR-342 into colon cancer cell lines has been shown to lead to apoptosis that can be inhibited by methylation (Grady et al., 2008). Four miRNAs (miR-124a, let-7a-3, miR-10a, and miR-342) have been shown to be silenced by DNA hypomethylation (Lujambio et al., 2007). The miR-17-92 miRNA family has been associated with MSI+ tumors (Lanza et al., 2007). Of these, only miR-10a was upregulated significantly and only for colon tumors compared with normal tissue. MiR-10a was

upregulated with two probes being significantly associated with CIMP+ tumors (ratios of 2.5 and 2.3 when compared with normal tissue). However, it should be noted that while this finding is replicated, it was ranked number 54 and 69 in terms of miRNAs that were differently expressed in CIMP+ tumors.

Several studies have focused on *TP53* and miRNA, in part because of the hypothesized role with inflammation (Schetter et al., 2010). Work focusing on inflammation has shown links between nuclear factor kappa B (NF κ B), inflammatory cytokines, prostaglandins, reactive oxygen and nitrogen species and specific miRNAs (Schetter, et al.). MiR-155 and miR-146 have been associated with NF κ B, suppressor of cytokine signaling 1 (SOCS1), interleukin 6 (IL6), and tumor necrosis factor-alpha (TNF- α); let-7i has been associated with IL6 pathways; miR-21 has been associated with IL6, signal transducer and activator of transcription 3 (STAT3), phosphatase and tensin homolog (PTEN), and Interleukin 12 (IL12). Polymorphisms in some of these inflammatory-related genes also have been associated with miRNAs (Schetter et al.; Taganov et al., 2006). Many of the miRNAs associated with inflammation-related genes also have been associated with *TP53*-mutated tumors. MiRNA expression levels have been associated with the vascular endothelial growth factor (VEGF) and angiogenesis signaling pathways in studies of nasopharyngeal carcinomas (Chen et al., 2009). VEGF and other angiogenesis genes are intertwined with an inflammation-related pathway that is hypothesized as being important to the etiology of colon and rectal cancer (Slattery and Fitzpatrick, 2009). Of these previously reported associations, only miR-21 was associated with both colon and rectal cancer, with a more prominent role in rectal cancer. For rectal cancer, miR-21 was upregulated at two probes, ranking number 2 and number 8 on the list, with a ratio of 4.16 and 3.74 compared with normal tissue. For colon cancers compared with normal two probes also were upregulated at rank number 17 and 66 and corresponding ratios of 2.85 and 2.45. Let-7i also was upregulated in rectal tumors, ranking number 74 and 117 (upregulated ratios of 2.18 and 1.89).

Most other studies have targeted a few preselected miRNAs with a hypothesized mechanism of action. In a field where there is rapid increase in knowledge, dismissing the potential spectrum of associations to only a handful of previously examined miRNAs will miss many important associations, as shown here. In this study, we evaluated all 866 known human miRNAs; we identified hundreds of miRNAs that were differently expressed in tumors relative to normal tissue. Many of these miRNAs have been linked to specific pathways and gene targets but have not been studied with colon and rectal cancer.

Assessing target genes of miRNAs that were the most differentially expressed showed several important targets (Betel et al., 2008). MiR-135b has been shown to target genes such as signal transducer and activator of transcription 6 (*STAT6*), insulin receptor substrate 2 (*IRS2*), bone morphogenetic protein receptor, type II (*BMPR2*) and bone morphogenetic protein receptor, type IA (*BMPR1A*) which are members of the TGF- β -signaling pathway and recently shown to be associated with colon and rectal cancer (Slattery et al., 2010). These genes were further linked to important phenotypes of ulcerative colitis, obesity, and CRC. MiR-224 was linked to important phenotypes of obesity and insulin resistance and targeted genes such as adiponectin (*ADIPOQ*), mothers against decapentaplegic 4 (*SMAD4*), breast cancer 1 (*BRCA1*), alcohol dehydrogenase 1D (*ADH1D*), and interleukin 1 receptor (*IL1R*). MiR-146a also appeared to be important for inflammation and obesity related mechanisms and targeted genes such as C-reactive protein (*CRP*), *SMAD4*, leptin (*LEP*), *IRS2*, *BMPR1A*, and prostaglandin-endoperoxide synthase 2 (*PTGS2* or *COX2*). MiR-139-3p associated with colon cancer in our study appears to target adiposity, insulin, and colon cancer-related genes such as lipoprotein lipase (*LPL*), kinase insert domain receptor (*KDR*, a *VEGF* receptor), and cyclin D1 (*CCND1*). MiR-451 also appears to target

important genes within obesity, inflammation, and insulin-related pathways, including *IL6R* and tumor necrosis factor ligand superfamily member 15 (*TNFSF15*). MiR-183 appears to target insulin receptor substrate 1 (*IRS1*), *PTEN*, methylenetetrahydrofolate reductase (*MTHFR*), *NFKB1*, *PTGS2*, insulin-like growth factor (*IGF*), transforming growth factor beta receptor 1 (*TGFBR1*), all important genes in colon and rectal cancer. MiR-187 also targets important genes involved in obesity, insulin, and inflammation-related pathways including tumor necrosis factor (*TNF*), *ADIPOQ*, *LEPR*, insulin receptor (*INSR*), peroxisome proliferator-activated receptor gamma (*PPARG*), and ornithine decarboxylase (*ODC*). Further examination of miRNAs at a more global level may provide important information into how genes and pathways are linked together to influence cancer risk.

The data presented here are limited in the number of samples examined and their selection was based on known tumor characteristics. Thus, this does not represent the importance of miRNAs to all colon and rectal cancers, but rather to colon and rectal cancers with specific tumor characteristics. In addition, although technical replicates and independent probes for specific miRNAs showed a high level of correlation of expression levels, it is important to validate these findings using methods such as TaqMan assays to verify quantitative miRNA expression levels. Another limitation to this study is that we used a subset of normal tissue from cases with tumor tissue for comparison. Ideally, all cases would have had normal tissue samples analyzed as paired samples, but such an approach was cost prohibitive and we believe that the normal tissues chosen were representative. However, despite these limitations, the study is the most extensive evaluation of miRNA in colon and rectal cancers to date. Given our ability to separate colon and rectal tumors, we can gain insight into important differences between these two diseases that can help substantiate our previous observations of differences in risk factors for the two cancers.

It has been stated that “Every cellular process is likely to be regulated by microRNAs, and an aberrant microRNA expression signature is a hallmark of several diseases, including cancer” (Iorio and Croce, 2009). The importance of understanding how miRNAs function as well as what causes their expression to be increased or decreased is only beginning to be understood. However, given their importance to colon and rectal cancer, as demonstrated here by the number of miRNAs that are differentially expressed in tumor tissue, is it imperative that we attempt to understand how they function in the carcinogenic process from disease development to survival after diagnosis, using powerful samples with the capability of exploring the spectrum of the disease process.

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Figure 1.
Comparison of miRNA expression from normal, proximal, and distal colon and rectal cancers.

Table 1

Associations Between MSI and miRNA Expression Levels

Colon MSI vs. normal tissue				Colon MSI vs. other tumor changes			
Gene name	Ratio	Direction	adj. P-value	Gene name	Ratio	Direction	adj. P-value
hsa-miR-7	4.08	Up	0.000	hsa-miR-139-3p	2.50	Up	0.028
hsa-miR-183	3.33	Up	0.001	hsa-miR-223	1.99	Up	0.028
hsa-miR-195	3.25	Down	0.001	hsa-let-7g*	1.87	Down	0.028
hsa-miR-21	2.98	Up	0.008	hsa-miR-552	1.78	Down	0.028
hsa-miR-183	2.95	Up	0.002	hsa-miR-223	1.72	Up	0.028
hsa-miR-21	2.91	Up	0.015	hsa-miR-424*	1.67	Down	0.029
hsa-miR-145	2.71	Down	0.002	hsa-miR-24-2*	1.58	Down	0.047
hsa-miR-93	2.69	Up	0.001	hsa-miR-370	1.57	Up	0.028
hsa-miR-451	2.67	Down	0.036				
hsa-miR-1	2.67	Down	0.001				
hsa-miR-497	2.58	Down	0.001				
hsa-miR-17	2.57	Up	0.007				
hsa-miR-195	2.57	Down	0.001				
hsa-let-7i	2.57	Up	0.002				
hsa-miR-34a	2.56	Up	0.005				
hsa-miR-451	2.49	Down	0.045				
hsa-let-7i	2.49	Up	0.003				
hsa-miR-34a	2.47	Up	0.002				
hsa-miR-650	2.42	Down	0.001				
hsa-miR-215	2.39	Down	0.016				
hsa-miR-223	2.37	Up	0.016				
hsa-miR-150	2.35	Down	0.027				
hsa-miR-224	2.32	Up	0.000				
hsa-miR-215	2.3	Down	0.016				
hsa-miR-20a	2.26	Up	0.019				
hsa-miR-145	2.26	Down	0.002				
hsa-miR-25	2.22	Up	0.009				

Colon MSI vs. normal tissue				Colon MSI vs. other tumor changes			
Gene name	Ratio	Direction	adj. P-value	Gene name	Ratio	Direction	adj. P-value
hsa-miR-130b	2.19	Up	0.001				
hsa-miR-20a	2.19	Up	0.019				
hsa-miR-92a	2.15	Up	0.013				

Table 2

Associations Between CIMP+ Tumors and miRNA Expression Levels

Colon CIMP+ vs normal				CIMP+ vs. other tumor markers				Rectal CIMP+ vs normal				Rectal CIMP+ vs other tumor markers			
Gene name	Ratio	Direction	adj. P-value	Gene name	Ratio	Direction	adj. P-value	Gene name	Ratio	Direction	adj. P-value	Gene name	Ratio	Direction	adj. P-value
hsa-miR-183	3.81	Up	0.0000	hsa-miR-24-2*	3.25	Down	0.030	hsa-miR-135b	5.18	Up	0.0001	hsa-miR-146a	3.21	Up	0.026
hsa-miR-650	3.69	Down	0.0000	hsa-miR-3 1	1.92	Up	0.030	hsa-miR-224	4.81	Up	0.0001	hsa-miR-146a	2.69	Up	0.047
hsa-miR-183	3.4	Up	0.0002	hsa-miR-892b	1.84	Down	0.035	hsa-miR-135b	4.74	Up	0.0007	hsa-miR-625	1.74	Up	0.047
hsa-miR-224	3.37	Up	0.0001	hsa-miR-646	1.69	Down	0.030	hsa-miR-21	4.25	Up	0.0029	hsa-miR-551b*	1.63	Down	0.047
hsa-miR-892b	3.27	Down	0.0004	hsa-miR-3 1*	1.62	Up	0.030	hsa-miR-375	3.70	Down	0.0477	hsa-miR-1 297	1.60	Up	0.047
hsa-miR-1305	3.21	Down	0.0002	hsa-miR-151-3p	1.55	Up	0.035	hsa-miR-7	3.65	Up	0.0132	hsa-miR-625	1.57	Up	0.047
hsa-miR-21	3.2	Up	0.0005	hsa-miR-338-3p	1.53	Up	0.094	hsa-miR-224	3.54	Up	0.0005	hsa-miR-769-3p	1.53	Down	0.047
hsa-miR-892b	3.18	Down	0.0003	hsa-miR-492	1.53	Up	0.030	hsa-miR-146a	3.42	Up	0.0030	hsa-miR-1 87*	1.53	Down	0.047
hsa-miR-29a	3.17	Up	0.0001					hsa-miR-375	3.41	Down	0.0149	hsa-miR-1 537	1.53	Down	0.047
hsa-miR-34a	3.12	Up	0.0006					hsa-miR-1	3.41	Down	0.0043	hsa-miR-635	1.52	Up	0.047
hsa-miR-139-3p	3.11	Down	0.0204					hsa-miR-21	3.40	Up	0.0146				
hsa-miR-24-2*	3.11	Down	0.0335					hsa-miR-1 33b	3.30	Down	0.0008				
hsa-miR-21	3.07	Up	0.0016					hsa-miR-145	3.28	Down	0.0094				
hsa-miR-29b	3.01	Up	0.0003					hsa-miR-17	3.10	Up	0.0014				
hsa-miR-1288	2.99	Down	0.0003					hsa-miR-17	3.07	Up	0.0017				
hsa-miR-29a	2.96	Up	0.0002					hsa-miR-20a	3.04	Up	0.0014				
hsa-miR-92a	2.94	Up	0.0003					hsa-miR-183	3.04	Up	0.0053				
hsa-miR-7	2.91	Up	0.0006					hsa-miR-20a	2.98	Up	0.0013				
hsa-miR-34a	2.88	Up	0.0004					hsa-miR-650	2.95	Down	0.0094				
hsa-miR-25	2.88	Up	0.0004					hsa-miR-183	2.80	Up	0.0048				
hsa-miR-92a	2.88	Up	0.0005					hsa-miR-20b	2.78	Up	0.0048				
hsa-miR-21*	2.86	Up	0.0001					hsa-miR-145	2.75	Down	0.0139				
hsa-miR-3 1	2.86	Up	0.0066					hsa-miR-146a	2.73	Up	0.0115				
hsa-miR-20a	2.84	Up	0.0007					hsa-miR-183	2.71	Up	0.0056				
hsa-miR-17	2.83	Up	0.0007					hsa-miR-34a	2.69	Up	0.0013				
hsa-miR-1305	2.76	Down	0.0006					hsa-miR-552	2.53	Up	0.0030				
hsa-miR-93	2.73	Up	0.0006					hsa-miR-93	2.51	Up	0.0013				

Colon CIMP+ vs normal				CIMP + vs. other tumor markers				Rectal CIMP+ vs normal				Rectal CIMP+ vs other tumor markers			
Gene name	Ratio	Direction	adj. <i>P</i> -value	Gene name	Ratio	Direction	adj. <i>P</i> -value	Gene name	Ratio	Direction	adj. <i>P</i> -value	Gene name	Ratio	Direction	adj. <i>P</i> -value
hsa-miR-96	2.72	Up	0.0002					hsa-miR-29a	2.47	Up	0.021				
hsa-miR-1288	2.64	Down	0.0004					hsa-miR-96	2.44	Up	0.0043				
hsa-miR-892b	2.64	Down	0.0002					hsa-miR-497	2.41	Down	0.0133				

Table 3
Associations Between *KRAS2* Mutations in Colon and Rectal Tumors and miRNA Expression Levels

Colon <i>KRAS2</i> vs. normal tissue				Rectal <i>KRAS2</i> vs. normal tissue				Rectal <i>KRAS2</i> vs. other tumor markers			
Gene name	Ratio	Direction	adj. P-value	Gene name	Ratio	Direction	adj. P-value	Gene name	Ratio	Direction	adj. P-value
hsa-miR-650	3.02	Down	0.001	hsa-miR-224	5.33	Up	0.0000	hsa-miR-146a	2.63	Down	0.028
hsa-miR-150	2.99	Down	0.010	hsa-miR-135b	4.42	Up	0.0000	hsa-miR-146a	2.43	Down	0.028
hsa-miR-139-3p	2.92	Down	0.039	hsa-miR-135b	4.29	Up	0.0000	hsa-miR-451	2.09	Down	0.036
hsa-miR-195	2.75	Down	0.006	hsa-miR-451	4.27	Down	0.0001	hsa-miR-451	2.06	Down	0.036
hsa-miR-183	2.7	Up	0.001	hsa-miR-451	4.15	Down	0.0001	hsa-miR-615-5p	1.89	Down	0.050
hsa-miR-20a	2.63	Up	0.003	hsa-miR-552	4.13	Up	0.0001	hsa-miR-146b-5p	1.78	Down	0.034
hsa-miR-20a	2.58	Up	0.003	hsa-miR-224	4.08	Up	0.0001	hsa-miR-572	1.75	Up	0.034
hsa-miR-17	2.55	Up	0.003	hsa-miR-1	4.05	Down	0.0005	hsa-miR-572	1.65	Up	0.028
hsa-miR-92a	2.54	Up	0.003	hsa-miR-552	3.93	Up	0.0003	hsa-miR-638	1.65	Up	0.036
hsa-miR-183	2.54	Up	0.003	hsa-miR-133b	3.79	Down	0.0001	hsa-miR-342-3p	1.59	Down	0.034
hsa-miR-215	2.53	Down	0.014	hsa-miR-183	3.66	Up	0.0007	hsa-miR-23b	1.58	Down	0.036
hsa-miR-34a	2.51	Up	0.006	hsa-miR-552	3.65	Up	0.0001	hsa-miR-491-5p	1.55	Down	0.036
hsa-miR-34a	2.48	Up	0.003	hsa-miR-375	3.65	Down	0.0116	hsa-miR-638	1.51	Up	0.036
hsa-miR-92a	2.48	Up	0.003	hsa-miR-650	3.59	Down	0.0013	hsa-miR-342-3p	1.50	Down	0.036
hsa-miR-29a	2.46	Up	0.006	hsa-miR-195	3.57	Down	0.0017				
hsa-miR-21	2.45	Up	0.018	hsa-miR-183	3.5	Up	0.0004				
hsa-miR-195	2.43	Down	0.003	hsa-miR-195	3.39	Down	0.0017				
hsa-miR-96	2.35	Up	0.001	hsa-miR-497	3.38	Down	0.0012				
hsa-miR-224	2.33	Up	0.003	hsa-miR-145	3.27	Down	0.0015				
hsa-miR-215	2.31	Down	0.018	hsa-miR-375	3.25	Down	0.0029				
hsa-miR-125b-2*	2.29	Down	0.054	hsa-miR-17	3.08	Up	0.0005				
hsa-miR-451	2.29	Down	0.039	hsa-miR-92a	3.04	Up	0.0000				
hsa-miR-150	2.28	Down	0.015	hsa-miR-150	2.97	Down	0.0005				
hsa-miR-451	2.22	Down	0.039	hsa-miR-92a	2.93	Up	0.0000				
hsa-miR-29a	2.22	Up	0.010	hsa-miR-145	2.91	Down	0.0016				
hsa-miR-17	2.22	Up	0.003	hsa-miR-21	2.86	Up	0.0029				
hsa-miR-552	2.17	Up	0.010	hsa-miR-20a	2.79	Up	0.0011				

Colon <i>KRAS2</i> vs. normal tissue				Rectal <i>KRAS2</i> vs. normal tissue				Rectal <i>KRAS2</i> vs. other tumor markers			
Gene name	Ratio	Direction	adj. P-value	Gene name	Ratio	Direction	adj. P-value	Gene name	Ratio	Direction	adj. P-value
hsa-miR-125b-2*	2.14	Down	0.054	hsa-miR-96	2.67	Up	0.0001				
hsa-miR-21	2.13	Up	0.053	hsa-miR-183	2.64	Up	0.0016				
hsa-miR-139-3p	2.09	Down	0.049	hsa-miR-20a	2.62	Up	0.0017				

There were no miRNAs with adjusted *P* value above 0.05 when comparing.

Table 4
Associations Between *TP53* and miRNA Expression Levels

Colon <i>TP53</i> vs. normal tissue				Colon <i>TP53</i> vs. other tumor markers				Rectal <i>TP53</i> vs. normal tissue				Rectal <i>TP53</i> vs. other tumor markers			
Gene name	Ratio	Direction	adj. <i>P</i> -value	Gene name	Ratio	Direction	adj. <i>P</i> -value	Gene name	Ratio	Direction	adj. <i>P</i> -value	Gene name	Ratio	Direction	adj. <i>P</i> -value
hsa-miR-224	5.97	Up	0.0000	hsa-miR-224	2.20	Up	0.033	hsa-miR-224	4.90	Up	0.0000	hsa-miR-187	1.98	Down	0.017
hsa-miR-650	4.61	Down	0.0000	hsa-miR-224	2.00	Up	0.035	hsa-miR-21	4.16	Up	0.0002	hsa-miR-135b	1.94	Down	0.050
hsa-miR-17	4.54	Up	0.0000	hsa-miR-548a-3p	1.92	Down	0.030	hsa-miR-183	4.02	Up	0.0001	hsa-miR-135b	1.81	Down	0.041
hsa-miR-139-3p	4.18	Down	0.0044	hsa-miR-17	1.91	Up	0.043	hsa-miR-224	3.87	Up	0.0000	hsa-miR-302b*	1.53	Down	0.006
hsa-miR-17	4.18	Up	0.0000	hsa-miR-1226	1.82	Down	0.050	hsa-miR-183	3.81	Up	0.0000	hsa-miR-541*	1.53	Down	0.022
hsa-miR-20a	4.08	Up	0.0000	hsa-miR-532-5p	1.69	Up	0.025	hsa-miR-1	3.78	Down	0.0004	hsa-miR-302b	1.52	Down	0.022
hsa-miR-224	4.02	Up	0.0000	hsa-miR-17	1.69	Up	0.034	hsa-miR-17	3.74	Up	0.0000				
hsa-miR-20a	3.92	Up	0.0000	hsa-miR-573	1.68	Down	0.043	hsa-miR-21	3.68	Up	0.0014				
hsa-miR-92a	3.64	Up	0.0001	hsa-miR-574-5p	1.67	Down	0.050	hsa-miR-133b	3.68	Down	0.0000				
hsa-miR-92a	3.57	Up	0.0002	hsa-miR-424*	1.63	Up	0.043	hsa-miR-20a	3.48	Up	0.0000				
hsa-miR-29a	3.53	Up	0.0000	hsa-miR-501-3p	1.61	Up	0.030	hsa-miR-17	3.46	Up	0.0000				
hsa-miR-93	3.18	Up	0.0000	hsa-miR-890	1.57	Down	0.043	hsa-miR-20a	3.42	Up	0.0000				
hsa-miR-1305	3.06	Down	0.0001	hsa-miR-16-1*	1.54	Down	0.030	hsa-miR-375	3.40	Down	0.0107				
hsa-miR-1288	3.02	Down	0.0002	hsa-miR-424*	1.54	Up	0.035	hsa-miR-92a	3.35	Up	0.0000				
hsa-miR-150	3.01	Down	0.0030					hsa-miR-92a	3.26	Up	0.0000				
hsa-miR-1305	3	Down	0.0002					hsa-miR-7	3.08	Up	0.0022				
hsa-miR-29a	2.95	Up	0.0001					hsa-miR-145	2.98	Down	0.0017				
hsa-miR-21*	2.95	Up	0.0000					hsa-miR-552	2.97	Up	0.0007				
hsa-miR-552	2.93	Up	0.0001					hsa-miR-183	2.91	Up	0.0007				
hsa-miR-183	2.9	Up	0.0000					hsa-miR-17	2.90	Up	0.0002				
hsa-miR-221	2.9	Up	0.0001					hsa-miR-20b	2.88	Up	0.0002				
hsa-miR-552	2.89	Up	0.0001					hsa-miR-650	2.86	Down	0.0014				
hsa-miR-1288	2.86	Down	0.0001					hsa-miR-552	2.83	Up	0.0019				
hsa-miR-17	2.86	Up	0.0000					hsa-miR-455-3p	2.81	Up	0.0001				
hsa-miR-20b	2.86	Up	0.0000					hsa-miR-96	2.80	Up	0.0002				
hsa-miR-29b	2.85	Up	0.0002					hsa-miR-96	2.78	Up	0.0002				
hsa-miR-21	2.85	Up	0.0008					hsa-miR-375	2.78	Down	0.0035				

Colon <i>TP53</i> vs. normal tissue				Colon <i>TP53</i> vs. other tumor markers				Rectal <i>TP53</i> vs. normal tissue				Rectal <i>TP53</i> vs. other tumor markers			
Gene name	Ratio	Direction	adj. <i>P</i> -value	Gene name	Ratio	Direction	adj. <i>P</i> -value	Gene name	Ratio	Direction	adj. <i>P</i> -value	Gene name	Ratio	Direction	adj. <i>P</i> -value
hsa-miR-892b	2.77	Down	0.0010					hsa-miR-552	2.70	Up	0.0025				
hsa-miR-183	2.76	Up	0.0001					hsa-miR-17*	2.67	Up	0.0002				
hsa-miR-375	2.75	Down	0.0012					hsa-miR-424	2.59	Up	0.0018				