Modern Pathology (2016) 29, 915–927



# Colorectal tumor molecular phenotype and miRNA: expression profiles and prognosis

Martha L Slattery[1], Jennifer S Herrick[1], Lila E Mullany[1], Erica Wolff[1], Michael D Hoffman[1], Daniel F Pellatt[1], John R Stevens[2] and Roger K Wolff[1]

[1]*Department of Medicine, University of Utah, Salt Lake City, UT, USA and* [2]*Department of Mathematics and Statistics, Utah State University, Logan, UT, USA*

**MiRNAs regulate gene expression by post-transcriptionally suppressing mRNA translation or by causing mRNA degradation. It has been proposed that unique miRNAs influence specific tumor molecular phenotype. In this paper, we test the hypotheses that miRNA expression differs by tumor molecular phenotype and that those differences may influence prognosis. Data come from population-based studies of colorectal cancer conducted in Utah and the Northern California Kaiser Permanente Medical Care Program. A total of 1893 carcinoma samples were run on the Agilent Human miRNA Microarray V19.0 containing 2006 miRNAs. We assessed differences in miRNA expression between *TP53*-mutated and non-mutated, *KRAS*-mutated and non-mutated, *BRAF*-mutated and non-mutated, CpG island methylator phenotype (CIMP) high and CIMP low, and microsatellite instability (MSI) and microsatellite stable (MSS) colon and rectal tumors. Using a Cox proportional hazard model we evaluated if those miRNAs differentially expressed by tumor phenotype influenced survival after adjusting for age, sex, and AJCC stage. There were 22 differentially expressed miRNAs for *TP53*-mutated colon tumors and 5 for *TP53*-mutated rectal tumors with a fold change of $>1.49$ (or $<0.67$). Additionally, 13 miRNAS were differentially expressed for *KRAS*-mutated rectal tumors, 8 differentially expressed miRNAs for colon CIMP high tumors, and 2 differentially expressed miRNAs for *BRAF*-mutated colon tumors. The majority of differentially expressed miRNAS were observed between MSI and MSS tumors (94 differentially expressed miRNAs for colon; 41 differentially expressed miRNAs for rectal tumors). Of these miRNAs differentially expressed between MSI and MSS tumors, the majority were downregulated. Ten of the differentially expressed miRNAs were associated with survival; after adjustment for MSI status, five miRNAS, miR-196b-5p, miR-31-5p, miR-99b-5p, miR-636, and miR-192-3p, were significantly associated with survival. In summary, it appears that the majority of miRNAs that are differentially expressed by tumor molecular phenotype are MSI tumors. However, these miRNAs appear to have minimal effect on prognosis.**
*Modern Pathology* (2016) **29,** 915–927; doi:10.1038/modpathol.2016.73; published online 20 May 2016

MiRNAs are small non-protein-coding RNA molecules that regulate gene expression either by post-transcriptionally suppressing mRNA translation or by causing mRNA degradation.[1–6] We know that miRNAs play a critical role in regulation of proliferation, differentiation, apoptosis, and stress response and are involved in the majority of physiological processes.[7,8] In our previous work we have shown that colorectal carcinoma cells show widespread dysregulation from normal mucosa[9] and that differences by tumor molecular phenotype also exist.[10] Others also have explored miRNAs with tumor molecular phenotype to gain insight into unique pathways involved in colorectal carcinoma. Most work in this area has focused on microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) tumors,[11] although data also suggest that *TP53*-mutated tumors, *KRAS*-mutated tumors, and *BRAF*-mutated tumors may be uniquely associated with specific miRNAs.[12–14] Most studies to date have focused on specific miRNAs to see if they are associated with specific tumor molecular phenotype. Few have looked broadly across the range of miRNAs to identify associations with specific tumor molecular phenotype.

Survival differences by tumor phenotype also have been shown especially for MSI, CIMP high, and *BRAF*-mutated tumors.[15–18] We have observed differences in survival for microsatellite unstable colon

Correspondence: Professor ML Slattery, PhD, MPH, Internal Medicine, University of Utah, 383 Colorow, Salt Lake City, 84108 UT, USA.
E-mail: Marty.Slattery@hsc.utah.edu
Received 4 January 2016; revised 29 February 2016; accepted 6 March 2016; published online 20 May 2016

and rectal cancer when compared with microsatellite stable tumors, in that individuals diagnosed with colon cancer with microsatellite unstable tumors had improved survival while those diagnosed with rectal cancer and microsatellite unstable tumors had worse survival.[16,17] It is possible that miRNAs that differ by MSI could explain differences in survival observed for colon and rectal cancer.

In this study we examine differences in expression of miRNAs in colon and rectal carcinoma based on tumor molecular phenotype. We assess unique miRNA expression associated with *TP53*-mutated, *KRAS*-mutated, *BRAF*-mutated, CIMP, and microsatellite unstable tumors. An Agilent Array, containing over 2000 miRNAs, was used to fully evaluate the range of miRNAs expressed in colorectal cancer tissue. We consider the association with survival for those miRNAs that are differentially expressed by tumor molecular phenotype to better understand the role of these miRNAs on prognosis after diagnosis with colorectal cancer.

## Materials and methods

### Study Participants

The study was approved by the Institutional Review Board of the University of Utah; study participants signed informed consent. Study participants came from two population-based case–control studies that included all incident colon and rectal cancers between 30 and 79 years of age who resided along the Wasatch Front in Utah or were members of the Kaiser Permanente Medical Care Program in Northern California. Participants were white, Hispanic, or black for the colon cancer study; the rectal cancer study also included Asians and American Indians not living on reservations.[19,20] Cases had to have tumor registry verification of a first primary adenocarcinoma of the colon or rectum and were diagnosed between October 1991 and September 1994 for the colon cancer and between June 1997 and May 2001 for the rectal cancer. Tumor tissue was obtained for 97% of all Utah cases diagnosed and for 85% of all Kaiser Permanente Medical Care Program in Northern California study participants.[21] Detail study methods have been described.[9] Individuals with familial adenomatous polyposis coli, inflammatory bowel disease, Crohn's disease, and known Lynch syndrome were excluded.

### miRNA Processing

RNA was extracted from formalin-fixed paraffin embedded tissue using Ambion's RecoverAll Total Nucleic Acid isolation kit. Normal mucosa adjacent to the carcinoma tissue was used. The Agilent Human miRNA Microarray V19.0 was used, given the number of miRNAs, its high level of reliability (repeatability coefficient was 0.98 in our data), and the amount of RNA needed to run the platform. The microarray contains probes for 2006 unique human miRNAs. A total of 100 ng total RNA was labeled with Cy3 and hybridized to the Agilent Microarray and were scanned on an Agilent SureScan microarray scanner model G2600D. Data were extracted from the scanned image using Agilent Feature Extract software v.11.5.1.1. Data were required to pass stringent quality control parameters established by Agilent that included tests for excessive background fluorescence, excessive variation among probe sequence replicates on the array, and measures of the total gene signal on the array to assess low signal. If samples failed to meet quality standards for any of these parameters, the sample was re-labeled, hybridized to arrays, and scanned. If a sample failed quality control assessment a second time, the sample was deemed to be of poor quality and the individual was excluded from downstream analysis. The Agilent platform was shown to have high repeatability ($r=0.98$) and good correlation with other platforms such as Nanostring.[9] Other analysis of a subset of important miRNAs showed 100% agreement in terms of directionality of differential expression and fold change.[22]

### Tumor Molecular Phenotype

We have previously assessed *TP53 and KRAS* mutations, the CIMP using the classic panel,[18] and MSI on the mononucleotide repeats at *BAT26* and *TGFβRII* and a panel of 10 tetranucleotide repeats that were correlated highly with the Bethesda Panel;[23] our study was done prior to the development of the Bethesda Panel. The classic CIMP panel consisted of *MLH1*, *p16*, and MINT1, MINT2, and MINT31. Tumors were scored as CIMP high if two or more of the CpG islands were methylated, otherwise they were classified as CIMP low. This panel was run prior to the advent of more recent panels.[24,25] Additionally, the *BRAF* V600E mutation was assessed in colon carcinomas.[15]

### Tumor Registry Data

Both Utah and California are members of the National Cancer Institute funded Surveillance, Epidemiology, and End Disease Program. In addition to disease stage data, they provided follow-up data on all study participants which included total number of months survived, date of death or date of last follow-up, and cause of death. Follow-up was complete through 2006.

### Statistical Methods

Of the 2006 unique human miRNAs assessed, 1278 were expressed in colorectal carcinoma tissue. To normalize differences in miRNA expression that

could be attributed to the array, the amount of RNA, location on array, or other factors that could erroneously influence expression, total gene signal was normalized by multiplying each sample by a scaling factor[26] (http://genespring-support.com/files/gs_12_6/GeneSpring-manual.pdf), which was the median of the 75th percentiles of all the samples divided by the individual 75th percentile of each sample. We limited our analysis to miRNAs that were expressed in at least five carcinoma tissue samples and also had the mutated tumor phenotype of interest. Analysis ranged from 983 miRNAs assessed with *TP53* in colon cancer to 694 miRNAs assessed with MSI in rectal cancer.

We assessed differences between tumor phenotype mutated and non-mutated, microsatellite unstable *vs* microsatellite stable, and CIMP high *vs* CIMP low samples for carcinoma tissue. We used log base 2 transformed miRNA expression levels using the significance analysis of microarrays technique implemented in the R package siggenes;[27] *P*-values were based upon 1000 permutations. To adjust for multiple comparisons, we applied a false discovery rate level of significance of 0.05 based on Benjamini and Hochberg[28] as implemented in siggenes. For those miRNAs that were significantly differentially expressed, we report the mean level of expression and the fold change (on non-log-transformed data) between the tumor phenotypes. We present statistically significant data where the fold change was 1.50 or higher or 0.67 or lower.

We further assessed the impact of differentially expressed miRNAs between mutated and non-mutated tumors on survival for rectal cancer and colon cancer separately. We calculated Cox proportional hazard ratios and corresponding 95% confidence intervals adjusting for age, sex, and AJCC stage. We also adjusted for MSI status since microsatellite instability has been shown to be associated with survival. Our statistical end point for our survival analysis was CRC cancer-specific survival based on months between diagnosis date and date of death or lost to follow-up. Individuals dying of other causes or who were lost to follow-up were censored at the time of their death or date of last contact. Because several miRNAs were infrequently expressed we calculated *P*-values based on 1000 permutations for the HR using the R survival package.[29,30] For miRNAs that were infrequently expressed, which we defined as expressed in less than 50% of individuals, we calculated the HR based on any expression. SAS 9.4 (SAS Institute, Cary, NC, USA) was used to calculate the Cox proportional hazard ratios with the unit of change being the interquartile range. Adjustment for multiple comparisons was done by controlling the familywise error rate using the Sidak method;[31] we accounted for both the tests performed on the more commonly expressed miRNAs as well as on miRNAs infrequently expressed when calculating the familywise error rate.

### Bioinformatics

Experimentally verified miRNA-target genes were identified for miRNAs associated with colon microsatellite instability tumor phenotype. Target genes were identified using the Homo sapiens download from miRTarBase v6.0 (http://mirtarbase.mbc.nctu.edu.tw/).[32] R was used to filter the miRTarBase repository for genes associated with the miRNAs of interest, as well as compute the number of miRNAs each gene was associated with. The list of unique genes associated with these miRNAs comprised 9404 target genes, ranging from genes having one miRNA association with 29 associations. Genes that were associated with seven miRNAs or more were kept for functional analysis; this number was chosen as it created a sublist of 1048 genes, which is suitable for gene set enrichment analysis. This subset of target genes was used as input to Ingenuity Pathway Analysis (http://www.ingenuity.com/)[33] for functional analysis. Four genes were not mapped by Ingenuity Pathway Analysis: *C1ORF21*, *C11ORF57*, *C5ORF51*, and *NDUFA4P1*. We performed a core analysis using Ingenuity Pathway Analysis knowledgebase of genes only and direct relationships only, which included all sources, only experimentally verified results, mammalian species, and all tissues and mutations in the analysis with stringent filters. We exported the canonical pathways, where the enrichment score was corrected for multiple comparisons using the Benjamini Hochberg correction.

## Results

A total of 1893 individuals had miRNA assessed; 60.8% were colon carcinomas and 39.2% were rectal carcinomas (Table 1). The mean age of study participants was 64.2 years and 45.8% of participants were females. For the 1862 individuals with AJCC stage available, 30.0% were Stage 1, 26.3% were Stage 2, 29.4% were Stage 3, and 14.3% were Stage 4. At the end of 5 years, 550 of 1893 participants had died of CRC.

Several miRNAs were differentially expressed between mutated and non-mutated carcinomas using a false discovery rate of 0.05; those with a fold change of 1.5 or higher or 0.67 or lower in miRNA expression between mutated and non-mutated carcinomas are shown, although many more miRNAs were statistically significantly different in level of expression (Table 2). There were more differentially expressed miRNAs in *TP53*-mutated tumors than for *KRAS*-mutated, CIMP high, or *BRAF*-mutated tumors. The majority of miRNAs that were dysregulated were different between colon and rectal tumor subsite. MiR-4700-3p was downregulated in *TP53*-mutated tumors for both colon and rectal cancer. The majority of miRNAs dysregulated for *TP53*-mutated tumors were upregulated, were infrequently expressed, and had low levels of expression

**Table 1** Description of study population

| | *Overall* | *TP53-mutated* | | *KRAS-mutated* | | *CpG island methylator phenotype high* | | *Microsatellite instability* | | *BRAF-mutated* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *(N = 1893)* | *(N = 864)* | *(%[a])* | *(N = 569)* | *%* | *(N = 354)* | *(%)* | *(N = 170)* | *(%)* | *(N = 90)* | *(%)* |
| *Study center* | | | | | | | | | | | |
| Kaiser Permanente | 1144 | 507 | 0.44 | 336 | 0.29 | 222 | 0.19 | 107 | 0.09 | 58 | 0.05 |
| Utah | 749 | 357 | 0.48 | 233 | 0.31 | 132 | 0.18 | 47 | 0.06 | 32 | 0.04 |
| *Tumor site* | | | | | | | | | | | |
| Colon | 1150 | 500 | 0.43 | 347 | 0.30 | 275 | 0.24 | 154 | 0.13 | 90 | 0.08 |
| Rectal | 743 | 364 | 0.49 | 222 | 0.30 | 79 | 0.11 | 16 | 0.02 | NE | |
| Age (mean) | 64.2 | 63.8 | | 64.0 | | 66.2 | | 66.8 | | 67.6 | |
| Sex (% F) | 45.8 | 44.0 | | 45.5 | | 54.5 | | 63.0 | | 54.4 | |
| *AJCC stage* | | | | | | | | | | | |
| 1 | 559 | 274 | 0.49 | 139 | 0.25 | 85 | 0.15 | 34 | 0.06 | 15 | 0.03 |
| 2 | 489 | 195 | 0.40 | 160 | 0.33 | 93 | 0.19 | 59 | 0.12 | 23 | 0.05 |
| 3 | 548 | 258 | 0.47 | 168 | 0.31 | 123 | 0.22 | 50 | 0.09 | 35 | 0.06 |
| 4 | 266 | 123 | 0.46 | 89 | 0.33 | 50 | 0.19 | 10 | 0.04 | 16 | 0.06 |
| *5-year survival* | | | | | | | | | | | |
| Alive | 1081 | 487 | 0.45 | 307 | 0.28 | 194 | 0.18 | 107 | 0.10 | 49 | 0.05 |
| Dead: colorectal cancer | 550 | 253 | 0.46 | 194 | 0.35 | 117 | 0.21 | 23 | 0.04 | 33 | 0.06 |
| Dead: other | 198 | 93 | 0.47 | 57 | 0.29 | 34 | 0.17 | 20 | 0.10 | 7 | 0.04 |
| Lost to follow-up | 62 | 31 | 0.50 | 10 | 0.16 | 9 | 0.15 | 4 | 0.06 | 1 | 0.02 |

Abbreviation: NE, not evaluated.
[a]% refers to percentage of row.

when expressed. Unlike for *TP53*, *KRAS* differentially expressed miRNAs were only observed for rectal cancer. The majority of miRNAs associated with *KRAS*-specific mutations were downregulated in KRAS-mutated tumors, and were more frequently expressed than those differentially expressed for *TP53*. However, the three miRNAs that were upregulated in *KRAS* rectal tumors were seldom expressed and had low levels of expression. Differentially expressed miRNAs for CIMP high and *BRAF*-mutated tumors were only observed for colon cancer. MiR-196a-5p and miR-196b-5p were downregulated in both CIMP high and *BRAF*-mutated tumors.

Of the 94 differentially expressed miRNAs between microsatellite unstable and microsatellite stable colon tumors, all but eight were downregulated (Table 3). The majority of the downregulated miRNAs were frequently expressed. Additionally, the levels of expression appeared to be greater than those observed for most of the other tumor molecular phenotype differentially expressed miRNAs. Likewise, we observed that 41 miRNAs were significantly differentially expressed between microsatellite unstable and microsatellite stable rectal carcinomas with a fold change of 1.5 or greater or 0.67 or lower; almost all were downregulated (Table 4). Twenty-eight of the 41 miRNAs that were dysregulated between microsatellite unstable and microsatellite stable rectal carcinomas also were dysregulated between microsatellite unstable and microsatellite stable colon carcinomas. For the most part, the fold change observed between microsatellite unstable and microsatellite stable miRNA expression levels for rectal carcinomas was larger than observed for colon carcinomas.

We assessed if miRNAs significantly differentially expressed with a fold change of 1.5 or greater (or conversely 0.67 or less) were associated with survival (Table 5). Associations with survival could give insight into functionality as well as potentially explain differences in survival patterns for those with microsatellite unstable and microsatellite stable tumor phenotypes.[16,17] There were few miRNAs that were associated with survival after adjustment for multiple comparisons along with age, sex, disease stage at time of diagnosis and even fewer significant associations after adjustment for MSI tumor phenotype. Prior to adjustment for MSI status, there were four miRNAs associated with MSI status and one miRNA associated with CIMP status in colon tumors that was associated with colorectal cancer survival. For rectal cancer, there was one miRNA associated with KRAS, three associated with MSI, and one associated with *TP53* mutations in rectal tumors that also were associated with colorectal cancer survival after diagnosis with rectal cancer. After adjustment for MSI, there was one differentially expressed miRNA between colon *BRAF*-mutated and non-mutated tumors and two miRNAs differentially expressed between colon microsatellite unstable

**Table 2** Differences in miRNA expression by tumor molecular phenotype with a false discovery rate set at 0.05

| *miRNA* | *% Expressing* | *Mean expression* | *% Expressing* | *Mean expression* | *Siggenes P-value* | *Fold change (mutated/not mutated)* |
|---|---|---|---|---|---|---|
| *Colon cancer* | *TP53 not mutated* | | *TP53 mutated* | | | |
| hsa-let-7b-3p | 10.6 | 2.1 | 15.4 | 4.22 | 0.00148 | 2.01 |
| hsa-let-7d-3p | 8.7 | 2.45 | 14 | 4.45 | 0.00282 | 1.81 |
| hsa-miR-1226-3p | 0.2 | 0.36 | 2.4 | 2.13 | 0.00165 | 5.89 |
| hsa-miR-1227-3p | 4.2 | 1.3 | 7.8 | 2.82 | 0.00174 | 2.17 |
| hsa-miR-124-3p | 20.9 | 1.33 | 15 | 0.85 | 0.01242 | 0.64 |
| hsa-miR-1296 | 3.7 | 0.92 | 7.4 | 2.41 | 0.00154 | 2.61 |
| hsa-miR-133b | 12.4 | 1.47 | 19.4 | 2.34 | 0.00074 | 1.59 |
| hsa-miR-1976 | 0.2 | 0.53 | 2.6 | 9.05 | 0.00059 | 17.07 |
| hsa-miR-3190-5p | 0.3 | 1.32 | 2.6 | 4.61 | 0.00238 | 3.49 |
| hsa-miR-328 | 12.7 | 2.37 | 17.4 | 4.89 | 0.00213 | 2.06 |
| hsa-miR-34b-5p | 2.5 | 3.61 | 1.2 | 1.53 | 0.01979 | 0.42 |
| hsa-miR-3613-3p | 0.3 | 0.42 | 2.4 | 4.7 | 0.00306 | 11.27 |
| hsa-miR-4469 | 27.1 | 1.7 | 20.8 | 1.12 | 0.00334 | 0.66 |
| hsa-miR-449b-3p | 4.5 | 2.71 | 8.8 | 5.04 | 0.0031 | 1.86 |
| hsa-miR-4695-3p | 2.7 | 1.51 | 5.6 | 3.5 | 0.00273 | 2.32 |
| hsa-miR-4700-3p | 13.9 | 2.06 | 9.8 | 1.35 | 0.0479 | 0.65 |
| hsa-miR-4763-5p | 13.6 | 1.54 | 18.2 | 3.03 | 0.00211 | 1.96 |
| hsa-miR-513a-3p | 13.1 | 0.84 | 9.2 | 0.49 | 0.02807 | 0.59 |
| hsa-miR-625-5p | 11.1 | 1.77 | 6.2 | 1.09 | 0.00439 | 0.62 |
| hsa-miR-6509-3p | 0.2 | 0.71 | 2.2 | 4.91 | 0.00289 | 6.88 |
| hsa-miR-6511a-3p | 3.9 | 2.24 | 7.4 | 4.81 | 0.00198 | 2.15 |
| hsa-miR-885-5p | 7.9 | 4.16 | 12.4 | 7.44 | 0.00246 | 1.79 |
| *Rectal cancer* | | | | | | |
| hsa-miR-127-5p | 6.7 | 0.23 | 11.3 | 0.37 | 0.02098 | 1.65 |
| hsa-miR-192-3p | 23.9 | 1.42 | 36.5 | 2.21 | 0.00006 | 1.56 |
| hsa-miR-3180 | 4.8 | 0.86 | 11.8 | 1.54 | 0.00022 | 1.79 |
| hsa-miR-4700-3p | 9.6 | 3.03 | 5.5 | 0.48 | 0.00375 | 0.16 |
| hsa-miR-590-5p | 11.8 | 1.13 | 20.6 | 1.81 | 0.00159 | 1.6 |
| *Rectal cancer* | *KRAS not mutated* | | *KRAS-mutated* | | | |
| hsa-miR-192-3p | 32.9 | 2.1 | 21.6 | 1.1 | 0.00009 | 0.53 |
| hsa-miR-196b-5p | 77.9 | 16.36 | 73.4 | 10.54 | 0.00062 | 0.64 |
| hsa-miR-204-3p | 4.7 | 0.4 | 10.8 | 0.67 | 0.01043 | 1.66 |
| hsa-miR-324-5p | 48.6 | 4.37 | 32 | 2.74 | 0.00001 | 0.63 |
| hsa-miR-374b-5p | 38.2 | 6.09 | 30.2 | 3.57 | 0.00127 | 0.59 |
| hsa-miR-4255 | 3.3 | 0.34 | 7.2 | 0.56 | 0.04833 | 1.66 |
| hsa-miR-4290 | 28.7 | 3.92 | 18.5 | 2.48 | 0.00311 | 0.63 |
| hsa-miR-518e-5p | 0.2 | 1.23 | 2.7 | 2.05 | 0.03663 | 1.66 |
| hsa-miR-532-5p | 47.7 | 5.03 | 42.3 | 3.27 | 0.00491 | 0.65 |
| hsa-miR-552 | 13.6 | 4.19 | 6.8 | 2.31 | 0.00009 | 0.55 |
| hsa-miR-652-3p | 25.6 | 1.88 | 17.1 | 1.21 | 0.00323 | 0.64 |
| hsa-miR-660-5p | 14.7 | 3.11 | 7.7 | 2.05 | 0.0002 | 0.66 |
| hsa-miR-7-5p | 54.3 | 6.59 | 45 | 4.44 | 0.0002 | 0.67 |
| *Colon cancer* | *CpG island methylator phenotype low* | | *CpG island methylator phenotype high* | | | |
| hsa-miR-196a-5p | 66.3 | 6.85 | 50.9 | 4.6 | < 0.00001 | 0.67 |
| hsa-miR-196b-5p | 82.7 | 18.03 | 68 | 9.4 | < 0.00001 | 0.52 |
| hsa-miR-31-5p | 9.9 | 7.31 | 32.7 | 12.12 | < 0.00001 | 1.66 |
| hsa-miR-4267 | 3.7 | 0.59 | 7.6 | 1.37 | 0.026 | 2.33 |
| hsa-miR-513a-3p | 10.1 | 0.58 | 15.6 | 0.99 | 0.03441 | 1.71 |
| hsa-miR-548d-5p | 11.4 | 0.97 | 7.3 | 0.38 | 0.00389 | 0.39 |
| hsa-miR-590-5p | 23.6 | 2.28 | 16.4 | 1.14 | 0.0008 | 0.5 |
| hsa-miR-625-5p | 6.6 | 0.96 | 13.8 | 2.33 | 0.00063 | 2.43 |
| *Colon cancer* | *BRAF not mutated* | | *BRAF*-mutated | | | |
| hsa-miR-196a-5p | 63.5 | 6.48 | 42.2 | 3.18 | 0.00042 | 0.49 |
| hsa-miR-196b-5p | 79.7 | 16.39 | 67.8 | 7.28 | 0.00027 | 0.44 |

and microsatellite stable associated with survival after diagnosis with colon cancer. Additionally, one miRNA associated with microsatellite unstable and microsatellite stable in rectal tumors and one miRNA associated with *TP53* were associated with colorectal cancer survival after diagnosis with colon cancer. Interestingly, miR-196b-5p associated with *BRAF* mutations in colon cancer was only associated with colorectal cancer survival after adjustment for MSI status. For those that remained significant after

**Table 3** Associations between miRNAs differentially expressed between microsatellite stable and microsatellite unstable colon tumors with a false discovery rate set at 0.05

| *miRNA* | *Microsatellite stable* | | *Microsatellite unstable* | | | |
|---|---|---|---|---|---|---|
| | *% Expressing* | *Mean expression* | *% Expressing* | *Mean expression* | *Siggenes* P-*value* | *Fold change*[a] |
| hsa-let-7a-5p | 99.0 | 276.83 | 98.7 | 183.31 | < 0.001 | 0.66 |
| hsa-let-7e-5p | 94.1 | 24.07 | 90.3 | 15.57 | < 0.001 | 0.65 |
| hsa-let-7 f-5p | 98.5 | 155.97 | 97.4 | 96.19 | < 0.001 | 0.62 |
| hsa-let-7 g-5p | 97.1 | 53.66 | 95.5 | 34.33 | < 0.001 | 0.64 |
| hsa-miR-100-5p | 70.4 | 8.80 | 50.0 | 4.79 | < 0.001 | 0.54 |
| hsa-miR-125b-5p | 97.3 | 53.77 | 96.8 | 30.40 | < 0.001 | 0.57 |
| hsa-miR-127-3p | 15.0 | 2.70 | 5.2 | 1.15 | < 0.001 | 0.43 |
| hsa-miR-1291 | 76.8 | 5.92 | 59.1 | 3.86 | < 0.001 | 0.65 |
| hsa-miR-130a-3p | 49.3 | 5.88 | 25.3 | 2.79 | < 0.001 | 0.47 |
| hsa-miR-143-3p | 63.8 | 7.07 | 39.0 | 2.68 | < 0.001 | 0.38 |
| hsa-miR-145-5p | 99.8 | 134.19 | 99.4 | 77.28 | < 0.001 | 0.58 |
| hsa-miR-148a-3p | 79.4 | 17.83 | 60.4 | 8.17 | < 0.001 | 0.46 |
| hsa-miR-151a-3p | 51.1 | 4.83 | 23.4 | 2.33 | < 0.001 | 0.48 |
| hsa-miR-151a-5p | 91.2 | 14.36 | 80.5 | 6.85 | < 0.001 | 0.48 |
| hsa-miR-151b | 83.5 | 6.95 | 66.9 | 3.51 | < 0.001 | 0.51 |
| hsa-miR-15a-5p | 61.8 | 6.82 | 48.1 | 3.68 | < 0.001 | 0.54 |
| hsa-miR-17-5p | 98.1 | 53.02 | 98.1 | 31.47 | < 0.001 | 0.59 |
| hsa-miR-183-5p | 24.3 | 4.88 | 9.7 | 2.51 | < 0.001 | 0.51 |
| hsa-miR-185-5p | 12.3 | 3.49 | 5.8 | 2.23 | 0.001 | 0.64 |
| hsa-miR-1913 | 14.1 | 1.93 | 6.5 | 0.93 | 0.001 | 0.48 |
| hsa-miR-192-3p | 21.1 | 1.58 | 8.4 | 0.53 | < 0.001 | 0.33 |
| hsa-miR-192-5p | 97.2 | 87.65 | 91.6 | 50.24 | < 0.001 | 0.57 |
| hsa-miR-193b-3p | 84.6 | 8.50 | 64.9 | 4.59 | < 0.001 | 0.54 |
| hsa-miR-194-5p | 97.3 | 84.55 | 92.9 | 54.19 | < 0.001 | 0.64 |
| hsa-miR-195-5p | 44.1 | 3.23 | 20.8 | 1.12 | < 0.001 | 0.35 |
| hsa-miR-196a-5p | 64.3 | 6.72 | 42.2 | 3.36 | < 0.001 | 0.50 |
| hsa-miR-196b-5p | 81.0 | 17.51 | 51.9 | 4.72 | < 0.001 | 0.27 |
| hsa-miR-1973 | 99.8 | 793.25 | 100.0 | 512.18 | < 0.001 | 0.65 |
| hsa-miR-199a-3p | 94.1 | 38.36 | 91.6 | 21.28 | < 0.001 | 0.55 |
| hsa-miR-199a-5p | 89.5 | 17.08 | 81.8 | 8.86 | < 0.001 | 0.52 |
| hsa-miR-199b-5p | 37.9 | 3.72 | 12.3 | 1.62 | < 0.001 | 0.43 |
| hsa-miR-19b-3p | 91.9 | 25.37 | 87.7 | 14.21 | < 0.001 | 0.56 |
| hsa-miR-200a-3p | 93.5 | 27.08 | 90.9 | 17.29 | < 0.001 | 0.64 |
| hsa-miR-200b-3p | 98.4 | 151.75 | 98.1 | 97.72 | < 0.001 | 0.64 |
| hsa-miR-203a | 66.4 | 11.82 | 56.5 | 7.58 | < 0.001 | 0.64 |
| hsa-miR-204-3p | 15.0 | 1.12 | 9.7 | 0.71 | 0.044 | 0.64 |
| hsa-miR-20a-5p | 97.6 | 61.83 | 96.8 | 33.17 | < 0.001 | 0.54 |
| hsa-miR-20b-5p | 86.7 | 15.29 | 79.9 | 7.72 | < 0.001 | 0.50 |
| hsa-miR-214-3p | 88.7 | 11.97 | 81.2 | 7.47 | < 0.001 | 0.62 |
| hsa-miR-215 | 95.6 | 41.56 | 89.6 | 26.31 | < 0.001 | 0.63 |
| hsa-miR-221-3p | 84.6 | 12.18 | 70.8 | 7.46 | < 0.001 | 0.61 |
| hsa-miR-223-3p | 89.5 | 22.79 | 94.8 | 36.61 | < 0.001 | 1.61 |
| hsa-miR-224-5p | 67.6 | 13.82 | 17.5 | 4.55 | < 0.001 | 0.33 |
| hsa-miR-23a-3p | 99.1 | 157.85 | 98.7 | 93.21 | < 0.001 | 0.59 |
| hsa-miR-23b-3p | 98.2 | 65.43 | 96.1 | 35.36 | < 0.001 | 0.54 |
| hsa-miR-26a-5p | 98.7 | 96.40 | 98.1 | 55.09 | < 0.001 | 0.57 |
| hsa-miR-26b-5p | 89.3 | 18.41 | 84.4 | 11.01 | < 0.001 | 0.60 |
| hsa-miR-27a-3p | 97.6 | 49.86 | 96.8 | 30.52 | < 0.001 | 0.61 |
| hsa-miR-27b-3p | 95.5 | 32.03 | 92.2 | 17.97 | < 0.001 | 0.56 |
| hsa-miR-28-5p | 20.7 | 1.67 | 5.2 | 0.42 | < 0.001 | 0.25 |
| hsa-miR-29a-3p | 98.1 | 97.72 | 98.1 | 54.89 | < 0.001 | 0.56 |
| hsa-miR-29b-3p | 93.7 | 22.49 | 89.6 | 13.68 | < 0.001 | 0.61 |
| hsa-miR-29c-3p | 85.5 | 18.35 | 77.9 | 10.14 | < 0.001 | 0.55 |
| hsa-miR-30a-5p | 32.7 | 2.24 | 11.7 | 0.53 | < 0.001 | 0.24 |
| hsa-miR-30b-5p | 94.4 | 20.87 | 90.3 | 10.81 | < 0.001 | 0.52 |
| hsa-miR-30c-5p | 79.0 | 6.79 | 66.9 | 3.53 | < 0.001 | 0.52 |
| hsa-miR-30d-5p | 99.8 | 32.98 | 99.4 | 21.46 | < 0.001 | 0.65 |
| hsa-miR-30e-5p | 46.6 | 3.03 | 30.5 | 1.58 | < 0.001 | 0.52 |
| hsa-miR-31-5p | 14.5 | 10.05 | 37.0 | 15.68 | < 0.001 | 1.56 |
| hsa-miR-330-3p | 46.5 | 2.99 | 61.0 | 4.69 | < 0.001 | 1.57 |
| hsa-miR-3609 | 45.9 | 2.57 | 18.8 | 0.81 | < 0.001 | 0.31 |
| hsa-miR-361-3p | 11.5 | 1.36 | 4.5 | 0.56 | < 0.001 | 0.41 |
| hsa-miR-361-5p | 88.3 | 11.15 | 70.8 | 4.32 | < 0.001 | 0.39 |
| hsa-miR-362-5p | 33.4 | 3.63 | 13.0 | 2.34 | < 0.001 | 0.64 |
| hsa-miR-3651 | 99.0 | 55.13 | 98.7 | 35.97 | < 0.001 | 0.65 |

**Table 3 (Continued)**

| *miRNA* | *Microsatellite stable* | | *Microsatellite unstable* | | | |
|---|---|---|---|---|---|---|
| | *% Expressing* | *Mean expression* | *% Expressing* | *Mean expression* | *Siggenes P-value* | *Fold change*[a] |
| hsa-miR-3653 | 94.1 | 13.36 | 87.0 | 7.12 | < 0.001 | 0.53 |
| hsa-miR-365a-3p | 63.7 | 7.91 | 37.0 | 4.18 | < 0.001 | 0.53 |
| hsa-miR-374b-5p | 29.4 | 6.75 | 6.5 | 1.99 | < 0.001 | 0.29 |
| hsa-miR-424-5p | 33.3 | 4.08 | 13.6 | 2.50 | < 0.001 | 0.61 |
| hsa-miR-425-5p | 81.2 | 9.86 | 74.7 | 5.34 | < 0.001 | 0.54 |
| hsa-miR-4284 | 100.0 | 1352.71 | 100.0 | 873.15 | < 0.001 | 0.65 |
| hsa-miR-429 | 66.3 | 12.28 | 49.4 | 6.86 | < 0.001 | 0.56 |
| hsa-miR-4492 | 12.2 | 1.20 | 20.1 | 1.97 | 0.013 | 1.65 |
| hsa-miR-455-3p | 14.8 | 5.00 | 7.8 | 2.24 | 0.001 | 0.45 |
| hsa-miR-4681 | 3.5 | 0.70 | 8.4 | 1.45 | 0.024 | 2.06 |
| hsa-miR-483-3p | 41.5 | 9.94 | 33.1 | 3.80 | 0.002 | 0.38 |
| hsa-miR-497-5p | 23.3 | 1.51 | 7.1 | 0.44 | < 0.001 | 0.29 |
| hsa-miR-505-5p | 8.5 | 0.50 | 3.9 | 0.32 | 0.006 | 0.63 |
| hsa-miR-520d-3p | 12.4 | 1.62 | 22.1 | 3.05 | 0.014 | 1.88 |
| hsa-miR-532-3p | 27.9 | 3.10 | 6.5 | 1.57 | < 0.001 | 0.51 |
| hsa-miR-532-5p | 41.1 | 5.74 | 7.8 | 1.62 | < 0.001 | 0.28 |
| hsa-miR-590-5p | 23.0 | 2.14 | 5.8 | 0.34 | < 0.001 | 0.16 |
| hsa-miR-625-5p | 7.6 | 1.27 | 16.9 | 2.47 | 0.003 | 1.95 |
| hsa-miR-6515-5p | 27.4 | 1.14 | 35.7 | 1.79 | 0.010 | 1.56 |
| hsa-miR-652-3p | 19.5 | 2.50 | 5.8 | 1.12 | < 0.001 | 0.45 |
| hsa-miR-664a-3p | 39.5 | 4.27 | 18.2 | 2.27 | < 0.001 | 0.53 |
| hsa-miR-664a-5p | 93.0 | 11.72 | 81.8 | 6.73 | < 0.001 | 0.57 |
| hsa-miR-664b-3p | 99.7 | 31.27 | 100.0 | 16.29 | < 0.001 | 0.52 |
| hsa-miR-664b-5p | 99.7 | 59.42 | 98.7 | 30.99 | < 0.001 | 0.52 |
| hsa-miR-92a-3p | 99.8 | 107.15 | 99.4 | 52.95 | < 0.001 | 0.49 |
| hsa-miR-92b-3p | 11.2 | 1.03 | 8.4 | 0.48 | 0.042 | 0.47 |
| hsa-miR-98-5p | 34.5 | 3.59 | 9.1 | 1.29 | < 0.001 | 0.36 |
| hsa-miR-99a-5p | 49.8 | 5.52 | 22.1 | 2.07 | < 0.001 | 0.38 |
| hsa-miR-99b-5p | 44.0 | 5.10 | 31.8 | 3.33 | 0.001 | 0.65 |

[a]Fold change is microsatellite unstable/microsatellite stable.

adjustment for microsatellite status, the hazard ratio changed from 1.49 to 1.70 for miR-31-5p after adjustment for MSI status; most of the other hazard ratio only changed marginally if at all.

Assessment of canonical pathways associated with differentially expressed miRNAs between microsatellite unstable and microsatellite stable tumors showed hundreds of pathways significantly enriched by genes regulated by differentially expressed miRNAs (Supplementary Table S1). Targeted pathways include those related to molecular mechanisms of cancer, estrogen-mediated signaling, cell-cycle regulation, *PI3K/AKT* signaling, *PTEN* signaling, *TP53* signaling, IGF-1 signaling, and *TGFβ* signaling. The top 50 pathways are displayed in Figure 1.

## Discussion

There were few differentially expressed miRNAs by *TP53*, *KRAS*, CIMP, and *BRAF* molecular phenotype for either colon or rectal carcinoma. Conversely, 94 miRNAs were differentially expressed between microsatellite unstable and microsatellite stable tumors for colon carcinomas and 41 miRNAs were differentially expressed between microsatellite unstable and microsatellite stable tumors for rectal carcinomas. For the most part, miRNA expression was downregulated in microsatellite unstable tumors. Evaluation of those miRNAs that were associated with specific tumor molecular phenotype with survival showed few associations. Those that were associated with survival were originally identified as being differentially expressed between microsatellite stable and microsatellite unstable tumors. MiRNAs that influenced colon cancer survival increased the likelihood of dying, while those associated with rectal cancer improved survival.

In interpreting the findings from this study, several things should be considered. We only reported associations for miRNAs that were both statistically significant and had a fold change of 1.5 or greater or 0.67 or lower. This excluded numerous associations that were statistically significant, and it also excluded associations that had lower differences in expression between mutated and not-mutated tumors. While this is a common practice, it is not without problems. First, we do not know what level of fold change is biologically meaningful. Second, the level of miRNA expression can impact the fold change. This is most evident in our study when looking at fold changes for *TP53*-mutated,

*KRAS*-mutated, and CIMP high tumors. The larger fold changes are often associated with low levels of miRNA expression and miRNAs expressed in few individuals; this can yield a large fold change that may not be biologically meaningful. In our data, larger fold changes associated with MSI and microsatellite stable tumors may be more meaningful given miRNA expression levels for that specific tumor phenotype were more frequently expressed and had higher levels of expression. Additionally we are looking at miRNAs that are uniquely associated with tumor phenotype, thus some miRNAs, such as miR-21 being significantly differentially expressed in our overall colorectal cancer data,[9] was not associated with any one tumor molecular phenotype.

Comparison of our data to the literature on miRNA and tumor molecular phenotype is difficult given most studies that suggest associations between specific tumor phenotype and miRNAs have been conducted on cell lines or cells from cultured tissue and were limited to targeted miRNAs. Those conducted in populations have generally studied few people and often evaluated expression only compared to normal mucosa, often do not report specificity to a given phenotype, and may have samples limited to specific disease characteristics such as Crohn's Disease. Despite these differences in methodology, there are suggestions that certain miRNAs can be associated with tumor molecular phenotype. This study gives us an opportunity to test some of the previously hypothesized associations. A study of six patients with either Crohn's Disease or ulcerative colitis and expression of 88 miRNAs identified six differentially expressed miRNAs (miR-122, mIR-214, miR-372, miR-15b, let-7e, and miR-17) among *TP53*-mutated tumors.[34] Others have found miR-34 associated with the *TP53* network;[35,36] miRNA-16,[37] miR-1915,[38] miR-221,[39] and miR-148b[40] also have been linked to *TP53* mainly in cell lines or tissue cultures. We only found miRNA-34b-5p associated with *TP53*-mutated colon tumors in our data regardless of level of fold change. It is believed that miR-34 is involved in apoptosis and cell-cycle arrest.[41]

We examined *TP53*-mutated tumors compared with non-mutated tumors previously in a small set of cases and this study replicates some of the previously reported associations.[10] For those that did replicate, which included miR-135b for rectal carcinoma and miR-224, miR-17, miR-1226, miR-532-5p, miR-17, miR-574-5p, miR-424, and miR-16 for colon cancer, the direction of association was the same as previously reported. Inconsistent nomenclature between an earlier versions of the Agilent array we used previously in our pilot study and to the much larger platform used in this study prohibited comparison of some miRNAs. Likewise, we adjusted for more comparisons because of the larger platform in the current study and many of the previous associations that did not replicate were marginally significant with adjusted *P*-values between 0.04 and 0.05. We identified 22 miRNAs associated with *TP53*-mutated colon tumors and five miRNAs associated with *TP53*-mutated rectal tumors in this study. With one exception the differentially expressed miRNAs for colon and rectal *TP53*-mutated tumors were different.

Several miRNAs also have been examined in conjunction with *KRAS*-mutated and non-mutated tumors. Among these, let-7, miR-100, miR-126, miR-143, miR-145, miR-200c, miR-221, miR222, miR-224, miR-345, miR-4689, and miR-96-5p have been linked to KRAS mutations mainly in studies of cell lines and cell cultures.[42–52] MiR-31-5p has been associated with both *KRAS* and *BRAF* mutations;[14,53] miRNA-31-5p was seen as the most upregulated miRNA associated with the *BRAF* V600E mutation in a study of 760 miRNA in 29 CRC cases.[14] This miRNA also has been associated with CIMP status and serrated polyps.[53] *BRAF* also has been identified as a direct target of miR-378-5p.[54] In our data, miR-31-5p was significantly upregulated for CIMP high colon carcinomas with a fold change of 1.66 and in *BRAF*-mutated tumors; however, the fold change of 1.17, although statistically significant, may have less importance biologically. On the miRNAs previously linked to CIMP, we observed statistically significant small changes in expression levels resulting in small fold changes for miR-145-5p (fold change = 0.90), miR222-3p (fold change = 1.30), and miR-224-5p (fold change = 0.91). Our previous study identified no miRNAs that were associated with *KRAS* in colon carcinomas and 11 that were associated with rectal carcinoma when compared with non-*KRAS*-mutated tumors.[10] In this study only two miRNAs, miR-572 and miR-638, which although statistically significant in our data had low fold changes (fold change 1.16 and 1.11 respectively), showed any association with *KRAS*-mutated tumors. None of the other miRNAs linked in the literature to *KRAS* were associated with *KRAS*-mutated tumors in our data. Likewise, miR-378-5p was not associated with colon *BRAF*-mutated tumors. We replicated our previously identified association between CIMP with miR-31 and miR-492 from our smaller pilot data; however, the fold change for miR-492 was small (fold change = 1.26). We currently did not observe any CIMP specific associations for rectal tumors, whereas previously we identified eight miRNAs that were associated with CIMP high tumors, although the adjusted *P*-values were marginal at 0.047.

Most previous work with miRNAs and tumor molecular phenotype has been with microsatellite unstable tumors. In our data the majority of associations also were observed for differences in miRNA expression between microsatellite unstable and microsatellite stable carcinomas; this was true for both colon and rectal cancer. For the most part, miRNAs were downregulated in microsatellite unstable tumors. Others have shown that expression of miR-92, miR-223, miR-155, miR-196a, miR-31,

**Table 4** Associations between miRNAs differentially expressed between microsatellite unstable and microsatellite stable rectal tumors with a false discovery rate set at 0.05

| *miRNA* | *Microsatellite stable* | | *Microsatellite unstable* | | | |
|---|---|---|---|---|---|---|
| | *% Expressing* | *Mean expression* | *% Expressing* | *Mean expression* | *Siggenes* P-*value* | *Fold change*[a] |
| hsa-let-7a-5p | 99.9 | 272.16 | 100 | 160.81 | 0.010 | 0.59 |
| hsa-let-7 f-5p | 99.4 | 149.04 | 100 | 81.94 | 0.010 | 0.55 |
| hsa-let-7 g-5p | 99 | 52.05 | 100 | 27.61 | 0.010 | 0.53 |
| hsa-miR-100-5p | 80.8 | 9.71 | 50 | 2.56 | < 0.001 | 0.26 |
| hsa-miR-125b-5p | 98.9 | 62.09 | 100 | 28.37 | 0.010 | 0.46 |
| hsa-miR-1281 | 100 | 42.21 | 100 | 63.57 | 0.010 | 1.51 |
| hsa-miR-145-5p | 100 | 133.78 | 100 | 47.27 | < 0.001 | 0.35 |
| hsa-miR-151a-5p | 95.9 | 14.13 | 62.5 | 4.25 | < 0.001 | 0.3 |
| hsa-miR-151b | 88.7 | 6.81 | 50 | 2.12 | < 0.001 | 0.31 |
| hsa-miR-196b-5p | 77.6 | 14.95 | 37.5 | 1.33 | < 0.001 | 0.09 |
| hsa-miR-199a-5p | 93.8 | 18.26 | 75 | 6.39 | 0.010 | 0.35 |
| hsa-miR-221-3p | 90.6 | 11.27 | 62.5 | 4.57 | 0.010 | 0.41 |
| hsa-miR-23a-3p | 100 | 164.28 | 100 | 83.89 | < 0.001 | 0.51 |
| hsa-miR-23b-3p | 99.3 | 68.68 | 100 | 29.5 | < 0.001 | 0.43 |
| hsa-miR-24-3p | 100 | 97 | 100 | 57.89 | < 0.001 | 0.6 |
| hsa-miR-26a-5p | 100 | 97.29 | 100 | 40.86 | < 0.001 | 0.42 |
| hsa-miR-26b-5p | 94.2 | 16.93 | 62.5 | 6.01 | 0.010 | 0.35 |
| hsa-miR-27b-3p | 98.5 | 31.49 | 87.5 | 14.04 | 0.010 | 0.45 |
| hsa-miR-29c-3p | 90.9 | 15.69 | 56.3 | 5.74 | 0.010 | 0.37 |
| hsa-miR-30b-5p | 97.6 | 21.37 | 75 | 8.42 | 0.010 | 0.39 |
| hsa-miR-30c-5p | 88.5 | 7.56 | 62.5 | 2.7 | 0.010 | 0.36 |
| hsa-miR-30d-5p | 99.9 | 31.73 | 100 | 18.7 | < 0.001 | 0.59 |
| hsa-miR-3150b-5p | 97.1 | 12 | 100 | 18.48 | < 0.001 | 1.54 |
| hsa-miR-331-3p | 98.8 | 13.8 | 75 | 6.55 | 0.010 | 0.47 |
| hsa-miR-361-5p | 93.5 | 11.38 | 56.3 | 2.74 | < 0.001 | 0.24 |
| hsa-miR-3653 | 97.8 | 13.88 | 75 | 6.64 | 0.010 | 0.48 |
| hsa-miR-365a-3p | 74.8 | 8.09 | 37.5 | 3.27 | 0.010 | 0.4 |
| hsa-miR-424-3p | 100 | 37.2 | 100 | 23.46 | 0.010 | 0.63 |
| hsa-miR-425-5p | 89.8 | 10.87 | 62.5 | 5.37 | 0.010 | 0.49 |
| hsa-miR-4532 | 100 | 296.27 | 100 | 446.24 | 0.030 | 1.51 |
| hsa-miR-4655-3p | 98.5 | 14.89 | 100 | 22.35 | 0.010 | 1.5 |
| hsa-miR-4674 | 91 | 5.41 | 100 | 10.55 | 0.020 | 1.95 |
| hsa-miR-4769-3p | 92.3 | 7.77 | 100 | 12.21 | < 0.001 | 1.57 |
| hsa-miR-520e | 94.7 | 9.87 | 100 | 15 | < 0.001 | 1.52 |
| hsa-miR-5703 | 100 | 197.43 | 100 | 309.88 | < 0.001 | 1.57 |
| hsa-miR-636 | 99.7 | 8.97 | 100 | 13.59 | 0.010 | 1.51 |
| hsa-miR-664b-3p | 99.9 | 33.48 | 100 | 19.84 | < 0.001 | 0.59 |
| hsa-miR-664b-5p | 99.9 | 58.55 | 100 | 35.64 | < 0.001 | 0.61 |
| hsa-miR-877-3p | 100 | 24.65 | 100 | 37.46 | < 0.001 | 1.52 |
| hsa-miR-92a-3p | 100 | 116.97 | 100 | 58.86 | < 0.001 | 0.5 |
| hsa-miR-940 | 100 | 591.89 | 100 | 895.9 | 0.010 | 1.51 |

[a]Fold change is based on microsatellite unstable/microsatellite stable.

and miR-26b were significantly different in a group of 23 microsatellite stable and 16 microsatellite unstable tumors.[55] They also showed that miR-31 and miR-223 were overexpressed in colorectal cancer in patients with hereditary non-polyposis colorectal cancer syndrome and that the oncogenic miR-17-92 family was significantly upregulated in microsatellite stable cancers.[55] Similar findings have been reported for individuals with Lynch syndrome.[56] Others have reported that miR-484 expression was significantly decreased in microsatellite unstable colorectal cancers.[57] MiR-155 has been associated with MSI in several studies.[57–60] Several studies also have shown that miR-21 was overexpressed in microsatellite unstable tumors.[57,58] In our previous work we also evaluated microsatellite unstable *vs* microsatellite stable tumors and observed eight miRNAs that were significantly up or down-regulated;[10] all but one of those miRNAs (miR-552) was significantly associated with microsatellite unstable carcinomas in this larger study.

Microsatellite unstable carcinomas appear to have the most unique associations with miRNA expression of any tumor molecular phenotypes examined. Given the number of miRNAs that were differentially expressed between microsatellite unstable and microsatellite stable carcinomas for both colon and rectal cancer, we further evaluated canonical pathways that were influenced by genes regulated by miRNAs specific to microsatellite unstable carcinomas. While many of the pathways that were enriched by genes associated with miRNAs differentially

**Table 5** Associations between miRNAs dysregulated for colon and for rectal cancer and colorectal cancer survival

| *Survival association* | *Original tumor phenotype* | *miRNA* | *% Expressing* | *25th %ile*[a] | *75th %ile*[a] | *Hazard ratio*[b] | *(95% confidence intervals)* | P-*value* | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *Raw*[b] | *Adjust*[c] *ed* |
| Colon | CIMP and MSI | hsa-miR-31-5p | 14.8 | 0.00 | 0.00 | 1.49 | (1.16, 1.91) | 0.002 | 0.01 |
| Colon | MSI | hsa-miR-193b-3p | 86.8 | 1.92 | 3.40 | 1.28 | (1.14, 1.44) | 0.0001 | 0.009 |
| Colon | MSI | hsa-miR-214-3p | 90.9 | 2.36 | 3.99 | 1.26 | (1.11, 1.43) | 0.0003 | 0.03 |
| Colon | MSI | hsa-miR-28-5p | 21.0 | 0.00 | 0.00 | 1.62 | (1.26, 2.08) | 0.0002 | 0.02 |
| Colon | MSI | hsa-miR-99b-5p | 47.3 | 0.00 | 2.54 | 1.55 | (1.25, 1.92) | < .0001 | 0.005 |
| Rectal | *KRAS* | hsa-miR-552 | 9.7 | 0.00 | 0.00 | 0.47 | (0.28, 0.77) | 0.003 | 0.03 |
| Rectal | MSI | hsa-miR-3653 | 94.8 | 3.28 | 4.19 | 0.80 | (0.71, 0.90) | 0.0006 | 0.02 |
| Rectal | MSI | hsa-miR-520e | 91.4 | 3.00 | 3.68 | 1.21 | (1.07, 1.37) | 0.001 | 0.04 |
| Rectal | MSI | hsa-miR-636 | 99.4 | 3.00 | 3.44 | 1.25 | (1.11, 1.41) | 0.0005 | 0.02 |
| Rectal | *TP53* | hsa-miR-192-3p | 23.1 | 0.00 | 1.64 | 0.65 | (0.48, 0.88) | 0.005 | 0.03 |
| *Additional adjustment for MSI* | | | | | | | | | |
| Colon | *BRAF*[c] | hsa-miR-196b-5p | 76.8 | 1.58 | 4.24 | 0.83 | (0.71, 0.97) | 0.02 | 0.04 |
| Colon | CIMP and MSI | hsa-miR-31-5p | 14.8 | 0.00 | 0.00 | 1.70 | (1.32, 2.19) | < .0001 | 0.004 |
| Colon | MSI | hsa-miR-99b-5p | 47.3 | 0.00 | 2.54 | 1.59 | (1.28, 1.97) | < .0001 | 0.003 |
| Rectal | MSI | hsa-miR-636 | 99.4 | 3.00 | 3.44 | 1.23 | (1.09, 1.39) | 0.001 | 0.04 |
| Rectal | *TP53* | hsa-miR-192-3p | 23.1 | 0.00 | 1.64 | 0.65 | (0.48, 0.89) | 0.007 | 0.03 |

Abbreviations: CIMP, CpG island methylator phenotype; MSI, microsatellite instability status.
[a]Expression values for the 25th and 7th are expressed as log2 transformed values that were used in the Cox proportional hazard models.
[b]*P*-values adjusted for age, sex, and AJCC Stage.
[c]Adjusted for multiple comparisons.

expressed between microsatellite unstable and microsatellite stable carcinomas were directly cancer related, such as Molecular Mechanisms of Cancer, others were involved in estrogen signaling, TP53 signaling (noted by IPA as p53), PTEN signaling, IGF-1 (noted by IPA as IGF-1) signaling, TGFβ signaling, STAT3 pathway, (MAPK7) ERK5 signaling, and WNT/β catenin signaling. This reflects the wide range of pathways and mechanisms that could be associated with microsatellite unstable tumors. Many of these pathways have been previously identified as being associated with miRNAs.[61] Unfortunately, many of the bioinformatics databases available to assess functionality are limited in their specificity. Thus many of these pathways could be associated with miRNAs and colorectal as well as with microsatellite unstable tumors specifically.

For those miRNAs that were differentially expressed by tumor molecular phenotype, we tested their association with survival. The motivation for this was the observation that microsatellite unstable tumors are associated with survival differently than microsatellite stable tumors and that these associations differ for colon and rectal cancer.[16,17] For the most part, we observed few associations between differentially expressed miRNAs by tumor phenotype and impact on survival. Those associated with colon microsatellite unstable carcinomas had the greatest impact on survival. Differences in associations between colon and rectal cancer could be from importance of different pathways associated with miRNAs and microsatellite unstable carcinomas for colon and rectal cancer. Some of these miRNAs have previously been associated with either advanced stage or survival. Others have reported a direct association between miR-31 with advanced disease stage and worse survival.[14,61] Our replication of previously reported associations with disease stage and survival using half of the existing data set did not show an association with miR-31-5p after adjusting for disease stage while miR-99b-5p was associated with worse survival among individuals diagnosed with colon cancer.[62] Given the infrequent expression of miR-31-5p in colorectal carcinomas we had limited power to evaluate associations with the smaller sample size. Adjustment for MSI status attenuated some of the associations with survival, while others became stronger. We presented both adjusted and not-adjusted MSI associations. It is unclear however if MSI is a confounder of associations in that it is associated with both the exposure and the outcome, or if it is in the causal pathway given that these miRNAs were associated with different levels of expression between microsatellite stable and microsatellite unstable carcinomas which could lead to tumor phenotype which then influenced survival.

This study has strengths, including the use of the Agilent platform with over 2000 miRNAs. We have previously reported on the comparability of the

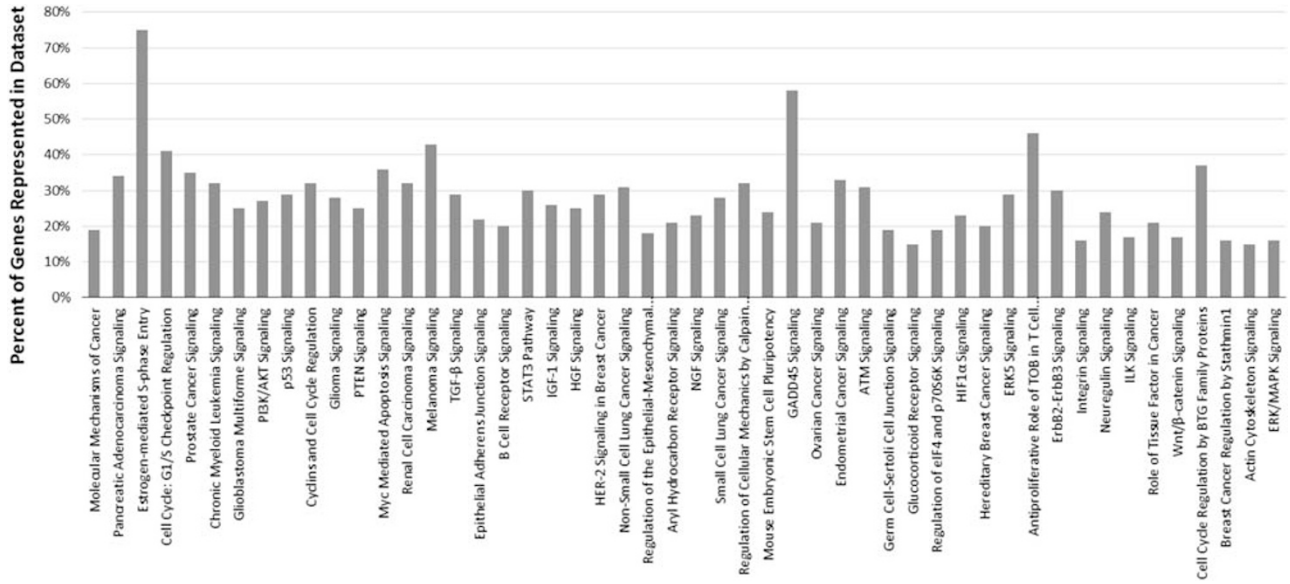


**Figure 1** Canonical pathway involvement of target genes associated with dysregulated miRNAs in microsatellite instability and microsatellite stable colorectal tumors.

Agilent to Nanostring and qPCR, with excellent results.[9] The reliability of the Agilent platform in our data was 0.98 and the overall platform had relatively good performance when compared with Nanostring.[9] Comparison of expression levels of a set of specific miRNAs with qPCR showed extremely high correlation in terms of directionality of expression and fold change observed between carcinoma and normal mucosa. Additionally, the study is large and contains detailed information on multiple tumor molecular phenotypes as well as survival. In this study, we applied an FDR of 0.05 to take into consideration multiple comparisons being made. However, ideally another large data set would replicate these findings. In previous analyses, we have split our data set to incorporate both discovery and testing; we chose not to do that in these analyses to maintain more power to identify associations for those tumor molecular phenotypes that are rarer, such as MSI. We thus encourage others to replicate these findings in a more targeted approach that focuses on specific miRNAs, especially those that have prognostic implications.

We believe that this study makes a unique contribution to the literature. First, it is large so that associations are measured with more precision. Second, we are able to incorporate discovery of new associations as well as test those previously identified as being associated with specific miRNAs. In doing this we have adjusted for multiple comparisons, where others looking at candidate miRNAs have not, and thus many previous associations may have been false positives that do not withstand the adjustment for multiple comparisons. This is not unlike the transition from candidate SNP and candidate pathways to genome-wide association studies, where more significant associations are identified when fewer adjustments for multiple comparisons are made.

In summary, our data suggest that most unique associations between tumor molecular phenotype and miRNAs are with microsatellite unstable *vs* microsatellite stable tumors. While several miRNAs were differentially expressed between these tumor phenotypes, few of the miRNAs were associated with survival. Microsatellite unstable colon carcinomas have previously been associated with better survival, while microsatellite unstable rectal tumors were associated with worse survival. Interestingly, miRNAs in this study uniformly show that higher levels of expression increase risk of dying for colon cancer, but improve survival if diagnosed with rectal cancer. We encourage others with similar data sets to confirm these associations.

## Acknowledgments

The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official view of the National Cancer Institute. We acknowledge Sandra Edwards for data oversight and Dr. Bette Caan and the staff at the KPMCP for their contribution to data collection and to Dr. Wade Samowitz for review of slides. This study was supported by NCI grants CA163683, CA48998, and CA61757 from the National Cancer Institute at the National Institutes of Health.

## Disclosure/conflict of interest

The authors declare no conflict of interest.

## References

1 Ambros V. The functions of animal microRNAs. Nature 2004;431:350–355.
2 Murray BS, Choe SE, Woods M *et al.* An in silico analysis of microRNAs: mining the miRNAome. Mol Biosyst 2010;6:1853–1862.
3 Arora S, Rana R, Chhabra A *et al.* miRNA-transcription factor interactions: a combinatorial regulation of gene expression. Mol Genet Genomics 2013;288:77–87.
4 Gartel AL, Kandel ES. miRNAs: little known mediators of oncogenesis. Semin Cancer Biol 2008;18:103–110.
5 Nam S, Li M, Choi K *et al.* MicroRNA and mRNA integrated analysis (MMIA): a web tool for examining biological functions of microRNA expression. Nucleic Acids Res 2009;37:W356–W362.
6 Drusco A, Nuovo GJ, Zanesi N *et al.* MicroRNA profiles discriminate among colon cancer metastasis. PLoS One 2014;9:e96670.
7 Acunzo M, Romano G, Wernicke D *et al.* MicroRNA and cancer—a brief overview. Adv Biol Regul 2015;57: 1–9.
8 Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. J Clin Oncol 2009;27: 5848–5856.
9 Slattery ML, Herrick JS, Pellatt DF *et al.* MicroRNA profiles in colorectal carcinomas, adenomas and normal colonic mucosa: variations in miRNA expression and disease progression. Carcinogenesis 2016;37:245–261.
10 Slattery ML, Wolff E, Hoffman MD *et al.* MicroRNAs and colon and rectal cancer: differential expression by tumor location and subtype. Genes Chromosomes Cancer 2011;50:196–206.
11 Valeri N, Vannini I, Fanini F *et al.* Epigenetics, miRNAs, and human cancer: a new chapter in human gene regulation. Mamm Genome 2009;20:573–580.
12 El-Murr N, Abidi Z, Wanherdrick K *et al.* MiRNA genes constitute new targets for microsatellite instability in colorectal cancer. PLoS One 2012;7:e31862.
13 Krell J, Stebbing J, Frampton AE *et al.* The role of TP53 in miRNA loading onto AGO2 and in remodelling the miRNA-mRNA interaction network. Lancet 2015;385 (Suppl 1):S15.
14 Nosho K, Igarashi H, Nojima M *et al.* Association of microRNA-31 with BRAF mutation, colorectal cancer survival and serrated pathway. Carcinogenesis 2014;35: 776–783.
15 Samowitz WS, Sweeney C, Herrick J *et al.* Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res 2005;65: 6063–6069.
16 Samowitz WS, Curtin K, Wolff RK *et al.* Microsatellite instability and survival in rectal cancer. Cancer Causes Control 2009;20:1763–1768.
17 Samowitz WS, Curtin K, Ma KN *et al.* Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. Cancer Epidemiol Biomarkers Prev 2001;10:917–923.
18 Samowitz WS, Albertsen H, Herrick J *et al.* Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology 2005;129:837–845.
19 Slattery ML, Potter J, Caan B *et al.* Energy balance and colon cancer—beyond physical activity. Cancer Res 1997;57:75–80.
20 Slattery ML, Caan BJ, Benson J *et al.* Energy balance and rectal cancer: an evaluation of energy intake, energy expenditure, and body mass index. Nutr Cancer 2003;46:166–171.
21 Slattery ML, Edwards SL, Palmer L *et al.* Use of archival tissue in epidemiologic studies: collection procedures and assessment of potential sources of bias. Mutat Res 2000;432:7–14.
22 Pellatt DF, Stevens JR, Wolff RK *et al.* Expression profiles of miRNA subsets distinguish human colorectal carcinoma and normal colonic mucosa. Clin Transl Gastroenterol 2016;17:e152.
23 Slattery ML, Curtin K, Anderson K *et al.* Associations between cigarette smoking, lifestyle factors, and microsatellite instability in colon tumors. J Natl Cancer Inst 2000;92:1831–1836.
24 Hinoue T, Weisenberger DJ, Lange CP *et al.* Genome-scale analysis of aberrant DNA methylation in colorectal cancer. Genome Res 2012;22:271–282.
25 Ogino S, Kawasaki T, Kirkner GJ *et al.* Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn 2007;9:305–314.
26 Agilent Technologies I. Agilent GeneSpring User Manual, Vol. 2015. Aglient Technologies: Santa Clara, CA, 2013.
27 Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 2001;98: 5116–5121.
28 Benjamini YH, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc 1995;57:289–300.
29 Therneau T, A Package for Survival Analysis in S_ vol. Version 2.38, 2015; http:/CRAN.R-project.org/package_ survival.
30 Therneau TM, Grambsch PM. Modeling Survival Data: Extending the Cox Model. Springer: New York, 2000.
31 Sidak Z. Rectangular confidence regions for the means of multivariate normal distributions. J Am Stat Assoc 1967;62:626–633.
32 Chou CH, Chang NW, Shrestha S *et al.* miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database. Nucleic Acids Res 2015; 44:D239–D247.
33 Kramer A, Green J, Pollard J Jr *et al.* Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics 2014;30:523–530.
34 Kanaan Z, Rai SN, Eichenberger MR *et al.* Differential microRNA expression tracks neoplastic progression in inflammatory bowel disease-associated colorectal cancer. Hum Mutat 2012;33:551–560.
35 Kim NH, Kim HS, Kim NG *et al.* p53 and microRNA-34 are suppressors of canonical Wnt signaling. Sci Signal 2011;4ra71.
36 Siemens H, Jackstadt R, Kaller M *et al.* Repression of c-Kit by p53 is mediated by miR-34 and is associated with reduced chemoresistance, migration and stemness. Oncotarget 2013;4:1399–1415.
37 Ma Q, Wang X, Li Z *et al.* microRNA-16 represses colorectal cancer cell growth in vitro by regulating the p53/survivin signaling pathway. Oncol Rep 2013;29: 1652–1658.
38 Nakazawa K, Dashzeveg N, Yoshida K. Tumor suppressor p53 induces miR-1915 processing to inhibit Bcl-2 in the apoptotic response to DNA damage. FEBS J 2014;281:2937–2944.
39 Pu XX, Huang GL, Guo HQ *et al.* Circulating miR-221 directly amplified from plasma is a potential diagnostic

and prognostic marker of colorectal cancer and is correlated with p53 expression. J Gastroenterol Hepatol 2010;25:1674–1680.
40 Wang G, Cao X, Lai S *et al.* Altered p53 regulation of miR-148b and p55PIK contributes to tumor progression in colorectal cancer. Oncogene 2015;34:912–921.
41 Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol 2014;9:287–314.
42 Amankwatia EB, Chakravarty P, Carey FA *et al.* MicroRNA-224 is associated with colorectal cancer progression and response to 5-fluorouracil-based chemotherapy by KRAS-dependent and -independent mechanisms. Br J Cancer 2015;112:1480–1490.
43 Cha DJ, Franklin JL, Dou Y *et al.* KRAS-dependent sorting of miRNA to exosomes. Elife 2015;4:e07197.
44 Chen X, Guo X, Zhang H *et al.* Role of miR-143 targeting KRAS in colorectal tumorigenesis. Oncogene 2009;28:1385–1392.
45 Fei BY, Wang XY, Fang XD. MicroRNA-143 replenishment re-sensitizes colorectal cancer cells harboring mutant, but not wild-type, KRAS to paclitaxel treatment. Tumour Biol; e-pub ahead of print 18 November 2015.
46 Hara T, Jones MF, Subramanian M *et al.* Selective targeting of KRAS-mutant cells by miR-126 through repression of multiple genes essential for the survival of KRAS-mutant cells. Oncotarget 2014;5:7635–7650.
47 Hiraki M, Nishimura J, Takahashi H *et al.* Concurrent targeting of KRAS and AKT by MiR-4689 is a novel treatment against mutant KRAS colorectal cancer. Mol Ther Nucleic Acids 2015;4:e231.
48 Kent OA, Fox-Talbot K, Halushka MK. RREB1 repressed miR-143/145 modulates KRAS signaling through downregulation of multiple targets. Oncogene 2013;32:2576–2585.
49 Luu C, Heinrich EL, Duldulao M *et al.* TP53 and let-7a micro-RNA regulate K-Ras activity in HCT116 colorectal cancer cells. PLoS One 2013;8:e70604.
50 Ota T, Doi K, Fujimoto T *et al.* KRAS up-regulates the expression of miR-181a, miR-200c and miR-210 in a three-dimensional-specific manner in DLD-1 colorectal cancer cells. Anticancer Res 2012;32:2271–2275.
51 Ruzzo A, Graziano F, Vincenzi B *et al.* High let-7a microRNA levels in KRAS-mutated colorectal carcinomas may rescue anti-EGFR therapy effects in patients with chemotherapy-refractory metastatic disease. Oncologist 2012;17:823–829.
52 Tsunoda T, Takashima Y, Yoshida Y *et al.* Oncogenic KRAS regulates miR-200c and miR-221/222 in a 3D-specific manner in colorectal cancer cells. Anticancer Res 2011;31:2453–2459.
53 Ito M, Mitsuhashi K, Igarashi H *et al.* MicroRNA-31 expression in relation to BRAF mutation, CpG island methylation and colorectal continuum in serrated lesions. Int J Cancer 2014;135:2507–2515.
54 Wang Z, Ma B, Ji X *et al.* MicroRNA-378-5p suppresses cell proliferation and induces apoptosis in colorectal cancer cells by targeting BRAF. Cancer Cell Int 2015; 15:40.
55 Lanza G, Ferracin M, Gafa R *et al.* mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer. Mol Cancer 2007;6:54.
56 Earle JS, Luthra R, Romans A *et al.* Association of microRNA expression with microsatellite instability status in colorectal adenocarcinoma. J Mol Diagn 2010;12:433–440.
57 Mei Q, Xue G, Li X *et al.* Methylation-induced loss of miR-484 in microsatellite-unstable colorectal cancer promotes both viability and IL-8 production via CD137L. J Pathol 2015;236:165–174.
58 Svrcek M, El-Murr N, Wanherdrick K *et al.* Overexpression of microRNAs-155 and 21 targeting mismatch repair proteins in inflammatory bowel diseases. Carcinogenesis 2013;34:828–834.
59 Valeri N, Gasparini P, Fabbri M *et al.* Modulation of mismatch repair and genomic stability by miR-155. Proc Natl Acad Sci USA 2010;107:6982–6987.
60 Yamamoto H, Adachi Y, Taniguchi H *et al.* Interrelationship between microsatellite instability and microRNA in gastrointestinal cancer. World J Gastroenterol 2012;18:2745–2755.
61 Slaby O, Svoboda M, Michalek J *et al.* MicroRNAs in colorectal cancer: translation of molecular biology into clinical application. Mol Cancer 2009;8:102.
62 Slattery ML, Herrick JS, Mullany LE *et al.* An evaluation and replication of miRNAs with disease stage and colorectal cancer-specific mortality. Int J Cancer 2015;137:428–438.

Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)