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Infrequently expressed miRNAs in colorectal cancer tissue and tumor molecular phenotype

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Abstract

We have previously shown that commonly expressed miRNAs influenced tumor molecular phenotype in colorectal cancer. We hypothesize that infrequently expressed miRNAs, when showing higher levels of expression, help to define tumor molecular phenotype. In this study we examine 304 miRNAs expressed in at least 30 individuals but in less than 50% of the population and with a mean level of expression above 1.0 relative florescent unit. We examine associations in 1893 individuals who have tumor molecular phenotype data as well as miRNA expression levels for both carcinoma and normal colorectal tissue. We compare miRNAs uniquely associated with tumor molecular phenotype to RNAseq data to identify genes associated with these miRNAs. This information is used to further identify unique pathways associated with tumor molecular phenotypes of *TP53*-mutated, *KRAS*-mutated, CpG island methylator phenotype, and microsatellite instability tumors. Thirty-seven miRNAs were uniquely associated with *TP53*-mutated tumors; 30 of these miRNAs had higher level of expression in *TP53*-mutated tumors while seven had lower levels of expression. Of the 34 miRNAs associated with CpG island methylator phenotype-high tumors, 16 were more likely to have a CpG island methylator phenotype-high tumor and 19 were less likely to be CpG island methylator phenotype-high. For microsatellite instability, 13 of the 22 infrequently expressed miRNAs were significantly less likely to be expressed in microsatellite unstable tumors. *KRAS*-mutated tumors were not

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MS obtained funding, planned study, oversaw study data collection and analysis, and wrote the manuscript.
AP and FL helped interpret findings and helped write the manuscript.
JS provided input into the statistical analysis
LM conducted bioinformatics analysis and helped write manuscript
RW oversaw laboratory analysis.
WS reviewed manuscript and did pathology overview for the study
JH conducted statistical analysis and managed data.
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associated with any miRNAs after adjustment for multiple comparisons. Of the dysregulated miRNAs, 17 were more likely to be *TP53*-mutated tumors while simultaneously being less likely to be CpG island methylator phenotype-high and/or microsatellite instability tumors. Genes regulated by these miRNAs were involved in numerous functions and pathways that influence cancer risk and progression. In summary, some infrequently expressed miRNAs, when expressed at higher levels appear to have significant biological meaning in terms of tumor molecular phenotype and gene expression profiles.

Keywords

Colorectal Cancer; TP53; CIMP; MSI; miRNA

Introduction

Molecular pathological epidemiology is a growing field of study that utilizes molecular information from tumors to better understand disease processes and progression (1). Assessment of tumor molecular phenotype in colorectal cancer has led to a better understanding of lifestyle factors that are uniquely associated with specific tumor phenotype (2–10). Tumor markers also have been examined with survival in an effort to identify biomarkers that can be used to predict prognosis and provide individualized treatment (11–16). While most studies have focused on common tumor molecular phenotype, such as *TP53*-mutated and *KRAS*-mutated tumors, microsatellite instability, and CpG Island Methylator Phenotype, studies are now examining other characteristics of tumors, such as gene expression and miRNA expression that may be important in identifying key disease pathways (11, 14, 17, 18).

MiRNAs are small, non-protein-coding RNA molecules that regulate gene expression either by post-transcriptionally suppressing mRNA translation or by causing mRNA degradation (18–23). We have previously shown that commonly expressed miRNAs influence tumor molecular phenotype in colorectal cancer, with the greatest number of differentially expressed miRNAs being observed for microsatellite unstable tumors compared to microsatellite stable tumors (24). MiRNAs were less frequently differentially expressed for *TP53*-mutated tumors, *KRAS*-mutated tumors, and CpG island methylator phenotype-high tumors. Most research focusing on miRNAs and tumor phenotype have focused on microsatellite unstable and CpG island methylator phenotype-high tumors (25) and on targeted miRNAs. Most targeted miRNAs studied, such as miR-21, are commonly expressed in tumors. Examination of infrequently expressed miRNAs may provide insight into unique pathways associated with tumor molecular phenotype.

In this study we focus on miRNAs that are infrequently expressed in normal colorectal mucosa and carcinoma tissue. We have previously shown that 34.5% of miRNAs expressed in colon tumor tissue are expressed in fewer than 10% of the population (26). Almost half of the miRNAs expressed in colorectal cancer tissue are expressed in less than half of the population. This presents two interesting questions: first, are low levels of expression purely noise in the data representing background expression levels; second, are infrequently

expressed miRNA meaningful when expressed at higher levels beyond what could be considered background noise? Since tumor molecular phenotype also varies in percentage of the population with a given phenotype, it is a logical question to determine if infrequently expressed miRNAs when expressed at higher levels are associated with unique tumor molecular phenotypes. In this study we examine associations between tumor molecular phenotype and infrequently expressed miRNA to determine if such associations exist. We further examine infrequently expressed miRNAs to determine genes they may be associated with gene expression when expressed at higher levels along with functions and pathways associated with those genes. The size and design of this study makes in uniquely powered to examine the role of infrequently expressed miRNAs as they relate to colorectal cancer.

Methods

Study Participants

Study participants were recruited as part of two population-based case-control studies that included all incident colon and rectal cancer between 30 to 79 years of age who resided in Utah or were from the Kaiser Permanente Medical Care Program in Northern California. Participants were white, Hispanic, or black for the colon cancer study and also included participants of Asian race for the rectal portion of the study (27, 28). Case diagnosis was verified by tumor registry data as a first primary adenocarcinoma of the colon or rectum and were diagnosed between October 1991 and September 1994 for the colon cancer study and between May 1997 and May 2001 for the rectal cancer study. Detailed study methods have been described (29). The study was approved by the Institutional Review Boards at the University of Utah and Kaiser Permanente Medical Care Program in Northern California.

RNA processing

Formalin-fixed paraffin embedded tissue from the initial biopsy or surgery was used to extract RNA. Both carcinoma tissue and adjacent normal mucosa were used. Tissue was micro-dissected from 1–4 sequential sections on aniline blue stained slides using an H&E slide for reference. Total RNA was extracted, isolated, and purified using the RecoverAll Total Nucleic Acid isolation kit (Ambion); NanoDrop spectrophotometer was used to determine RNA yields.

miRNA

The Agilent Human miRNA Microarray V19.0 containing probes for 2006 unique human miRNAs was used. Data were required to pass stringent quality control parameters established by Agilent to be included in the analyses. Quality control parameters 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 re-scanned. If a sample failed quality control assessment a second time, the sample was deemed to be of poor quality and the sample was excluded from analysis. Our previous analysis has shown that the repeatability associated with this microarray was extremely high (r=0.98) (29), and that comparison of miRNA expression levels obtained from the Agilent microarray to those obtained from qPCR had an

agreement of 100% in terms of directionality of findings and that the fold change calculated for the miRNA expression difference between carcinoma and normal colonic mucosa was almost identical (30). Of the 2006 unique human miRNAs assessed on the Agilent microarray, 1226 were expressed in colon carcinoma tissue and 1179 in normal colon mucosa.

To normalize differences in miRNA expression that could be attributed to the array, amount of RNA, location on array, or factors that could erroneously influence miRNA expression levels, total gene signal was normalized by multiplying each sample by a scaling factor (31), which was the median of the 75th percentiles of all the samples divided by the individual 75th percentile of each sample.

mRNA: RNA-Seq Sequencing Library Preparation and Data Processing

Total RNA was run on 245 carcinoma and normal mucosa pairs; of these 207 paired samples passed quality control and were used in analyses. Tissues samples taken from the study subjects at time of diagnosis were used for RNA extraction as previously described (32). For mRNA analysis, RNA library construction was done with the Illumina TruSeq Stranded Total RNA Sample Preparation Kit with Ribo-Zero. The samples were then fragmented and primed for cDNA synthesis, adapters were then ligated onto the cDNA, and the resulting samples were then amplified using PCR; the amplified library was then purified using Agencount AMPure XP beads. A more detailed description of the methods can be found in our previous work (33). Illumina TruSeq v3 single read flow cell and a 50 cycle single-read sequence run was performed on an Illumina HiSeq instrument. Reads were aligned to a sequence database containing the human genome (build GRCh37/hg19, February 2009 from genome.ucsc.edu) and alignment was performed using novoalign v2.08.01. Total gene counts were calculated for each exon and UTR of the genes using a list of gene coordinates obtained from http://genome.ucsc.edu. We dropped features that were not expressed in our RNA-Seq data or for which the expression was missing for the majority of samples, retaining 17,384 protein-coding genes (33).

Tumor Molecular Phenotype

We have previously assessed *TP53 and KRAS* mutations (4, 8, 34), the CpG island methylator phenotype using the classic panel that consisted of *MLH1*, *CDKN2A*, and MINT1, MINT2, and MINT31 (35), and microsatellite instability based on the mononucleotide repeats at *BAT26* and *TGFβR2* and a panel of 10 tetranucleotide repeats that were correlated highly with the Bethesda Panel (6); our original microsatellite instability studies were done prior to the development of the Bethesda Panel. Tumors were scored as CpG island methylator phenotype-high if two or more of the CpG islands were methylated for the five markers; otherwise they were classified as CpG island methylator phenotype -low/negative. This panel was run prior to the advent of more recent panels (36, 37).

Statistical Methods

The study focuses on infrequently expressed miRNAs which we define as being expressed in less than 50% of the study population for either normal mucosa or tumor. To be included in

the analysis, miRNAs also had to have a mean level of expression of 1.0 Agilent Relative Florescent Unit (ARFU) in tumors or normal mucosa and be expressed in at least 30 individuals. Each infrequently expressed miRNA could be considered expressed or not in each tumor and normal, resulting in three primary dysregulation groups based on the tumor-normal expression differences: up-regulated (expressed more in tumor than in normal), down-regulated (expressed more in normal than in tumor), and referent (neither up- nor down-regulated at the 25%tile/75%tile cutpoints). Rather than forcing the same number of subjects to fall into these three groups for all infrequently expressed miRNAs, cutpoints were selected based on the upper 25% and lower 25% of the tumor-normal differences for all infrequently expressed miRNAs. The resulting three-level dysregulation group factor (up, down, or referent) was used as a predictor in a per-miRNA logistic regression model also adjusting for age, study center, and sex and standardized what was considered true expression for all miRNAs. A total of 304 miRNAs were analyzed that fit these criteria. We used paired carcinoma and normal mucosa miRNA expression, evaluating differential expression between the two tissue types to control for differences in expression by tumor site and other potential confounding factors. Analyses were run separately for overall colorectal cancer, colon cancer, and rectal cancer. We analyzed difference in association for infrequently expressed miRNAs by *TP53*-mutated versus non-*TP53*-mutated, *KRAS*-mutated versus non-*KRAS*-mutated, CpG island methylator phenotype-high relative to CpG island methylator phenotype-low/negative, and microsatellite unstable compared to microsatellite stable. Adjustment for multiple comparisons was done using the positive false discovery rate Q value (38); given the infrequent expression of these miRNA, we report any associations for which the Q value was less than 0.05.

We compared those miRNA with a Q value of <0.05 (58=7 miRNAs) to RNAseq data to identify genes whose expression was associated with these infrequently expressed miRNAs. To determine statistical significance between the miRNA::mRNA associations, we ran a Fisher-Pitman Monte Carlo test with 10,000 permutation comparing differences in mean levels of gene expression across miRNA dysregulation groups of ≤75%ile vs >75%ile in R using the ‘coin’ package. RPKM (Reads Per Kilobase of transcript per Million mapped reads) mRNA expression level data were used in these analyses. Identification of networks and functions associated with genes whose mean expression was altered by miRNAs was done using Ingenuity Pathway Analysis®; adjustments for multiple comparisons were made using the Benjamini and Hochberg method (39). Both causal and interaction networks were generated. Interaction networks were limited to 35 molecules per network and 25 networks per analysis, and excluded endogenous chemicals. We focused on algorithmically derived interaction networks, which are assigned a score based on their relevance to the genes in the input dataset, the number of focus genes (i.e. dysregulated genes in our data that are in that network), and their connectivity (40). The score is calculated as $-\log_{10}P$, where P is generated using a Fisher’s exact test (41). Studies have found scores >3 to be significant, with a score of 3 indicating a 1/1000 chance that the focus genes are in a network due to random chance (42–44). Other studies have opted to utilize more stringent criteria and higher scores to ensure that their discovered networks are highly significant (45, 46); we utilized highly stringent criteria, only including networks with scores over 20.

Results

The study population is described in Table 1. Over half of the population were males. There were approximately equal numbers of individuals enrolled with proximal and distal colon tumors. Slightly less than half, 47.6%, of tumors had a *TP53* mutation, 31.7% had a *KRAS* mutation, 21.2% were classified as CpG island methylator phenotype-high and 9.1% were microsatellite unstable.

Assessment of *TP53*-mutated tumors associated with infrequently expressed miRNAs showed that 30 miRNAs were more likely to have a *TP53* mutation if they were upregulated in tumors while seven miRNAs were associated with a lower likelihood of having a *TP53* mutation if they were upregulated in tumors (Table 2). Most of the miRNAs (20 of the 37 miRNAs) were associated with a high level of differential expression in less than 20% of the. While some miRNAs were associated with a high level of differential expression in a large percentage of the population, these miRNAs were not expressed or extremely infrequently expressed in normal mucosa but were expressed to a greater degree in tumor tissue. There were no miRNAs more likely to have a *TP53* mutation if down-regulated after adjusting for multiple comparisons. Site-specific associations for colon and rectal cancer generally had Q values of >0.05. However many of these miRNAs with a Q value of 0.03 to 0.04 overall had a Q value of 0.07 for colon cancer specifically, most likely reflecting the decrease in power when analyzing colon cancer specifically rather than colorectal cancer combined. The lowest Q values for miRNAs for rectal cancer were 0.083. There were no unique associations with *KRAS*-mutated tumors.

Thirty-five infrequently expressed miRNAs were associated with CpG island methylator phenotype-high tumors (Table 3). Of these 35 miRNAs, 19 were less likely to be associated with a CpG island methylator phenotype-high tumor when up-regulated in tumor tissue, while 16 were more likely to have a CpG island methylator phenotype-high tumor if the miRNA was up-regulated in the tumor. Nine of these 35 miRNAs had over 20% of the population in the higher level of differential miRNA expression. As with *TP53*, many of these miRNAs had similar findings for colon cancer specifically as we observed for overall colorectal cancer, although the lowest FDR was 0.078 for colon cancer even when the raw p values were <0.0001 and comparable for both overall colorectal cancer and colon cancer specifically. Also like for *TP53*, after adjustment for multiple comparisons there were no significant findings between CIMP-high tumors and down-regulated miRNAs.

MSI was associated with 22 infrequently expressed miRNAs (Table 4). Of these miRNAs, the majority (13 of 22) were less likely to be associated with a microsatellite unstable tumor if up-regulated in the tumor. Only two of the 22 miRNAs had over 20% of the population in the group of dysregulation. There were no significant associations with microsatellite unstable tumors and down-regulated infrequently expressed miRNAs.

We determined which genes were associated with each of the 57 miRNAs that had a Q value of <0.05 using our RNAseq data. Those associations for all genes whose expression was altered by significant miRNAs are summarized in Supplemental Table 1. There was considerable overlap in miRNAs associated with tumor molecular phenotype. For instance,

19 miRNAs were associated with both CpG island methylator phenotype-high tumors and *TP53*-mutated tumors; 9 of these miRNAs also were associated with microsatellite unstable tumors. For each miRNAs where a higher level of expression increased the likelihood of having a *TP53*-mutated tumor, there was a decreased the likelihood of having a CpG island methylator phenotype-high or microsatellite unstable tumor.

We have summarized the top three networks (Supplemental Table 2 has all networks with Scores of over 20) derived from genes linked to the 19 miRNAS that were associated with multiple tumor molecular phenotypes of *TP53*, CpG island methylator phenotype high, and/or microsatellite unstable (Figure 1). Network 1 (Immunological Disease, Inflammatory Disease, and Inflammatory Response) had a Score of 28 and 35 focus molecules including genes that were influenced by the miRNAs; Network 2 (Cell Cycle, Cancer, Cell-To-Cell Signaling and Interaction) had a Score of 25 and 34 Focus molecules influenced by the genes associated with these miRNAs; Network 3 (Amino Acid Metabolism, Small Molecule Biochemistry, Drug Metabolism) also had a Score of 25 and 34 Focus Molecules associated with genes linked to these miRNAs. The majority of genes in these networks were up-regulated (indicated in red) when the miRNAs were expressed at higher levels. The genes that were down-regulated (indicated in green *NR3C1*, *TRPM6*, *GLP2R*, *ZFYVE28*, *FGD4*, RNF112, *TNFRSF17*, *TNFSF13*, and *CLEC3B)* were all down-regulated in the presence of high levels of miR-224-5p. Higher levels of miR-224-5p were more likely to be present in *TP53*-mutated tumors and less likely to be present in CpG island methylator phenotype-high tumors. *PHGDH* was up-regulated at high levels of miR-19a-3p and *KCND3* was up-regulated at high levels of miR-424-5p; high levels of miR-424-5p were more likely to have a *TP53*-mutated tumor and less likely to have a CpG island methylator phenotype-high tumor. *MYC* expression was associated with six miRNA, miR-151a-3p, miR-19a-3p, miR-3687, miR-374b-5p, miR-4533, and miR-7-5p. Higher levels of miRNA expression for all but miR-4533 were associated with *TP53*-mutated tumors, while miR-4533 was associated with tumors that were more likely to have microsatellite instability and CpG island methylator phenotype-high.

Discussion

Our data suggest that some miRNAs although infrequently expressed, when expressed at higher levels or up-regulated, are associated with specific tumor molecular phenotype. We did not have similar associations for down-regulated miRNAs. Of those infrequently expressed miRNAs significantly associated with tumor molecular phenotype when expressed at high levels were more likely to be highly expressed in *TP53*-mutated tumors and less likely to be associated with CpG island methylator phenotype-high or microsatellite unstable tumors. Many of these miRNAs were associated with altered gene mRNA expression in colorectal cancer tissue when expressed at high levels.

Many miRNAs are expressed infrequently in the population and often have low levels of expression when detected (29). Many of the miRNAs that have levels of expression around 0 could be considered background noise from slight differences in RNA samples despite high quality control. Additionally, although the data were normalized, picking a scale to normalize on is arbitrary and a different scale could have slightly altered what was

considered background levels of expression. The Agilent Platform that we used to collect miRNA data in this study has been noted as being able to detect low levels of expression (47, 48). Based on our findings, it appears that very low levels of expression are similar to no expression for most miRNAs, and that distinct associations for specific tumor molecular phenotype can only be seen when examining expression of these miRNAs at higher levels. These higher levels of expression are less likely to be the result of background expression, especially considering associations with tumor molecular phenotype.

To gain insight into pathways and functions of infrequently expressed miRNAs, we utilized our colorectal gene expression data from RNAseq. We assessed which genes were associated with miRNAs when miRNAs were more highly expressed. Since most of these miRNAs are infrequently expressed, there is less information regarding gene associations in existing databases, and even less information for colorectal tissue-specific expression, thus making use of our data imperative. Examining gene expression provided some insight into how these infrequently expressed miRNAs could be associated with various disease pathways. A limitation of RNAseq data, although a common method to determine miRNA::mRNA associations (49), is that miRNA targeted genes could be missed since gene expression studies more likely capture associations with transcription better than translation. However, we believe that our having RNAseq data in conjunction with miRNA data provides insight into colon-specific direct and indirect functions and pathways associated with these infrequently expressed miRNAs.

Given their infrequent expression, many of the miRNAs evaluated in our study have no known association with colorectal tumor molecular phenotype in the literature. However, our findings suggest that some infrequently expressed miRNAs, when they have high levels of expression in a tumor, may play an important role in tumorigenesis and the development of specific tumor phenotype. For instance, miR-19a-3p, which had about 25% to 30% of the population with high differential expression, was included previously in a miRNA cluster that functioned alongside Epstein-Barr Virus to control gene expression in human B cells through a *TP53*-induced mechanism (50). While we could find no reported association between this miRNA, or the others evaluated in this study, and colorectal cancer-specific tumor molecular phenotype, these findings are consistent with our finding that high levels of miR-19a-3p is associated with a *TP53* phenotype in colorectal cancer.

It has been shown that *TP53* mutations are inversely related to CpG island methylator phenotype-high and microsatellite unstable in colorectal cancer; *TP53* mutations are present in higher rates in microsatellite stable tumors while CpG island methylator phenotype-high tumors also are frequently microsatellite unstable tumors (34, 51). Our findings support this pattern by demonstrating that certain infrequently expressed miRNAs when upregulated in *TP53*-mutated tumors are simultaneously more likely to be down-regulated in CpG island methylator phenotype-high and microsatellite unstable tumors.

To further put these findings in perspective, we identified three major networks that represented the genes associated with those miRNAs that were up-regulated in *TP53*-mutated tumors and down-regulated in CpG island methylator phenotype-high and microsatellite unstable tumors. The first network has *NR3C1* as one of its central

components (See Figure 1). NR3C1 is a glucocorticoid receptor that induces apoptotic cell death, via decreased expression of anti-apoptotic proteins, such as *BCL2* and *MCL1*, and induces expression of pro-apoptotic proteins like BCL2-like apoptosis initiator 11 (52). In earlier studies, *NR3C1* has been associated with proximal microsatellite unstable tumors, with hypermethylation of *NR3C1* being identified as a marker for microsatellite unstable tumors and a marker to differentiate between CpG island methylator phenotype-high and CpG island methylator phenotype-low/negative phenotypes (53). These findings correlate with our identified association between the NR3C1 pathway and tumor phenotype; *NR3C1* was down-regulated in our data, suggesting less likely association with CpG island methylator phenotype-high and microsatellite unstable tumors. Our findings suggest that differential methylation of *NR3C1*, and its subsequent role in tumorigenesis and phenotype, may be in part due to the dysregulation of previously unstudied, infrequently expressed miRNAs.

The NFkB complex is central in our second Ingenuity Pathway Analysis network and is well known in literature for up-regulating and promoting various pro-inflammatory cytokines and linking various gastrointestinal conditions such as inflammatory bowel disease, diabetes mellitus, and colorectal cancer (54). The classical NFkB pathway plays a major role in linking inflammation to the onset and progression of malignancy in various tissues (55). One pro-inflammatory stimulus includes red meat consumption which has been linked to colon cancer and *TP53*-mutated tumors specifically (56, 57). A prospective study in Denmark has shown that the combination of polymorphisms in NFκB that down-regulate its expression, and high red meat consumption increases the likelihood of developing colorectal cancer (58). They proposed that lower NFkB activity leads to higher loads of reactive oxygen species secondary to heme degradation, contributing to colorectal carcinogenesis. Moreover, other studies have found that the NFκB pathway to be linked with the *TP53* pathway in hepatocellular carcinoma; the crosstalk between these two pathways is critical for the survival of HCC cells in the setting of reactive oxygen species (59). These previous findings further support an association between the NFkB complex and a *TP53* molecular phenotype in certain cancers, especially in the setting of pro-inflammatory stimuli. Here we suggest that the up-regulation of infrequently expressed miRNAs may provide an important link between NFκB and its related genes and *TP53* phenotype in colorectal cancer.

In our third Ingenuity Pathway Analysis network, *MYC* encodes for c-myc, a transcription factor often constitutively amplified leading to tumor progression of many cancers. In colorectal cancer, aberrant WNT/b-catenin pathway influences the amplification of MYC, leading to increased cellular proliferation (60). In our data *MYC* was up-regulated in conjunction with miRNAs that were up-regulated in *TP53*-mutated tumors. Furthermore, the consensus molecular subtype 2 subtype of colorectal cancer is canonically known to have strong WNT/MYC activation in microsatellite stable tumors; this subtype was also found to be highly correlated with *TP53*-mutated tumors (61). This suggests that miRNA dysregulation from infrequently expressed miRNAs, may play an important role in MYC's function in *TP53*-mutated molecular phenotype.

The study has several strengths and weaknesses. First, given the size of the study and the Agilent Platform used, we can identify and examine the impact of infrequently expressed

miRNAs. Many studies are too small to be able to determine associations with infrequently expressed miRNAs. Our dataset is rich, in that we have information on tumor molecular phenotype as well as RNAseq for a subset of these samples to improve our understanding of how miRNAs alter specific genes in colorectal tissue. One of the limitations of the study, which applies to the field of miRNA research, is the difficulty in understanding the pathways and genes associated with miRNA expression, especially when miRNAs alter multiple genes and genes are modified by multiple miRNAs. We have attempted to address this weakness in part by using our colorectal RNAseq data in conjunction with our miRNA data to identify genes that are up- or down-regulated by infrequently expressed miRNAs. In this study we have used adjacent tissue to the tumor as our comparison tissue. However there are limitation that the “normal” tissue is not true normal, although the best tissue available for comparison.

In summary, our data suggest that a large percentage of miRNAs expressed in colorectal tissue are infrequently expressed. However, some of the infrequently expressed miRNAs, when expressed at higher levels influence tumor molecular phenotype. This information is important for consideration pathways associated with cancer as well as examining lifestyle and environmental factors that may alter those pathways. Genes associated with these infrequently expressed miRNAs are involved in a variety of functions that may impact cancer development and prognosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hamada T, Keum N, Nishihara R, Ogino S. Molecular pathological epidemiology: new developing frontiers of big data science to study etiologies and pathogenesis. J Gastroenterol. 2016
2. Nishihara R, Lochhead P, Kuchiba A, Jung S, Yamauchi M, Liao X, et al. Aspirin use and risk of colorectal cancer according to BRAF mutation status. JAMA. 2013; 309(24):2563–71. [PubMed: 23800934]
3. Nishihara R, Wang M, Qian ZR, Baba Y, Yamauchi M, Mima K, et al. Alcohol, one-carbon nutrient intake, and risk of colorectal cancer according to tumor methylation level of IGF2 differentially methylated region. Am J Clin Nutr. 2014; 100(6):1479–88. [PubMed: 25411283]
4. Slattery ML, Anderson K, Curtin K, Ma K, Schaffer D, Edwards S, et al. Lifestyle factors and Ki-ras mutations in colon cancer tumors. Mutat Res. 2001; 483(1–2):73–81. [PubMed: 11600135]
5. Slattery ML, Anderson K, Curtin K, Ma KN, Schaffer D, Samowitz W. Dietary intake and microsatellite instability in colon tumors. Int J Cancer. 2001; 93(4):601–7. [PubMed: 11477566]

6. Slattery ML, Curtin K, Anderson K, Ma KN, Ballard L, Edwards S, et al. Associations between cigarette smoking, lifestyle factors, and microsatellite instability in colon tumors. J Natl Cancer Inst. 2000; 92(22):1831–6. [PubMed: 11078760]
7. Slattery ML, Curtin K, Anderson K, Ma KN, Edwards S, Leppert M, et al. Associations between dietary intake and Ki-ras mutations in colon tumors: a population-based study. Cancer Res. 2000; 60(24):6935–41. [PubMed: 11156393]
8. Slattery ML, Curtin K, Ma K, Edwards S, Schaffer D, Anderson K, et al. Diet activity, and lifestyle associations with p53 mutations in colon tumors. Cancer Epidemiol Biomarkers Prev. 2002:541–8. [PubMed: 12050095]
9. Slattery ML, Curtin K, Sweeney C, Levin TR, Potter J, Wolff RK, et al. Diet and lifestyle factor associations with CpG island methylator phenotype and BRAF mutations in colon cancer. Int J Cancer. 2007; 120(3):656–63. [PubMed: 17096326]
10. Bernstein AM, Song M, Zhang X, Pan A, Wang M, Fuchs CS, et al. Processed and Unprocessed Red Meat and Risk of Colorectal Cancer: Analysis by Tumor Location and Modification by Time. PLoS One. 2015; 10(8):e0135959. [PubMed: 26305323]
11. Slattery ML, Herrick JS, Mullany LE, Gertz J, Wolff RK. Improved survival among colon cancer patients with increased differentially expressed pathways. BMC Med. 2015; 13:75. [PubMed: 25890236]
12. Samowitz WS, Curtin K, Ma KN, Schaffer D, Coleman LW, Leppert M, et al. Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. Cancer Epidemiol Biomarkers Prev. 2001; 10(9):917–23. [PubMed: 11535541]
13. Samowitz WS, Curtin K, Schaffer D, Robertson M, Leppert M, Slattery ML. Relationship of Ki-ras mutations in colon cancers to tumor location, stage, and survival: a population-based study. Cancer Epidemiol Biomarkers Prev. 2000; 9(11):1193–7. [PubMed: 11097226]
14. Dou R, Nishihara R, Cao Y, Hamada T, Mima K, Masuda A, et al. MicroRNA let-7, T Cells, and Patient Survival in Colorectal Cancer. Cancer Immunol Res. 2016; 4(11):927–35. [PubMed: 27737877]
15. Hanyuda A, Kim SA, Martinez-Fernandez A, Qian ZR, Yamauchi M, Nishihara R, et al. Survival Benefit of Exercise Differs by Tumor IRS1 Expression Status in Colorectal Cancer. Ann Surg Oncol. 2016; 23(3):908–17. [PubMed: 26577117]
16. Liao X, Morikawa T, Lochhead P, Imamura Y, Kuchiba A, Yamauchi M, et al. Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. Clin Cancer Res. 2012; 18(8):2257–68. [PubMed: 22357840]
17. Ogino S, Meyerhardt JA, Kawasaki T, Clark JW, Ryan DP, Kulke MH, et al. CpG island methylation, response to combination chemotherapy, and patient survival in advanced microsatellite stable colorectal carcinoma. Virchows Arch. 2007; 450(5):529–37. [PubMed: 17372756]
18. Drusco A, Nuovo GJ, Zanesi N, Di Leva G, Pichiorri F, Volinia S, et al. MicroRNA profiles discriminate among colon cancer metastasis. PLoS One. 2014; 9(6):e96670. [PubMed: 24921248]
19. Ambros V. The functions of animal microRNAs. Nature. 2004; 431(7006):350–5. [PubMed: 15372042]
20. Murray BS, Choe SE, Woods M, Ryan TE, Liu W. An in silico analysis of microRNAs: mining the miRNAome. Mol Biosyst. 2010; 6(10):1853–62. [PubMed: 20539892]
21. Arora S, Rana R, Chhabra A, Jaiswal A, Rani V. miRNA-transcription factor interactions: a combinatorial regulation of gene expression. Mol Genet Genomics. 2013; 288(3–4):77–87. [PubMed: 23334784]
22. Gartel AL, Kandel ES. miRNAs: Little known mediators of oncogenesis. Semin Cancer Biol. 2008; 18(2):103–10. [PubMed: 18295504]
23. Nam S, Li M, Choi K, Balch C, Kim S, Nephew KP. MicroRNA and mRNA integrated analysis (MMIA): a web tool for examining biological functions of microRNA expression. Nucleic Acids Res. 2009; 37:W356–62. Web Server issue. [PubMed: 19420067]
24. Slattery ML, Herrick JS, Mullany LE, Wolff E, Hoffman MD, Pellatt DF, et al. Colorectal tumor molecular phenotype and miRNA: expression profiles and prognosis. Mod Pathol. 2016; 29(8): 915–27. [PubMed: 27198570]

25. Valeri N, Vannini I, Fanini F, Calore F, Adair B, Fabbri M. Epigenetics, miRNAs, and human cancer: a new chapter in human gene regulation. Mamm Genome. 2009; 20(9–10):573–80. [PubMed: 19697081]
26. Slattery ML, Herrick JS, Pellatt DF, Stevens JR, Mullany LE, Wolff E, et al. MicroRNA profiles in colorectal carcinomas, adenomas and normal colonic mucosa: variations in miRNA expression and disease progression. Carcinogenesis. 2016
27. Slattery ML, Potter J, Caan B, Edwards S, Coates A, Ma KN, et al. Energy balance and colon cancer–beyond physical activity. Cancer research. 1997; 57(1):75–80. [PubMed: 8988044]
28. Slattery ML, Caan BJ, Benson J, Murtaugh M. Energy balance and rectal cancer: an evaluation of energy intake, energy expenditure, and body mass index. Nutr Cancer. 2003; 46(2):166–71. [PubMed: 14690792]
29. Slattery ML, Herrick JS, Pellatt DF, Stevens JR, Mullany LE, Wolff E, et al. MicroRNA profiles in colorectal carcinomas, adenomas and normal colonic mucosa: variations in miRNA expression and disease progression. Carcinogenesis. 2016; 37(3):245–61. [PubMed: 26740022]
30. Pellatt DF, Stevens JR, Wolff RK, Mullany LE, Herrick JS, Samowitz W, et al. Expression Profiles of miRNA Subsets Distinguish Human Colorectal Carcinoma and Normal Colonic Mucosa. Clin Transl Gastroenterol. 2016; 7:e152. [PubMed: 26963002]
31. Agilent Technologies I. Agilent GeneSpring User Manual. Santa Clara, CA: Aglient Technologies Inc; 2013. cited 2015 July 16
32. Slattery ML, Herrick JS, Mullany LE, Valeri N, Stevens J, Caan BJ, et al. An evaluation and replication of miRNAs with disease stage and colorectal cancer-specific mortality. Int J Cancer. 2015; 137(2):428–38. [PubMed: 25484364]
33. Slattery ML, Pellatt DF, Mullany LE, Wolff RK, Herrick JS. Gene expression in colon cancer: A focus on tumor site and molecular phenotype. Genes, chromosomes & cancer. 2015; 54(9):527–41. [PubMed: 26171582]
34. Samowitz WS, Holden JA, Curtin K, Edwards SL, Walker AR, Lin HA, et al. Inverse relationship between microsatellite instability and K-ras and p53 gene alterations in colon cancer. Am J Pathol. 2001; 158(4):1517–24. [PubMed: 11290569]
35. Samowitz WS, Albertsen H, Herrick J, Levin TR, Sweeney C, Murtaugh MA, et al. Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology. 2005; 129(3):837–45. [PubMed: 16143123]
36. Hinoue T, Weisenberger DJ, Lange CP, Shen H, Byun HM, Van Den Berg D, et al. Genome-scale analysis of aberrant DNA methylation in colorectal cancer. Genome Res. 2012; 22(2):271–82. [PubMed: 21659424]
37. Ogino S, Kawasaki T, Kirkner GJ, Kraft P, Loda M, Fuchs CS. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn. 2007; 9(3):305–14. [PubMed: 17591929]
38. Storey JD. A direct approach to false discovery rates. Journal of the Royal Statistical Society: Series B. 2002; 64S(8):479–98.
39. Benjamini YH,Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. 1995; 57:289–300.
40. Savli H, Szendroi A, Romics I, Nagy B. Gene network and canonical pathway analysis in prostate cancer: a microarray study. Experimental & molecular medicine. 2008; 40(2):176–85. [PubMed: 18446056]
41. Li Y, Carrillo JA, Ding Y, He Y, Zhao C, Zan L, et al. Ruminal Transcriptomic Analysis of Grass-Fed and Grain-Fed Angus Beef Cattle. PLoS One. 2015; 10(6):e0116437. [PubMed: 26090810]
42. Baranzini SE, Galwey NW, Wang J, Khankhanian P, Lindberg R, Pelletier D, et al. Pathway and network-based analysis of genome-wide association studies in multiple sclerosis. Human molecular genetics. 2009; 18(11):2078–90. [PubMed: 19286671]
43. Yan-Fang T, Dong W, Li P, Wen-Li Z, Jun L, Na W, et al. Analyzing the gene expression profile of pediatric acute myeloid leukemia with real-time PCR arrays. Cancer cell international. 2012; 12(1):40. [PubMed: 22958424]

44. Naito Y, Kuroda M, Mizushima K, Takagi T, Handa O, Kokura S, et al. Transcriptome Analysis for Cytoprotective Actions of Rebamipide against Indomethacin-Induced Gastric Mucosal Injury in Rats. Journal of clinical biochemistry and nutrition. 2007; 41(3):202–10. [PubMed: 18299717]
45. Reyes-Gibby CC, Yuan C, Wang J, Yeung SC, Shete S. Gene network analysis shows immune-signaling and ERK1/2 as novel genetic markers for multiple addiction phenotypes: alcohol, smoking and opioid addiction. BMC systems biology. 2015; 9:25. [PubMed: 26044620]
46. Jia P, Kao CF, Kuo PH, Zhao Z. A comprehensive network and pathway analysis of candidate genes in major depressive disorder. BMC Syst Biol. 2011; 5(Suppl 3):S12.
47. Sah S, McCall MN, Eveleigh D, Wilson M, Irizarry RA. Performance evaluation of commercial miRNA expression array platforms. BMC Res Notes. 3:80. [PubMed: 20298588]
48. Mestdagh P, Hartmann N, Baeriswyl L, Andreasen D, Bernard N, Chen C, et al. Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. Nat Methods. 2014; 11(8):809–15. [PubMed: 24973947]
49. Chou CH, Chang NW, Shrestha S, Hsu SD, Lin YL, Lee WH, et al. miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database. Nucleic Acids Res. 2016; 44(D1):D239–47. [PubMed: 26590260]
50. Riley KJ, Rabinowitz GS, Yario TA, Luna JM, Darnell RB, Steitz JA. EBV and human microRNAs co-target oncogenic and apoptotic viral and human genes during latency. EMBO J. 2012; 31(9): 2207–21. [PubMed: 22473208]
51. Bond CE, Umapathy A, Ramsnes I, Greco SA, Zhen Zhao Z, Mallitt KA, et al. p53 mutation is common in microsatellite stable, BRAF mutant colorectal cancers. Int J Cancer. 2012; 130(7): 1567–76. [PubMed: 21557216]
52. Wei TT, Lin YT, Chen WS, Luo P, Lin YC, Shun CT, et al. Dual Targeting of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase and Histone Deacetylase as a Therapy for Colorectal Cancer. EBioMedicine. 2016; 10:124–36. [PubMed: 27448759]
53. Ahlquist T, Lind GE, Costa VL, Meling GI, Vatn M, Hoff GS, et al. Gene methylation profiles of normal mucosa, and benign and malignant colorectal tumors identify early onset markers. Mol Cancer. 2008; 7:94. [PubMed: 19117505]
54. Jurjus A, Eid A, Al Kattar S, Zeenny MN, Gerges-Geagea A, Haydar H, et al. Inflammatory bowel disease, colorectal cancer and type 2 diabetes mellitus: The links. BBA Clin. 2016; 5:16–24. [PubMed: 27051585]
55. Li S, Pinard M, Wang Y, Yang L, Lin R, Hiscott J, et al. Crosstalk between the TNF and IGF pathways enhances NF-kappaB activation and signaling in cancer cells. Growth Horm IGF Res. 2015; 25(5):253–61. [PubMed: 26239406]
56. Pellatt AJ, Slattery ML, Mullany LE, Wolff RK, Pellatt DF. Dietary intake alters gene expression in colon tissue: possible underlying mechanism for the influence of diet on disease. Pharmacogenet Genomics. 2016; 26(6):294–306. [PubMed: 26959716]
57. Slattery ML, Curtin K, Ma K, Edwards S, Schaffer D, Anderson K, et al. Diet activity, and lifestyle associations with p53 mutations in colon tumors. Cancer Epidemiol Biomarkers Prev. 2002; 11(6): 541–8. [PubMed: 12050095]
58. Andersen V, Christensen J, Overvad K, Tjonneland A, Vogel U. Polymorphisms in NFkB, PXR, LXR and risk of colorectal cancer in a prospective study of Danes. BMC Cancer. 2010; 10:484. [PubMed: 20836841]
59. Huang Q, Zhan L, Cao H, Li J, Lyu Y, Guo X, et al. Increased mitochondrial fission promotes autophagy and hepatocellular carcinoma cell survival through the ROS-modulated coordinated regulation of the NFKB and TP53 pathways. Autophagy. 2016; 12(6):999–1014. [PubMed: 27124102]
60. Rennoll S, Yochum G. Regulation of MYC gene expression by aberrant Wnt/beta-catenin signaling in colorectal cancer. World J Biol Chem. 2015; 6(4):290–300. [PubMed: 26629312]
61. Rodriguez-Salas N, Dominguez G, Barderas R, Mendiola M, Garcia-Albeniz X, Maurel J, et al. Clinical relevance of colorectal cancer molecular subtypes. Crit Rev Oncol Hematol. 2017; 109:9–19. [PubMed: 28010901]

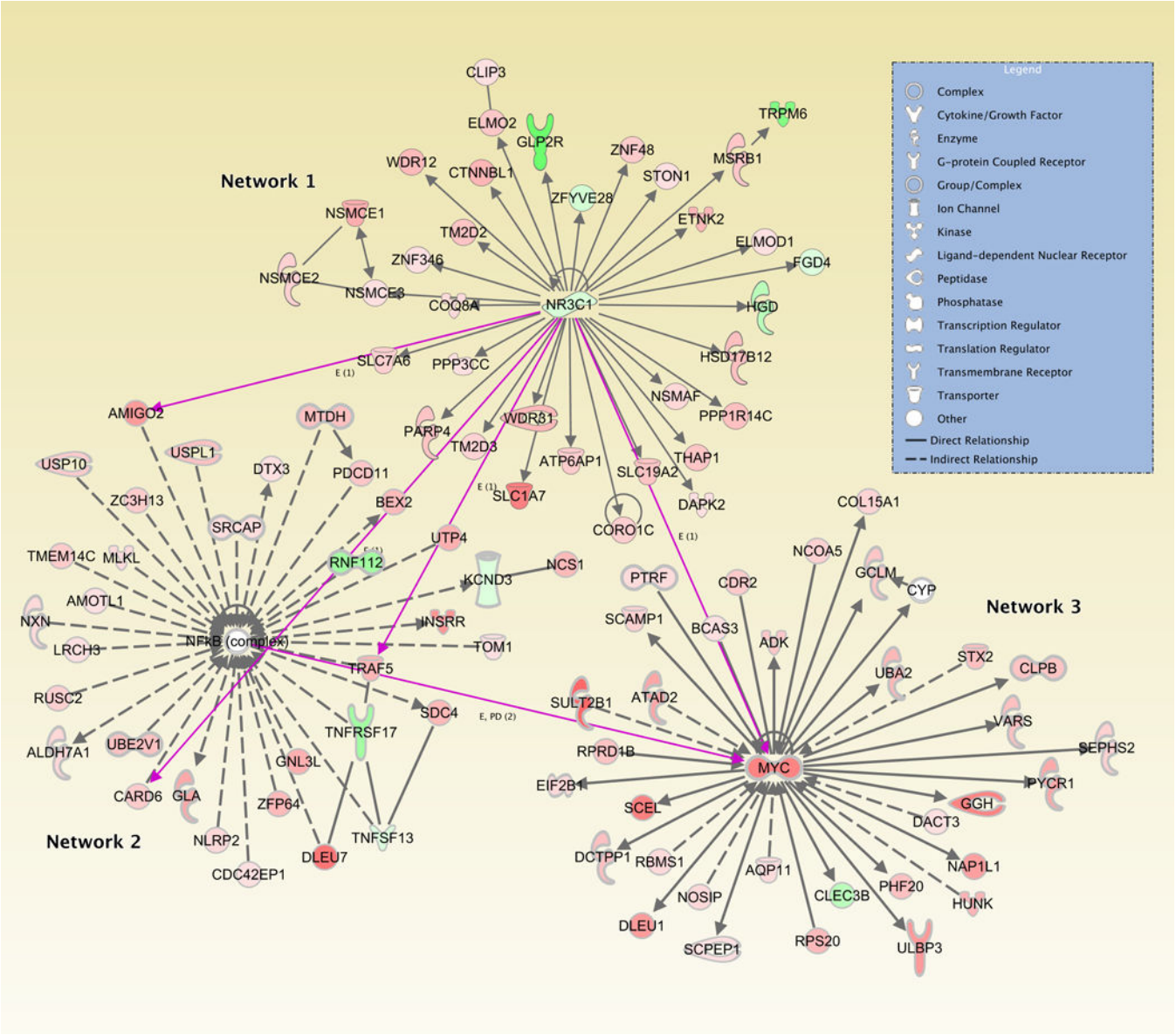

Figure 1.

Top Ingenuity Pathway Analysis networks associated with genes whose expression is altered by high levels of miRNA expression associated with both *TP53* and CpG island methylator phenotype-high and/or microsatellite instability

Table 1

Description of Study Population and miRNA expression

		Overall		Colon		Rectal	
		Subject N	**%**	**Subject N**	**%**	**Subject**	**%**
Sex							
	Male	1028	54.3	608	52.8	420	56.5
	Female	866	45.7	543	47.2	323	43.5
Center							
	Kaiser	1144	60.4	740	64.3	404	54.4
	Utah	750	39.6	411	35.7	339	45.6
Site							
	Proximal Colon	569	49.5	569	49.4	0	0.0
	Distal Colon	580	50.5	580	50.4	0	0.0
Study							
	Stage I	559	30.0	259	22.7	300	41.5
	Stage II	489	26.3	350	30.7	139	19.2
	Stage III	548	29.4	340	29.9	208	28.8
	Stage IV	266	14.3	190	16.7	76	10.5
TP53							
	Not Mutated	953	52.4	597	54.4	356	49.4
	Mutated	864	47.6	500	45.6	364	50.6
KRAS							
	Not Mutated	1240	68.5	724	67.6	516	69.9
	Mutated	569	31.5	347	32.4	222	30.1
CpG Island Methylator Phenotype							
	Low	1312	78.8	700	71.8	612	88.6
	High	354	21.2	275	28.2	79	11.4
Microsatellite Instability							
	Stable	1688	90.9	965	86.2	723	97.8
	Unstable	170	9.1	154	13.8	16	2.2

	Overall		Colon		Rectal	
	Subject N	%	Subject N	%	Subject	%
	Mean	STD	Mean	STD	Mean	STD
Age	64.2	10.2	65.4	9.5	62.3	11.0

Table 2

Associations between infrequently expressed miRNAs in colorectal cancer and *TP53* mutations

		Not-mutated		*TP53*-Mutated				P-value	
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**unadjusted**	**Q value**
hsa-miR-1207-3p	Down-regulated[1]	68	7.1	80	9.3	1.31	(0.94, 1.84)	0.115	0.919
	Referent	849	89.1	773	89.5	1.00			
	Up-regulated	36	3.8	11	1.3	0.35	(0.17, 0.69)	0.002	0.030
hsa-miR-1243	Down-regulated	161	16.9	140	16.2	0.92	(0.72, 1.18)	0.519	0.981
	Referent	710	74.5	677	78.4	1.00			
	Up-Regulated	82	8.6	47	5.4	0.60	(0.41, 0.88)	0.008	0.042
hsa-miR-1296	Down-regulated	51	5.4	44	5.1	0.95	(0.63, 1.45)	0.824	0.981
	Referent	878	92.1	777	89.9	1.00			
	Up-Regulated	24	2.5	43	5.0	2.02	(1.22, 3.37)	0.007	0.039
hsa-miR-133a	Down-regulated	35	3.7	38	4.4	1.19	(0.74, 1.91)	0.469	0.981
	Referent	910	95.5	805	93.2	1.00			
	Up-Regulated	8	0.8	21	2.4	2.99	(1.32, 6.80)	0.009	0.043
hsa-miR-133b	Down-regulated	483	50.7	458	53.0	1.17	(0.96, 1.43)	0.116	0.919
	Referent	415	43.5	326	37.7	1.00			
	Up-Regulated	55	5.8	80	9.3	1.85	(1.27, 2.69)	0.001	0.030
hsa-miR-151a-3p	Down-regulated	43	4.5	34	3.9	1.03	(0.64, 1.65)	0.900	0.982
	Referent	583	61.2	430	49.8	1.00			
	Up-Regulated	327	34.3	400	46.3	1.63	(1.35, 1.98)	<.0001	0.030
hsa-miR-184	Down-regulated	197	20.7	204	23.6	1.29	(1.02, 1.62)	0.031	0.919
	Referent	623	65.4	511	59.1	1.00			
	Up-Regulated	133	14.0	149	17.2	1.40	(1.08, 1.82)	0.012	0.047
hsa-miR-192-3p	Down-regulated	222	23.3	189	21.9	0.97	(0.77, 1.22)	0.793	0.981
	Referent	650	68.2	555	64.2	1.00			
	Up-Regulated	81	8.5	120	13.9	1.73	(1.27, 2.34)	0.000	0.030

		Not-mutated		*TP53*-Mutated				P-value	
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**unadjusted**	**Q value**
hsa-miR-19a-3p	Down-regulated	11	1.2	8	0.9	0.91	(0.36, 2.27)	0.836	0.981
	Referent	745	78.2	597	69.1	1.00			
	Up-Regulated	197	20.7	259	30.0	1.64	(1.33, 2.04)	<.0001	0.030
hsa-miR-224-5p	Down-regulated	24	2.5	14	1.6	0.93	(0.47, 1.84)	0.843	0.981
	Referent	364	38.2	222	25.7	1.00			
	Up-Regulated	565	59.3	628	72.7	1.79	(1.46, 2.19)	<.0001	0.030
hsa-miR-3190-5p	Down-regulated	11	1.2	11	1.3	1.14	(0.49, 2.65)	0.758	0.981
	Referent	937	98.3	837	96.9	1.00			
	Up-Regulated	5	0.5	16	1.9	3.62	(1.32, 9.93)	0.013	0.047
hsa-miR-31-5p	Down-regulated	14	1.5	11	1.3	0.83	(0.37, 1.83)	0.637	0.981
	Referent	775	81.3	762	88.2	1.00			
	Up-Regulated	164	17.2	91	10.5	0.57	(0.43, 0.75)	<.0001	0.030
hsa-miR-3607-3p	Down-regulated	53	5.6	63	7.3	1.36	(0.93, 1.99)	0.111	0.919
	Referent	835	87.6	715	82.8	1.00			
	Up-Regulated	65	6.8	86	10.0	1.52	(1.09, 2.14)	0.014	0.0495
hsa-miR-3609	Down-regulated	193	20.3	142	16.4	0.86	(0.67, 1.11)	0.247	0.981
	Referent	588	61.7	492	56.9	1.00			
	Up-Regulated	172	18.0	230	26.6	1.60	(1.27, 2.02)	<.0001	0.030
hsa-miR-3615	Down-regulated	207	21.7	201	23.3	1.04	(0.83, 1.31)	0.708	0.981
	Referent	640	67.2	602	69.7	1.00			
	Up-Regulated	106	11.1	61	7.1	0.63	(0.45, 0.88)	0.007	0.039
hsa-miR-3622b-3p	Down-regulated	22	2.3	26	3.0	1.48	(0.83, 2.64)	0.186	0.966
	Referent	746	78.3	615	71.2	1.00			
	Up-Regulated	185	19.4	223	25.8	1.48	(1.19, 1.85)	0.001	0.030
hsa-miR-362-5p	Down-regulated	26	2.7	13	1.5	0.60	(0.31, 1.18)	0.141	0.919
	Referent	730	76.6	587	67.9	1.00			

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		Not-mutated		*TP53*-Mutated				P-value	
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**unadjusted**	**Q value**
	Up-Regulated	197	20.7	264	30.6	1.66	(1.34, 2.06)	<.0001	0.030
hsa-miR-3687	Down-regulated	12	1.3	14	1.6	1.42	(0.65, 3.11)	0.376	0.981
	Referent	726	76.2	580	67.1	1.00			
	Up-Regulated	215	22.6	270	31.3	1.57	(1.27, 1.93)	<.0001	0.030
hsa-miR-374a-5p	Down-regulated	15	1.6	7	0.8	0.55	(0.22, 1.35)	0.189	0.966
	Referent	781	82.0	664	76.9	1.00			
	Up-Regulated	157	16.5	193	22.3	1.43	(1.13, 1.81)	0.003	0.031
hsa-miR-374b-5p	Down-regulated	31	3.3	16	1.9	0.62	(0.34, 1.15)	0.131	0.919
	Referent	711	74.6	563	65.2	1.00			
	Up-Regulated	211	22.1	285	33.0	1.68	(1.36, 2.08)	<.0001	0.030
hsa-miR-424-5p	Down-regulated	17	1.8	14	1.6	0.98	(0.48, 2.00)	0.950	0.997
	Referent	701	73.6	572	66.2	1.00			
	Up-Regulated	235	24.7	278	32.2	1.41	(1.15, 1.74)	0.001	0.030
hsa-miR-4251	Down-regulated	72	7.6	76	8.8	1.35	(0.95, 1.90)	0.090	0.919
	Referent	639	67.1	514	59.5	1.00			
	Up-Regulated	242	25.4	274	31.7	1.42	(1.15, 1.75)	0.001	0.030
hsa-miR-4296	Down-regulated	79	8.3	68	7.9	1.01	(0.71, 1.42)	0.975	0.997
	Referent	728	76.4	625	72.3	1.00			
	Up-Regulated	146	15.3	171	19.8	1.38	(1.08, 1.76)	0.011	0.047
hsa-miR-4421	Down-regulated	216	22.7	179	20.7	0.95	(0.75, 1.19)	0.640	0.981
	Referent	664	69.7	589	68.2	1.00			
	Up-Regulated	73	7.7	96	11.1	1.51	(1.09, 2.09)	0.013	0.047
hsa-miR-4654	Down-regulated	169	17.7	163	18.9	1.21	(0.95, 1.56)	0.128	0.919
	Referent	569	59.7	450	52.1	1.00			
	Up-Regulated	215	22.6	251	29.1	1.48	(1.19, 1.85)	0.001	0.030
hsa-miR-4695-3p	Down-regulated	25	2.6	25	2.9	1.14	(0.65, 2.01)	0.644	0.981

		Not-mutated		*TP53*-Mutated				P-value	
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**unadjusted**	**Q value**
	Referent	912	95.7	808	93.5	1.00			
	Up-Regulated	16	1.7	31	3.6	2.20	(1.19, 4.06)	0.012	0.047
hsa-miR-4711-5p	Down-regulated	22	2.3	19	2.2	0.94	(0.50, 1.75)	0.849	0.981
	Referent	905	95.0	840	97.2	1.00			
	Up-Regulated	26	2.7	5	0.6	0.22	(0.08, 0.57)	0.002	0.030
hsa-miR-484	Down-regulated	106	11.1	86	10.0	0.91	(0.67, 1.24)	0.550	0.981
	Referent	763	80.1	659	76.3	1.00			
	Up-Regulated	84	8.8	119	13.8	1.59	(1.18, 2.15)	0.002	0.030
hsa-miR-5095	Down-regulated	134	14.1	105	12.2	0.89	(0.67, 1.17)	0.401	0.981
	Referent	710	74.5	624	72.2	1.00			
	Up-Regulated	109	11.4	135	15.6	1.42	(1.08, 1.87)	0.013	0.047
hsa-miR-532-3p	Down-regulated	61	6.4	44	5.1	0.83	(0.55, 1.25)	0.368	0.981
	Referent	739	77.5	622	72.0	1.00			
	Up-Regulated	153	16.1	198	22.9	1.51	(1.19, 1.91)	0.001	0.030
hsa-miR-532-5p	Down-regulated	48	5.0	22	2.5	0.60	(0.35, 1.00)	0.052	0.919
	Referent	655	68.7	482	55.8	1.00			
	Up-Regulated	250	26.2	360	41.7	1.94	(1.59, 2.37)	<.0001	0.030
hsa-miR-5685	Down-regulated	184	19.3	173	20.0	1.01	(0.80, 1.28)	0.922	0.993
	Referent	683	71.7	645	74.7	1.00			
	Up-Regulated	86	9.0	46	5.3	0.57	(0.39, 0.83)	0.003	0.031
hsa-miR-625-5p	Down-regulated	17	1.8	17	2.0	1.09	(0.55, 2.15)	0.806	0.981
	Referent	898	94.2	831	96.2	1.00			
	Up-Regulated	38	4.0	16	1.9	0.47	(0.26, 0.84)	0.012	0.047
hsa-miR-652-3p	Down-regulated	39	4.1	25	2.9	0.72	(0.43, 1.20)	0.209	0.966
	Referent	824	86.5	702	81.3	1.00			
	Up-Regulated	90	9.4	137	15.9	1.75	(1.32, 2.33)	0.000	0.030

		Not-mutated		*TP53*-Mutated				P-value	
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**unadjusted**	**Q value**
hsa-miR-664a-3p	Down-regulated	201	21.1	186	21.5	1.13	(0.89, 1.43)	0.314	0.981
	Referent	569	59.7	455	52.7	1.00			
	Up-Regulated	183	19.2	223	25.8	1.50	(1.19, 1.89)	0.001	0.030
hsa-miR-7-5p	Down-regulated	14	1.5	10	1.2	0.98	(0.43, 2.22)	0.957	0.997
	Referent	580	60.9	421	48.7	1.00			
	Up-Regulated	359	37.7	433	50.1	1.67	(1.38, 2.01)	<.0001	0.030
hsa-miR-98-5p	Down-regulated	26	2.7	18	2.1	0.82	(0.44, 1.51)	0.522	0.981
	Referent	727	76.3	592	68.5	1.00			
	Up-Regulated	200	21.0	254	29.4	1.54	(1.24, 1.91)	<.0001	0.030

[1] Down-regulated have differential expression <-1.77; referent has differential expression between -1.77, 2.08; up- regulated has differential expression >2.08

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Table 3

Overall associations between differential miRNA expression in infrequently expressed miRNA and CIMP High tumors

		CIMP-Low/Negative		CIMP-High					
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**P value**	**Q value**
hsa-miR-151a-3p	Down-regulated[1]	45	3.4	20	5.6	1.46	(0.84, 2.56)	0.18	0.727
	Referent	696	53.0	221	62.4	1.00			
	Up-Regulated	571	43.5	113	31.9	0.64	(0.50, 0.83)	0.007	0.033
hsa-miR-1915-5p	Down-regulated	109	8.3	24	6.8	0.83	(0.52, 1.31)	0.42	0.773
	Referent	1138	86.7	298	84.2	1.00			
	Up-Regulated	65	5.0	32	9.0	1.90	(1.21, 2.98)	0.005	0.036
hsa-miR-193a-3p	Down-regulated	24	1.8	8	2.3	1.06	(0.46, 2.42)	0.90	0.895
	Referent	908	69.2	273	77.1	1.00			
	Up-Regulated	380	29.0	73	20.6	0.63	(0.47, 0.84)	0.002	0.033
hsa-miR-199b-5p	Down-regulated	80	6.1	29	8.2	1.25	(0.79, 1.99)	0.34	0.753
	Referent	804	61.3	247	69.8	1.00			
	Up-Regulated	428	32.6	78	22.0	0.61	(0.46, 0.81)	0.002	0.033
hsa-miR-19a-3p	Down-regulated	14	1.1	5	1.4	1.21	(0.42, 3.43)	0.73	0.803
	Referent	924	70.4	281	79.4	1.00			
	Up-Regulated	374	28.5	68	19.2	0.59	(0.44, 0.79)	0.0005	0.033
hsa-miR-2110	Down-regulated	68	5.2	17	4.8	0.94	(0.54, 1.63)	0.83	0.826
	Referent	1212	92.4	317	89.5	1.00			
	Up-Regulated	32	2.4	20	5.6	2.48	(1.39, 4.44)	0.002	0.033
hsa-miR-224-5p	Down-regulated	27	2.1	5	1.4	0.38	(0.14, 1.02)	0.05	0.436
	Referent	330	25.2	174	49.2	1.00			
	Up-Regulated	955	72.8	175	49.4	0.35	(0.27, 0.45)	<.0001	0.033
hsa-miR-30e-5p	Down-regulated	445	33.9	140	39.5	1.14	(0.88, 1.48)	0.31	0.753
	Referent	644	49.1	177	50.0	1.00			
	Up-Regulated	223	17.0	37	10.5	0.61	(0.41, 0.89)	0.01	0.041

		CIMP-Low/Negative		CIMP-High					
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**P value**	**Q value**
hsa-miR-31-5p	Down-regulated	17	1.3	7	2.0	1.80	(0.73, 4.44)	0.20	0.727
	Referent	1176	89.6	244	68.9	1.00			
	Up-Regulated	119	9.1	103	29.1	4.17	(3.08, 5.65)	<.0001	0.033
hsa-miR-3609	Down-regulated	219	16.7	83	23.4	1.34	(0.99, 1.80)	0.05	0.436
	Referent	757	57.7	228	64.4	1.00			
	Up-Regulated	336	25.6	43	12.1	0.43	(0.30, 0.61)	<.0001	0.033
hsa-miR-3615	Down-regulated	295	22.5	88	24.9	1.22	(0.92, 1.62)	0.17	0.727
	Referent	904	68.9	220	62.1	1.00			
	Up-Regulated	113	8.6	46	13.0	1.65	(1.13, 2.41)	0.01	0.038
hsa-miR-362-5p	Down-regulated	27	2.1	5	1.4	0.63	(0.24, 1.67)	0.35	0.753
	Referent	917	69.9	283	79.9	1.00			
	Up-Regulated	368	28.0	66	18.6	0.58	(0.43, 0.79)	0.0004	0.033
hsa-miR-3687	Down-regulated	20	1.5	5	1.4	0.95	(0.35, 2.58)	0.92	0.916
	Referent	908	69.2	273	77.1	1.00			
	Up-Regulated	384	29.3	76	21.5	0.66	(0.50, 0.88)	0.004	0.035
hsa-miR-374a-5p	Down-regulated	14	1.1	7	2.0	1.65	(0.65, 4.19)	0.29	0.753
	Referent	1003	76.4	305	86.2	1.00			
	Up-Regulated	295	22.5	42	11.9	0.48	(0.34, 0.68)	<.0001	0.033
hsa-miR-374b-5p	Down-regulated	27	2.1	15	4.2	1.96	(1.01, 3.80)	0.05	0.436
	Referent	878	66.9	272	76.8	1.00			
	Up-Regulated	407	31.0	67	18.9	0.54	(0.40, 0.72)	<.0001	0.033
hsa-miR-3938	Down-regulated	44	3.4	12	3.4	1.03	(0.54, 2.00)	0.92	0.919
	Referent	1239	94.4	324	91.5	1.00			
	Up-Regulated	29	2.2	18	5.1	2.51	(1.36, 4.65)	0.003	0.033
hsa-miR-3944-5p	Down-regulated	308	23.5	68	19.2	0.85	(0.62, 1.15)	0.29	0.753
	Referent	790	60.2	206	58.2	1.00			

		CIMP-Low/Negative		CIMP-High					
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**P value**	**Q value**
	Up-Regulated	214	16.3	80	22.6	1.47	(1.08, 1.99)	0.01	0.044
hsa-miR-424-5p	Down-regulated	21	1.6	8	2.3	1.33	(0.58, 3.07)	0.50	0.803
	Referent	875	66.7	280	79.1	1.00			
	Up-Regulated	416	31.7	66	18.6	0.52	(0.39, 0.70)	<.0001	0.033
hsa-miR-4492	Down-regulated	74	5.6	18	5.1	0.87	(0.51, 1.49)	0.62	0.803
	Referent	1190	90.7	309	87.3	1.00			
	Up-Regulated	48	3.7	27	7.6	2.18	(1.33, 3.59)	0.002	0.033
hsa-miR-4533	Down-regulated	169	12.9	61	17.2	1.52	(1.09, 2.11)	0.01	0.184
	Referent	1036	79.0	243	68.6	1.00			
	Up-Regulated	107	8.2	50	14.1	2.07	(1.43, 3.01)	0.0001	0.033
hsa-miR-4709-3p	Down-regulated	122	9.3	32	9.0	0.99	(0.65, 1.50)	0.96	0.965
	Referent	1045	79.6	265	74.9	1.00			
	Up-Regulated	145	11.1	57	16.1	1.55	(1.10, 2.18)	0.01	0.041
hsa-miR-4722-5p	Down-regulated	437	33.3	107	30.2	0.96	(0.73, 1.26)	0.75	0.803
	Referent	683	52.1	173	48.9	1.00			
	Up-Regulated	192	14.6	74	20.9	1.53	(1.11, 2.10)	0.01	0.038
hsa-miR-484	Down-regulated	131	10.0	46	13.0	1.26	(0.86, 1.83)	0.23	0.753
	Referent	1013	77.2	283	79.9	1.00			
	Up-Regulated	168	12.8	25	7.1	0.55	(0.35, 0.85)	0.008	0.038
hsa-miR-513a-3p	Down-regulated	59	4.5	18	5.1	1.16	(0.67, 2.01)	0.58	0.803
	Referent	1229	93.7	321	90.7	1.00			
	Up-Regulated	24	1.8	15	4.2	2.42	(1.24, 4.74)	0.01	0.038
hsa-miR-532-3p	Down-regulated	70	5.3	20	5.6	0.93	(0.55, 1.57)	0.77	0.803
	Referent	956	72.9	291	82.2	1.00			
	Up-Regulated	286	21.8	43	12.1	0.51	(0.36, 0.72)	0.0001	0.033
hsa-miR-532-5p	Down-regulated	38	2.9	17	4.8	1.38	(0.76, 2.53)	0.29	0.753

		CIMP-Low/Negative		CIMP-High					
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**P value**	**Q value**
	Referent	774	59.0	258	72.9	1.00			
	Up-Regulated	500	38.1	79	22.3	0.47	(0.36, 0.63)	<.0001	0.033
hsa-miR-5685	Down-regulated	265	20.2	63	17.8	0.89	(0.65, 1.22)	0.48	0.798
	Referent	969	73.9	252	71.2	1.00			
	Up-Regulated	78	5.9	39	11.0	1.96	(1.29, 2.97)	0.001	0.033
hsa-miR-590-5p	Down-regulated	173	13.2	61	17.2	1.28	(0.92, 1.77)	0.14	0.727
	Referent	983	74.9	269	76.0	1.00			
	Up-Regulated	156	11.9	24	6.8	0.55	(0.35, 0.87)	0.01	0.038
hsa-miR-6071	Down-regulated	151	11.5	37	10.5	0.89	(0.60, 1.30)	0.54	0.803
	Referent	1135	86.5	301	85.0	1.00			
	Up-Regulated	26	2.0	16	4.5	2.49	(1.31, 4.75)	0.006	0.036
hsa-miR-625-5p	Down-regulated	26	2.0	7	2.0	1.07	(0.46, 2.51)	0.88	0.876
	Referent	1261	96.1	323	91.2	1.00			
	Up-Regulated	25	1.9	24	6.8	3.55	(1.98, 6.37)	<.0001	0.033
hsa-miR-652-3p	Down-regulated	41	3.1	15	4.2	1.35	(0.72, 2.51)	0.35	0.753
	Referent	1085	82.7	310	87.6	1.00			
	Up-Regulated	186	14.2	29	8.2	0.56	(0.37, 0.86)	0.007	0.038
hsa-miR-664a-3p	Down-regulated	301	22.9	63	17.8	0.64	(0.47, 0.87)	<0.0001	0.184
	Referent	687	52.4	234	66.1	1.00			
	Up-Regulated	324	24.7	57	16.1	0.52	(0.38, 0.72)	<.0001	0.033
hsa-miR-873-3p	Down-regulated	42	3.2	10	2.8	0.94	(0.46, 1.90)	0.86	0.858
	Referent	1226	93.4	318	89.8	1.00			
	Up-Regulated	44	3.4	26	7.3	2.17	(1.30, 3.61)	0.001	0.033
hsa-miR-98-5p	Down-regulated	25	1.9	16	4.5	2.44	(1.26, 4.72)	0.008	0.184
	Referent	927	70.7	269	76.0	1.00			
	Up-Regulated	360	27.4	69	19.5	0.68	(0.51, 0.91)	0.01	0.038

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Table 4

Overall associations between differential miRNA expression in infrequently expressed miRNA and Microsatellite Instability tumors

		Microsatellite Stable		Microsatellite Unstable				P-value	
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**unadjusted**	**Q value**
hsa-miR-1207-3p	Down-regulated[1]	140	8.3	13	7.6	0.96	(0.53, 1.75)	0.90	0.90
	Referent	1512	89.6	145	85.3	1.00			
	Up-Regulated	35	2.1	12	7.1	3.28	(1.65, 6.53)	0.0007	0.04
hsa-miR-133b	Down-regulated	891	52.8	68	40.0	0.57	(0.40, 0.79)	.001	0.19
	Referent	664	39.4	96	56.5	1.00			
	Up-Regulated	132	7.8	6	3.5	0.31	(0.13, 0.74)	0.08	0.04
hsa-miR-151a-3p	Down-regulated	70	4.1	8	4.7	0.85	(0.39, 1.82)	0.67	0.71
	Referent	896	53.1	138	81.2	1.00			
	Up-Regulated	721	42.7	24	14.1	0.22	(0.14, 0.35)	<.0001	0.04
hsa-miR-192-3p	Down-regulated	384	22.8	35	20.6	0.86	(0.57, 1.29)	0.47	0.67
	Referent	1107	65.6	128	75.3	1.00			
	Up-Regulated	196	11.6	7	4.1	0.31	(0.14, 0.68)	0.004	0.04
hsa-miR-199b-5p	Down-regulated	107	6.3	18	10.6	1.55	(0.89, 2.71)	0.12	0.41
	Referent	1036	61.4	137	80.6	1.00			
	Up-Regulated	544	32.2	15	8.8	0.22	(0.13, 0.38)	<.0001	0.04
hsa-miR-203a	Down-regulated	174	10.3	17	10.0	0.70	(0.40, 1.23)	0.21	0.52
	Referent	474	28.1	71	41.8	1.00			
	Up-Regulated	1039	61.6	82	48.2	0.52	(0.37, 0.73)	0.0002	0.04
hsa-miR-28-3p	Down-regulated	186	11.0	16	9.4	0.95	(0.55, 1.64)	0.85	0.85
	Referent	1380	81.8	131	77.1	1.00			
	Up-Regulated	121	7.2	23	13.5	1.88	(1.16, 3.06)	0.01	0.04
hsa-miR-30a-5p	Down-regulated	604	35.8	64	37.6	1.00	(0.71, 1.41)	1.00	1.00
	Referent	900	53.3	101	59.4	1.00			
	Up-Regulated	183	10.8	5	2.9	0.24	(0.10, 0.61)	0.003	0.04

		Microsatellite Stable		Microsatellite Unstable				P-value	
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**unadjusted**	**Q value**
hsa-miR-30e-5p	Down-regulated	578	34.3	70	41.2	1.22	(0.87, 1.71)	0.24	0.53
	Referent	835	49.5	88	51.8	1.00			
	Up-Regulated	274	16.2	12	7.1	0.43	(0.23, 0.80)	0.007	0.04
hsa-miR-3609	Down-regulated	289	17.1	52	30.6	1.79	(1.25, 2.58)	0.002	0.19
	Referent	1000	59.3	108	63.5	1.00			
	Up-Regulated	398	23.6	10	5.9	0.23	(0.12, 0.45)	<.0001	0.04
hsa-miR-3615	Down-regulated	378	22.4	41	24.1	1.24	(0.84, 1.83)	0.27	0.54
	Referent	1173	69.5	99	58.2	1.00			
	Up-Regulated	136	8.1	30	17.6	2.41	(1.53, 3.79)	0.0001	0.04
hsa-miR-374b-5p	Down-regulated	44	2.6	5	2.9	0.97	(0.37, 2.52)	0.95	0.95
	Referent	1146	67.9	160	94.1	1.00			
	Up-Regulated	497	29.5	5	2.9	0.08	(0.03, 0.19)	<.0001	0.04
hsa-miR-3922-5p	Down-regulated	192	11.4	8	4.7	0.45	(0.22, 0.94)	0.03	0.28
	Referent	1318	78.1	127	74.7	1.00			
	Up-Regulated	177	10.5	35	20.6	2.07	(1.37, 3.13)	0.0005	0.04
hsa-miR-4492	Down-regulated	93	5.5	11	6.5	1.18	(0.61, 2.26)	0.63	0.71
	Referent	1526	90.5	143	84.1	1.00			
	Up-Regulated	68	4.0	16	9.4	2.29	(1.28, 4.10)	0.005	0.04
hsa-miR-4533	Down-regulated	231	13.7	27	15.9	1.27	(0.81, 1.98)	0.30	0.56
	Referent	1311	77.7	115	67.6	1.00			
	Up-Regulated	145	8.6	28	16.5	2.09	(1.32, 3.30)	0.002	0.04
hsa-miR-484	Down-regulated	182	10.8	11	6.5	0.57	(0.30, 1.08)	0.09	0.38
	Referent	1307	77.5	151	88.8	1.00			
	Up-Regulated	198	11.7	8	4.7	0.37	(0.18, 0.78)	0.008	0.04
hsa-miR-513a-3p	Down-regulated	81	4.8	7	4.1	0.84	(0.38, 1.86)	0.67	0.71
	Referent	1575	93.4	154	90.6	1.00			

		Microsatellite Stable		Microsatellite Unstable				P-value	
miRNA		**N**	**%**	**N**	**%**	**OR**	**(95% CI)**	**unadjusted**	**Q value**
	Up-Regulated	31	1.8	9	5.3	2.87	(1.32, 6.24)	0.01	0.04
hsa-miR-520d-3p	Down-regulated	124	7.4	16	9.4	1.33	(0.76, 2.32)	0.32	0.58
	Referent	1495	88.6	140	82.4	1.00			
	Up-Regulated	68	4.0	14	8.2	2.23	(1.21, 4.11)	0.008	0.04
hsa-miR-532-5p	Down-regulated	59	3.5	10	5.9	1.31	(0.64, 2.65)	0.46	0.67
	Referent	1016	60.2	153	90.0	1.00			
	Up-Regulated	612	36.3	7	4.1	0.08	(0.04, 0.17)	<.0001	0.04
hsa-miR-5685	Down-regulated	337	20.0	27	15.9	0.83	(0.53, 1.29)	0.41	0.66
	Referent	1243	73.7	116	68.2	1.00			
	Up-Regulated	107	6.3	27	15.9	2.75	(1.71, 4.40)	<.0001	0.04
hsa-miR-664a-3p	Down-regulated	360	21.3	28	16.5	0.59	(0.38, 0.91)	0.02	0.26
	Referent	923	54.7	130	76.5	1.00			
	Up-Regulated	404	23.9	12	7.1	0.22	(0.12, 0.40)	<.0001	0.04
hsa-miR-98-5p	Down-regulated	40	2.4	6	3.5	1.34	(0.55, 3.26)	0.52	0.67
	Referent	1189	70.5	158	92.9	1.00			
	Up-Regulated	458	27.1	6	3.5	0.10	(0.04, 0.23)	<.0001	0.04