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An evaluation and replication of miRNAs with disease stage and colorectal cancer-specific mortality

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Abstract

MiRNAs have been implicated in CRC development and associated with prognostic indicators such as disease stage and survival. Prognostic associations are often based on few individuals and imprecise. In this study, we utilize population-based data from 1141 colorectal cancer (CRC) cases to replicate previously reported associations between 121 miRNAs and disease stage and survival. The Agilent Human miRNA Microarray V19.0 was used to generate miRNA data following a stringent quality control protocol. Assessment of survival was done using Cox Proportional Hazard models adjusting for age, disease stage, and tumor molecular phenotype.

Five miRNAs were associated with more advanced disease stage; hsa-miR-145-5p and hsa-miR-31-5p showed increased expression with more advanced tumor stage, while hsa-miR-200b-3p, hsa-miR-215, and hsa-miR-451a had decreased expression with more advanced tumors. Thirteen miRNAs were associated with colorectal cancer mortality among individuals diagnosed with colon cancer while fourteen were associated with colorectal cancer mortality after a diagnosis with rectal cancer. Strongest associations were observed for those miRNAs that were expressed in a small subset of tumors. Most notable associations were for hsa-miR-145-3p (HR 2.94 95% CI 1.54, 5.61), and hsa-miR-9-3p (HR 10.28 95% CI1.31 80.84) with colon cancer and hsa-miR-335-5p (HR 0.17 95% CI 0.05, 0.54) for rectal cancer. Hsa-miR-374a-5p, hsa-miR-570-3p and hsa-miR-18a-5p significantly reduced the hazard of dying for all cases, regardless of tumor site.

Our findings illustrate the need for a large sample to evaluate the association of miRNAs with survival and disease stage in order to determine associations by tumor site.

Conflict of Interest. Dr. Samowitz may receive royalties in the future related to sales of the Ventana BRAF V600E antibody.

In 2003, it was first reported that miRNAs were associated with colorectal cancer (CRC)[1]. At that time, Michael et al. illustrated tumor suppressor-like activity for miR-143 and miR-145 in colon cancers and hypothesized that these miRNAs were targeting *ERK5* and *IRS1*. Since then, over 100 miRNAs have been implicated in CRC development while others have been examined with prognostic indicators. MiR-21, miR-143, miR-145, and miR-200c have been associated with survival after diagnosis with CRC[2, 3] MiR-21 has been associated with over a two-fold increased likelihood of death[4]; miR-145 could have potential clinical importance given its ability to predict survival with 81% accuracy[3]; and miR-200c has been correlated with poorer prognosis independent of tumor stage.[5]

Many of the reported studies have had small sample sizes, thus being limited in their ability to evaluate stage-specific associations and colon and rectal cancers separately. In this study, we utilize population-based data from 1141 CRC cases to replicate previously reported associations between 121 miRNAs and disease stage and survival; over or under differential expression between normal and tumor tissue was replicated previously (unpublished manuscript). We evaluate risk by level of miRNA expression in tumors and any expression for those miRNAs that are infrequently expressed.

Methods

Study participants come from two population-based case-control studies that included all incident colon and rectal cancers between 30 to 79 years of age who resided along the Wasatch Front in Utah or were members of the Kaiser Permanente Medical Care Program (KPMCP) in Northern California. Participants were white, Hispanic, or black for the colon cancer study; the rectal cancer study included Asians and American Indians not living on reservations were included[6, 7]. Cases had to have tumor registry verification of a first primary adenocarcinoma of the colon or rectum and diagnosed between October 1991 and September 1994 for the colon cancer group and between June 1997 and May 2001 for the rectal cancer group. Tumor tissue was obtained for 97% of all Utah cases diagnosed and for 85% of all KPMCP study participants[8] and included those who signed informed consent and those retrieved by local tumor registries and sent to study investigators without personal identifiers. Local tumor registry data were used to obtain date of birth, date of diagnosis, and tumor information for those individuals who were not interviewed. The study was approved by the Institutional Review Board of the University of Utah.

We have previously assessed these tumors for *TP53, KRAS,* and *BRAF* mutations, the CpG island methylator phenotype (CIMP) using the classic panel[9], and microsatellite instability (MSI) based on the mononucleotides *BAT26* and *TGFβRII* and a panel of 10 tetranucleotide repeats that were correlated highly with the Bethesda Panel [10]; our study was done prior to the Bethesda Panel development.

miRNA processing

RNA (miRNA) was extracted from formalin-fixed paraffin embedded tissues. We assessed slides and tumor blocks that were prepared over the duration of the study prior to the time of miRNA isolation to determine their suitability. The study pathologist (WS) reviewed slides to delineate tumor, normal, and polyp tissue. Cells were dissected from 1–4 sequential

sections on aniline blue stained slides using an H&E slide for reference. Total RNA containing miRNA was extracted, isolated, and purified using the RecoverAll Total Nucleic Acid isolation kit (Ambion), RNA yields were determined using a NanoDrop spectrophotometer. 100 ng total RNA was labeled with cy3 and hybridized to Agilent Human miRNA Microarray V19.0 and were scanned on an Agilent SureScan microarray scanner model G2600D. The Agilent Human microarray was generated using known miRNA sequence information compiled in the Sanger miRBASE database v19.0. The microarray contains probes for 2006 unique human miRNAs, with one to four unique probes for each of the known miRNAs. The miRNA array contains 60,000 unique human sequences and averages 30 replicates per probe sequence. Data were extracted from the scanned image using Agilent Feature Extract software v.11.5.1.1. Data were required to pass stringent QC 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 QC assessment a second time the sample was deemed to be of poor quality and the individual was excluded from down-stream analysis. Of the 1171 initial cases, 30 were excluded at this stage. A quantile normalization across arrays was done using preprocessCore[11] (www.bioconductor.org) to minimize differences that could be attributed to the array, amount of RNA, location on array, or other factors that could erroneously influence expression.

We refer to miRNAs using standard nomenclature used in the miRBase database[12]. Briefly, the first three letters signifies the organism, followed by a unique number. The number is followed by a dash and number (i.e., 1) if more than one loci codes for the miRNA. A lettered suffix denotes closely related miRNAs. If two miRNAs are coded by the same precursor product then the minor product is assigned the suffix (*). If predominant/minor product status is not known then the suffix 5p and 3p are used to denote 5′ and 3′ arm respectively. Many of the miRNAs being replicated in this study were reported prior to current nomenclature. For instance, let-7 may be reported in the literature as being associated with tumor stage, however Let-7 has since been further delineated to several closely related mature sequences and genomic loci, for example let-7a-3p, let-7a-5p, and let-7b-3p. A complete list of the 121 miRNAs previously reported with stage and/or survivals that are being evaluated in this replication is included in Supplemental Table 1.

Statistical Methods

To determine whether tumor stage was associated with miRNA expression levels, we used the significance analysis of microarrays as implemented in siggenes[13] after log base 2 transforming the data. We grouped stages 1 and 2 together and 3 and 4 together to maximize power and performed a two-class comparison with 1000 permutations to detect significant differences in miRNA expression by stage. Survival months were calculated from date of diagnosis to date of death from CRC or censoring on date of last follow-up or death from other causes. We used SAS 9.4 (SAS Institute, Cary, NC), to perform a Cox proportional hazards regression adjusted for age at diagnosis, gender, AJCC tumor stage, and tumor molecular phenotype to assess CRC-specific mortality. Analysis was done separately for

those with an expression interquartile range greater than 0 and those for whom few individuals had the miRNA expressed in their tumor. For miRNA's with an interquartile range greater than zero, we report the units of change as the interquartile range of the miRNA. For miRNA's with an interquartile range of zero, we created a "yes" vs. "no" variable and report the Hazard Ratio (HR) for any miRNA expression.

Bioinformatics analysis was performed on the list of miRNAs found to be strongly significantly associated with CRC-specific mortality to better understand the miRNAs role in pathway responses. Both miRTarBase[14] and miRWalk[15] databases were utilized; only validated gene targets were considered in the analysis. MiRTarBase, version 4.5 as of November 2013, provided target genes for hsa-miR-20a-3p, hsa-miR-335-5p, hsa-miR-374a-5p, and hsa-miR-9-3p, and miRWalk, updated as of March 2013, provided validated target genes for hsa-miR-145-3p (hsa-miR-145* in miRWalk, hsa-miR-145-3p in miRBase[16] release 21 as of June 2014) and hsa-miR-570 (did not differentiate between 5p and 3p in the database). MirTarBase provides a downloadable list of all the validated gene targets for Homo sapiens miRNAs, and miRWalk lists are available for an individually queried miRNA.

All of the validated gene target lists were downloaded in text file format, combined into one master list, and sorted first by miRNA and then by gene. This gene list was uploaded to the Database for Annotation, Visualization, and Integrated Discovery (DAVID)[17], which provides functional annotation tools to aid in understanding the biological meaning of a large gene list. The Pathway Viewer feature in the DAVID Functional Annotation Tool provides links between the functionally annotated genes to pathway databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG) database that was used in this analysis. Using DAVID's EASE Score, each pathway is associated with a subset of the genes from the uploaded list and assigned a raw *P*-value, an adjusted *P*-value (the Bonferroni value), and a fold-enrichment score. The fold enrichment score measures the magnitude of the enrichment of the genes; it compares the number of the genes of a particular functional annotation in the users list to the expected number of genes with this function in the human genome. Fold enrichment of 1.5 and higher are considered to be of interest. Pathway terms specific to other types of cancer (such as 'renal cell carcinoma') were not considered.

Results

The 1141 cases included in these analyses are described in Table 1. Slightly more than 50% of cases were men, with 65.3% of tumors located in the colon. *TP53* mutations were identified in 47.6% of tumors, 28.4% had a *KRAS* mutation, 9.0% were MSI unstable, and 19.9% had CIMP. At the time of last follow-up, 53.2% were alive. Twenty-seven miRNAs had no expression in tumors (Supplemental Table 1); 28 miRNAs were expressed in less than five percent of tumors.

Five miRNAs were associated with more advanced disease stage (Table 2). Hsa-miR-145-5p and hsa-miR-31-5p showed increased expression with more advanced tumor stage, while

hsa-miR-200b-3p, hsa-miR-215, and hsa-miR-451a had decreased expression with more advanced tumors.

Thirteen miRNAs were associated with colorectal cancer mortality among individuals diagnosed with colon cancer while fourteen were associated with colorectal cancer mortality after a diagnosis with rectal cancer (Table 3). As expression level increased for these miRNAs, the likelihood of dying decreased. Exceptions to this pattern were hsa-miR-145-5p (HR 1.29 95% CI 1.05, 1.59), hsa-miR-330-3p (HR 1.38 95% CI 1.03, 1.83), hsa-miR-500a-5p (HR 1.26 95% CI 1.04, 1.52), and hsa-miR-99a-5p (HR 1.29, 95% CI 1.01, 1.65). For the most part associations were in the same direction for both colon and rectal cancer, although the magnitude of associations were slightly stronger for rectal cancer.

Given the number of miRNAs infrequently expressed, we explored the possibility that, of these miRNAs, any expression could have either a better or worse survival than those without expression. Six of these miRNAs were highly significant associations with survival (Table 4). Four were associated with colon cancer and one with rectal cancer. Most notable associations were for hsa-miR-145-3p (HR 2.94 95% CI 1.54, 5.61), and hsa-miR-9-3p (HR 10.28 95% CI1.31 80.84) with colon cancer and hsa-miR-335-5p (HR 0.17 95% CI 0.05, 0.54) for rectal cancer. Hsa-miR-374a-5p, hsa-miR-570-3p and hsa-miR-18a-5p significantly reduced the hazard of dying for all cases, regardless of tumor site.

We further evaluated associations with miR-21 (Table 5). We saw no significant increased risk of mortality after diagnosis with colon cancer for miR-21 by tumor stage at diagnosis or by tumor molecular phenotype. An inverse association with mortality was observed for rectal cancer with the strongest inverse associations for lower disease stage and CIMP+ and MSI tumors.

Discussion

Of the 121 miRNAs included in this replication study, we were able to evaluate 92 miRNAs which showed tumor expression, however 26 of these showed minimal expression for most tumors. We replicated associations for five miRNAs with advanced stage at diagnosis and 25 with survival (17 with colon cancer and 15 with rectal cancer). Only seven miRNAs were associated with survival for both colon and rectal cancer. Of interest is the finding that miRNAs that showed expression in only a small subset of the population were strongly associated with survival, however given the infrequent expression in the population we would be able to mainly detect strong associations.

Interpretation of these results requires consideration of several factors. These findings build on our previous replication of differential expression between tumor and normal tissue (unpublished data). First, more advanced nomenclature is available for our study than for many of the previously reported studies. Since few studies have reported at the 3p and 5p level, we would expect that a large number of 121 miRNAs would not replicate. Secondly, reports in the literature include evaluation of both differential expression as well as absolute levels of expression in tumors. We report absolute expression in tumors because of greater ease in interpretation, although our findings were similar when we examined differential

expression (data not shown). The replication findings we present are not adjusted for multiple comparisons since the previous reports in the literature, which evaluated one or a few miRNA did not adjust for multiple comparisons. To enable us to replicate findings, we use a significance threshold of 0.05, however when more stringent adjustments were made adjusting for all comparisons with Bonferroni, only miRNA 215 remained significantly associated with survival among rectal cancers. Small differences in associations could stem from our use of the Agilent array while others have evaluated only a few miRNAs using qRT-PCR methods. We report findings for colon and rectal cancer separately, while others generally report CRC combined, however combining colon and rectal did not change the results. We believe that it is important to show site-specific associations as we have previously shown differences in expression based on tumor location [18] and others have reported similar differences by tumor location[19]. Additionally, we adjusted for AJCC stage to obtain unbiased survival estimates. However, the greatest difference in the results reported here and in the literature is the sample size utilized. Because of our considerably greater sample size we have more precision to estimate associations.

Several miRNAs have been reported as being associated with advanced disease stage or promoting metastasis[4, 20–30]. Many of these studies have been small, often based on data from cell lines or few study participants. We replicated five of the 55 miRNAs previously associated with advanced disease stage. Our findings were consistent with those reported by Arndt and colleagues[24] who showed that up-regulation of miR-145 was associated with advanced disease stage. Our finding of up-regulation of miR-31 and advanced stage was supported by several studies[24, 30, 31]. Likewise, our finding of significant down-regulation of miR-215 with advanced tumor stage replicates previous reports[21]. However, we observed that miR-200b-3p and miR-451a were significantly down-regulated in tumors with a higher stage, unlike other reports show these miRNAs to be up-regulated with greater tumor stage (miR-200b) or poorer survival (miR-451a).[25,32]

While more miRNAs replicated with survival, a considerable number did not, including let-7, miR-100, miR-10b, miR-126, miR-151a, miR-151b, miR-16, miR-197, miR-200a-3p, miR-200b-5p, miR-200c, miR-206, miR-223, miR-302c, miR-30c, miR-320a, miR-324, miR-34, miR-451a, miR-483, miR-490, miR-500a, miR-518c, miR-526b, miR-615, and miR-92a. Many that were statistically significantly associated with survival were associated in a different direction than reported. However, inconsistencies in terms of the direction of the expression with the associations exist in the literature. MiRNAs that we replicated with CRC-mortality and in the same direction as previously reported, include miR-141-3p, miR-148a-3p, miR-20b-5p, miR-215, miR-29a-3p, miR-29c-3p, miR-425-5p, and miR-145-3p. Others miR-570 were statistically significant inversely associated with survival in our data whereas they were directly associated before[33]. Several miRNAs have inconsistent associations with survival in the literature including miR-141-3p[23, 34], miR-148a-3p[26, 33], and miR-20a[4, 21]; in our data they were significantly inversely associated with colorectal cancer-specific mortality.

MiR-21 is one of the most frequently studied miRNA.[35] The majority of studies show that miR-21 is directly associated both with advanced tumor stage and poor survival[4, 22, 25, 30, 36, 37], although at least three studies have shown inverse associations

with survival with higher levels of miR-21[38–40]. Although miR-21 was up regulated in both colon and rectal tumors relative to normal, we did not see a significant difference in CRC-specific mortality overall (for colon cancer), by tumor stage at diagnosis or by tumor molecular phenotype. However, we saw a strong protective effect for higher levels of miR-21 expression for survival after rectal cancer diagnosis. Several studies have shown associations for stage 2 CRC only[41]; we examined stage 2 and saw a non-significant increase of around 1.3. This is similar to the meta-analysis reported by Ye and colleagues in an Asian population.[42] Meta-analysis reported over a two-fold increase risk of dying; miR-21 expression in serum from a sample of 282 CRC patients also has been associated with survival[43, 44]. Another meta-analysis of seven studies reported a HR of 1.76 (95% CI of 1.34, 2.32)[45]; although one of the studies that was included reported colon and rectal cancer separately and showed a 17% increased risk of colon cancer and a non-significant protective effect for rectal cancer for stage 2 cancers.[46]

We further explored those miRNAs that showed the strongest associations. Those that were infrequently expressed, but had a strong impact on survival when expressed were evaluated using bioinformatics tools. Evaluating target genes of these six miRNAs yielded an initial list of 2,721 genes, with over 2,500 associated with miR-335-5p. MiR-335-5p was only significant for rectal tumors that showed any expression. Considering only the genes hsa-miR-335-5p regulated that were also regulated by the other miRNAs produced a list of 206 genes. Originally 29 pathway terms were generated, however only pathways that had both a raw *P*-value and a Bonferroni corrected *P*-value of < 0.05 are shown in Table 6. Several of these pathways have been associated with CRC survival and other miRNA have been linked to them, including miR-137 with MAPK signaling pathway[47]; miR-181a with TGFβ[48]; miR-155 with innate immune response[49]; *TP53* and cell cycle with several miRNAs including miR-200c[50,51]; let-7b, let-7e, miR-145, miR-148, and miR-22 with insulin pathways[21]; and miR-145 with neuregulin pathways[24].

While many miRNAs have been associated with CRC disease stage and mortality, we replicated five miRNAs with disease stage and 25 with survival. We have analyzed colon and rectal cancer separately and have shown many unique associations. Our findings illustrate the need for a large enough sample to evaluate associations by tumor site and to generate sufficiently stable estimates of association adjusting for disease stage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty Impact Statement

This is a large study that examines miRNAs that have been previously associated with more advanced colorectal cancers, with disease stage and survival. Given our large sample size and data that incorporates both disease stage and tumor molecular phenotype, we are able to robustly evaluate associations with colorectal cancer-specific mortality.

Table 1

Description of Study Population

	Overall		Stage I		Stage II		Stage III		Stage IV	
	N	%	N	%	N	%	N	%	N	%
Sex										
Male	615	53.9	183	55.6	159	51.1	189	54.3	84	54.9
Female	526	46.1	146	44.4	152	48.9	159	45.7	69	45.1
Center										
Kaiser	718	62.9	210	63.8	199	64.0	209	60.1	100	65.4
Utah	423	37.1	119	36.2	112	36.0	139	39.9	53	34.6
Site										
Proximal	362	50.1	102	31.0	102	32.8	104	29.9	54	35.3
Distal	361	49.9	64	19.5	121	38.9	125	35.9	51	33.3
Study										
Colon	745	65.3	171	52.0	227	73.0	237	68.1	110	71.9
Rectal	396	34.7	158	48.0	84	27.0	111	31.9	43	28.1
TP53										
Wt	575	52.4	152	47.9	180	59.8	162	48.5	81	55.5
Mutated	523	47.6	165	52.1	121	40.2	172	51.5	65	44.5
Kras										
Wt	784	71.6	243	77.4	201	67.2	238	71.5	102	68.5
Mutated	311	28.4	71	22.6	98	32.8	95	28.5	47	31.5
MSI										
Stable	1019	91.0	294	90.7	264	87.1	314	91.5	147	98.0
Unstable	101	9.0	30	9.3	39	12.9	29	8.5	3	2.0
CIMP										
Low	815	80.1	245	83.1	222	79.3	245	78.0	103	80.5
High	202	19.9	50	16.9	58	20.7	69	22.0	25	19.5
Vital Status										
Dead	533	46.8	78	23.7	122	39.4	188	54.2	145	94.8
Alive	606	53.2	251	76.3	188	60.6	159	45.8	8	5.2

	Overall		Stage I		Stage II		Stage III		Stage IV	
	N	%	N	%	N	%	N	%	N	%
	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD
Age	64.2	10.4	63.0	11.0	65.8	9.9	64.3	10.0	63.2	10.7

Table 2

Associations between miRNAs and disease stage

	STAGE1/2 level		Stage 3/4 level			
miRNA	**Mean[1]**	**Std**	**Mean[1]**	**Std**	**Fold Change**	**P-value**
hsa-miR-145-5p	6.471	1.236	6.747	1.288	1.211	0.0007
hsa-miR-200b-3p	7.033	1.296	6.852	1.244	0.882	0.0227
hsa-miR-215	5.142	1.284	4.890	1.487	0.953	0.0044
hsa-miR-31-5p	0.354	1.009	0.562	1.325	1.155	0.0057
hsa-miR-451a	3.062	1.935	2.736	2.024	0.798	0.0089

[1]Mean values are based on log transformed data

Table 3

Associations between miRNAs and colorectal cancer survival

	Colon				Rectal			
miRNA	**25th %tile[1]**	**75th %tile[1]**	**HR[2]**	**(95% CI)**	**25th %tile[1]**	**75th %tile[1]**	**HR[2]**	**(95% CI)**
hsa-let-7a-5p	7.22	8.56	0.96	(0.81, 1.14)	7.68	8.65	0.92	(0.72, 1.17)
hsa-let-7b-5p	7.44	8.67	1.02	(0.84, 1.23)	7.89	8.86	0.94	(0.71, 1.24)
hsa-let-7c	5.50	6.70	1.06	(0.88, 1.28)	5.90	6.83	0.99	(0.76, 1.28)
hsa-let-7e-5p	3.66	4.87	1.10	(0.95, 1.27)	3.84	5.03	1.12	(0.87, 1.43)
hsa-miR-100-5p	0.00	3.55	1.33	(0.95, 1.88)	1.83	4.06	1.03	(0.79, 1.35)
hsa-miR-10b-3p	5.04	5.77	0.97	(0.78, 1.19)	4.99	5.48	0.97	(0.80, 1.19)
hsa-miR-10b-5p	2.56	4.20	1.07	(0.89, 1.28)	2.24	3.67	0.99	(0.80, 1.23)
hsa-miR-126-3p	3.15	4.42	0.91	(0.80, 1.03)	3.49	4.49	0.86	(0.74, 1.00)
hsa-miR-141-3p	4.37	5.77	**0.84**	**(0.73, 0.98)**	4.40	5.53	**0.78**	**(0.63, 0.97)**
hsa-miR-145-5p	5.58	7.34	**1.29**	**(1.05, 1.59)**	6.09	7.53	1.24	(0.94, 1.63)
hsa-miR-148a-3p	2.34	4.59	**0.83**	**(0.69, 0.99)**	2.81	4.60	0.84	(0.69, 1.03)
hsa-miR-151a-3p	0.00	2.44	1.09	(0.82, 1.43)	0.00	2.61	0.76	(0.51, 1.13)
hsa-miR-151a-5p	2.82	4.24	1.04	(0.89, 1.23)	3.24	4.40	0.91	(0.75, 1.10)
hsa-miR-151b	1.80	3.28	1.07	(0.90, 1.28)	2.15	3.36	0.85	(0.70, 1.03)
hsa-miR-15b-5p	3.92	5.27	0.88	(0.77, 1.01)	4.46	5.63	0.90	(0.73, 1.11)
hsa-miR-16-5p	5.34	6.57	0.89	(0.77, 1.02)	5.73	6.71	0.90	(0.74, 1.09)
hsa-miR-17-5p	4.63	6.20	0.86	(0.72, 1.03)	5.04	6.33	**0.76**	**(0.61, 0.94)**
hsa-miR-197-3p	3.43	4.20	1.05	(0.85, 1.29)	3.70	4.53	1.26	(0.96, 1.65)
hsa-miR-200a-3p	3.71	5.24	0.89	(0.75, 1.05)	3.73	5.21	0.85	(0.71, 1.03)
hsa-miR-200a-5p	2.53	3.12	0.99	(0.91, 1.07)	2.53	3.11	**0.91**	**(0.83, 0.99)**
hsa-miR-200b-3p	6.27	7.76	0.90	(0.77, 1.05)	6.41	7.92	**0.71**	**(0.53, 0.95)**
hsa-miR-200b-5p	3.15	3.62	0.99	(0.88, 1.11)	3.09	3.62	0.92	(0.78, 1.09)
hsa-miR-200c-3p	6.28	7.51	0.93	(0.78, 1.11)	6.43	7.57	0.78	(0.58, 1.04)
hsa-miR-206	2.29	3.46	0.96	(0.84, 1.09)	1.85	3.24	1.14	(0.92, 1.42)
hsa-miR-20a-5p	4.78	6.41	**0.81**	**(0.69, 0.96)**	5.20	6.57	**0.77**	**(0.62, 0.95)**
hsa-miR-20b-5p	2.49	4.45	**0.73**	**(0.61, 0.89)**	3.00	4.59	**0.71**	**(0.57, 0.89)**
hsa-miR-215	4.43	5.86	**0.83**	**(0.72, 0.96)**	4.59	6.02	**0.68**	**(0.55, 0.83)**

	Colon				Rectal			
miRNA	**25th %tile[1]**	**75th %tile[1]**	**HR[2]**	**(95% CI)**	**25th %tile[1]**	**75th %tile[1]**	**HR[2]**	**(95% CI)**
hsa-miR-21-3p	3.68	4.67	0.98	(0.87, 1.11)	4.01	4.86	**0.75**	**(0.62, 0.92)**
hsa-miR-21-5p	7.58	9.21	0.91	(0.77, 1.09)	8.03	9.21	**0.75**	**(0.59, 0.96)**
hsa-miR-221-3p	2.13	4.10	0.98	(0.81, 1.19)	2.66	4.13	0.81	(0.66, 1.00)
hsa-miR-222-3p	3.32	4.47	1.08	(0.92, 1.28)	3.50	4.52	0.82	(0.66, 1.03)
hsa-miR-223-3p	3.13	4.97	1.02	(0.87, 1.20)	3.64	5.09	0.87	(0.71, 1.07)
hsa-miR-224-5p	0.00	3.89	0.87	(0.63, 1.21)	1.40	4.09	0.75	(0.55, 1.03)
hsa-miR-22-3p	3.25	4.55	0.97	(0.84, 1.12)	3.57	4.63	0.89	(0.75, 1.05)
hsa-miR-25-3p	3.75	5.06	0.90	(0.78, 1.03)	4.29	5.29	**0.81**	**(0.67, 0.98)**
hsa-miR-26b-5p	3.34	4.71	0.90	(0.78, 1.04)	3.53	4.68	0.89	(0.76, 1.04)
hsa-miR-29a-3p	5.50	7.04	**0.85**	**(0.73, 0.98)**	5.87	7.18	**0.71**	**(0.54, 0.93)**
hsa-miR-29c-3p	3.20	4.71	**0.85**	**(0.74, 0.98)**	3.38	4.55	**0.83**	**(0.71, 0.97)**
hsa-miR-302c-5p	0.00	1.73	0.97	(0.73, 1.28)	0.00	1.38	0.96	(0.67, 1.37)
hsa-miR-30c-5p	1.46	3.34	0.97	(0.78, 1.20)	2.21	3.64	0.91	(0.72, 1.15)
hsa-miR-320a	6.37	6.64	0.87	(0.73, 1.04)	6.31	6.60	0.93	(0.74, 1.16)
hsa-miR-324-5p	0.00	2.11	1.05	(0.82, 1.35)	0.00	2.49	1.24	(0.87, 1.76)
hsa-miR-330-3p	0.00	2.23	**1.38**	**(1.03, 1.83)**	0.00	2.25	0.91	(0.62, 1.35)
hsa-miR-34a-5p	3.62	4.84	**0.85**	**(0.74, 0.99)**	3.82	4.85	0.98	(0.80, 1.18)
hsa-miR-34c-3p	2.29	2.83	0.98	(0.89, 1.08)	2.36	2.81	0.94	(0.84, 1.06)
hsa-miR-370	5.16	5.51	1.22	(0.98, 1.50)	5.12	5.50	0.98	(0.75, 1.26)
hsa-miR-425-5p	2.02	3.90	0.98	(0.81, 1.19)	2.68	4.15	**0.76**	**(0.62, 0.93)**
hsa-miR-451a	0.00	4.00	0.87	(0.64, 1.19)	3.25	4.95	0.93	(0.77, 1.12)
hsa-miR-483-3p	0.00	2.71	0.88	(0.69, 1.12)	0.00	3.29	1.12	(0.76, 1.64)
hsa-miR-490-5p	3.98	4.49	1.02	(0.83, 1.26)	3.81	4.26	1.16	(0.88, 1.53)
hsa-miR-500a-3p	0.00	2.21	0.95	(0.68, 1.33)	0.00	2.12	0.95	(0.62, 1.46)
hsa-miR-500a-5p	3.94	4.55	**1.26**	**(1.04, 1.52)**	3.98	4.68	1.01	(0.79, 1.31)
hsa-miR-518c-5p	0.00	1.07	1.08	(0.88, 1.34)	0.00	0.95	0.88	(0.66, 1.18)
hsa-miR-526b-5p	1.48	2.37	0.97	(0.84, 1.12)	1.50	2.32	0.94	(0.79, 1.12)
hsa-miR-615-3p	0.00	1.51	1.14	(0.90, 1.45)	0.00	1.34	1.09	(0.82, 1.45)
hsa-miR-92a-1-5p	0.00	0.35	0.99	(0.88, 1.12)	0.00	0.35	0.88	(0.75, 1.03)
hsa-miR-92a-3p	5.64	7.10	0.99	(0.80, 1.23)	6.36	7.50	0.78	(0.58, 1.03)

	Colon				Rectal			
miRNA	**25th %tile[1]**	**75th %tile[1]**	**HR[2]**	**(95% CI)**	**25th %tile[1]**	**75th %tile[1]**	**HR[2]**	**(95% CI)**
hsa-miR-99a-5p	0.00	2.60	**1.29**	**(1.01, 1.65)**	0.00	3.36	1.21	(0.80, 1.84)

[1] Log transformed data for 25th and 75th percentile values presented

[2] Hazard Ratios (HR) and 95% Confidence Intervals (CI) adjusted for age, sex, AJCC stage, and tumor molecular phenotype; bold text indicates significant associations

Table 4

Associations between any miRNA expressed and survival

	Overall			Colon			Rectal		
miRNA	**% any expression**	**HR[1]**	**(95% CI)**	**% any expression**	**HR[1]**	**(95% CI)**	**% any expression**	**HR[1]**	**(95% CI)**
hsa-miR-145-3p	2.42	2.02	(1.17, 3.50)	2.90	2.94	(1.54, 5.61)	1.65	0.77	(0.23, 2.64)
hsa-miR-18a-5p	11.99	0.64	(0.42, 0.98)	12.10	0.61	(0.35, 1.05)	11.81	0.60	(0.29, 1.21)
hsa-miR-335-5p	9.78	0.66	(0.42, 1.03)	10.39	1.13	(0.69, 1.83)	8.79	0.17	(0.05, 0.54)
hsa-miR-374a-5p	26.50	0.64	(0.48, 0.86)	27.09	0.67	(0.46, 0.98)	25.55	0.65	(0.40, 1.05)
hsa-miR-570-3p	2.42	0.18	(0.06, 0.57)	3.75	0.26	(0.08, 0.83)	0.27		
hsa-miR-9-3p	0.11	16.14	(2.10, 123.78)	0.17	10.28	(1.31, 80.84)	0.00		

[1]Hazard Ratios (HR) and 95% Confidence Intervals (CI) adjusted for age, sex, AJCC stage, and tumor molecular phenotype

Table 5

Associations between miR-21 and survival

	TP53 mutated		*KRAS* mutated		CIMP		MSI	
	No. Deaths/ Person Yrs	HR (95% CI)[1]	No. Deaths/ Person Yrs	HR (95% CI)	No. Deaths/ Person Yrs	HR (95% CI)	No. Deaths/ Person Yrs	HR (95% CI)
Colon Cancer								
miR-21-3p	102 / 1621	1.07 (0.91, 1.26)	68 / 1124	0.97 (0.76, 1.24)	56 / 844	0.90 (0.72, 1.13)	13 / 528	1.22 (0.66, 2.25)
miR-21-5p	102 / 1621	1.02 (0.80, 1.30)	68 / 1124	0.87 (0.64, 1.18)	56 / 844	0.93 (0.70, 1.24)	13 / 528	1.00 (0.37, 2.73)
Rectal Cancer								
miR-21-3p	54 / 1047	0.79 (0.59, 1.06)	38 / 428	0.96 (0.64, 1.44)	14 / 194	0.87 (0.50, 1.53)	5 / 44	0.35 (0.07, 1.61)
miR-21-5p	54 / 1047	0.90 (0.59, 1.35)	38 / 428	0.92 (0.58, 1.46)	14 / 194	0.78 (0.40, 1.51)	5 / 44	0.33 (0.07, 1.59)

	Stage 2		Stage 1&2		Stage 3&4	
Colon Cancer						
miR-21-3p	30 / 1075	1.26 (0.84, 1.89)	34 / 1973	1.22 (0.86, 1.74)	137 / 1103	0.85 (0.75, 0.97)
miR-21-5p	30 / 1075	1.18 (0.77, 1.81)	34 / 1973	1.13 (0.75, 1.72)	137 / 1103	0.82 (0.68, 0.98)
Rectal Cancer						
miR-21-3p	21 / 417	0.65 (0.37, 1.12)	37 / 1322	0.75 (0.57, 0.99)	73 / 553	0.77 (0.58, 1.01)
miR-21-5p	21 / 417	0.47 (0.26, 0.85)	37 / 1322	0.60 (0.41, 0.87)	73 / 553	0.93 (0.69, 1.24)

[1] HR (Hazard ratio) and 95% CI (Confidence is reported for interquartile range; adjusted for age, sex, AJCC stage (when looking at tumor molecular phenotype) and tumor molecular phenotype (for stage)

Table 6

Assessment of pathway targets for miR-570, miR-145-3p, miR-9-3p, miR-3824a-5p, and miR-20a-3p

miR-570	miR-145-3p	miR-9-3p	miR-335-5p	miR-374a-5p	miR-20a-3p	miRNA count	Term	Gene Count	% of Total Genes[1]	Raw *P* - Value	Genes	Fold Enrichment[2]	Bonferroni
x	x	x	x	x		5	Pathways in cancer	41	20.5	8.27E-20	*E2F1, E2F3, PDGFB, MMP9, ERBB2, SPI1, ITGB1, PTEN, SHH, CTNNB1, AKT1, CDC42, IGF1R, KRAS, BCL2, PIK3CA, RARA, EGF, MYC, BMP4, EGFR, RXRA, TGFBR2, SMAD4, TP53, SMAD3, LEF1, IGF1, SMAD2, FZD5, STAT1, CDK4, BIRC2, NRAS, CDKN1A, CCND1, EP300, JUN, VEGFA, MAPK3, LAMC2*	5.3867	9.51E-18
x	x		x			3	Colorectal cancer	18	9	3.10E-12	*EGFR, TGFBR2, TP53, SMAD4, SMAD3, LEF1, SMAD2, FZD5, CTNNB1, AKT1, IGF1R, CCND1, KRAS, JUN, BCL2, MAPK3, PIK3CA, MYC*	9.2343	3.56E-10
	x	x	x			3	Focal adhesion	24	12	8.40E-11	*EGFR, PDGFB, ERBB2, IGF1, ITGB1, BIRC2, PTEN, SRC, CTNNB1, ACTG1, AKT1, CDC42, IGF1R, CCND1, PAK3, JUN, BCL2, VEGFA, MAPK3, PIK3CA, LAMC2, COL1A1, PAK1, EGF*	5.1455	9.67E-09
	x		x			2	ErbB signaling pathway	16	8	7.13E-10	*EGFR, ERBB2, RPS6KB1, SRC, AKT1, NRAS, EIF4EBP1, CDKN1A, KRAS, PAK3, JUN, MAPK3, PIK3CA, PAK1, EGF, MYC*	7.9252	8.20E-08
x	x		x	x		4	Adherens junction	15	7.5	1.32E-09	*EGFR, ERBB2, TGFBR2, SMAD4, SMAD3, LEF1, SMAD2, SRC, CTNNB1, ACTG1, IGF1R, CDC42, EP300, MAPK3, YES1*	8.3948	1.52E-07
	x		x			2	Neurotrophin signaling pathway	15	7.5	6.92E-07	*IRAK2, IRS2, TP53, IRS1, TP73, AKT1, NRAS, CDC42, KRAS, MAPK14, JUN, BCL2, MAPK3, PIK3CA, MAPK7*	5.2129	7.96E-05
	x		x	x		3	p53 signaling pathway	11	5.5	2.67E-06	*CDKN1A, CCND1, TSC2, TP53, IGF1, CDK4, CCNG1, GADD45A, PTEN, ATM, TP73*	6.971	3.07E-04
x	x		x	x		4	TGF-beta signaling pathway	12	6	3.83E-06	*BMP4, PPP2R1B, EP300, SMAD7, MAPK3, IFNG, TGFBR2, SMAD4, SMAD3, SMAD2, RPS6KB1, MYC*	5.9439	4.40E-04
x	x		x	x		4	Cell cycle	14	7	4.54E-06	*E2F1, E2F3, TP53, SMAD4, SMAD3, SMAD2, CDK4, ATM, CCND1, CDKN1A, EP300, SMC1A, GADD45A, MYC*	4.8264	5.22E-04
	x		x			2	T cell receptor signaling pathway	13	6.5	5.42E-06	*CDK4, AKT1, NRAS, CDC42, KRAS, PAK3, JUN, MAPK14, IFNG, MAPK3, PIK3CA, PAK1, PPP3CA*	5.1871	6.23E-04
x	x		x	x		4	Wnt signaling pathway	15	7.5	7.46E-06	*PPP2R1B, VANGL1, TP53, SMAD4, SMAD3, LEF1, SMAD2, FZD5, CTNNB1, CCND1, EP300, JUN, PPP3CA, FBXW11, MYC*	4.2808	8.57E-04
	x		x	x	x	4	MAPK signaling pathway	20	10	8.36E-06	*EGFR, PDGFB, NF1, TGFBR2, TP53, SRF, STK3, AKT1, CDC42, NRAS, KRAS, MAPK14, JUN,*	3.228	9.61E-04

miR-570	miR-145-3p	miR-9-3p	miR-335-5p	miR-374a-5p	miR-20a-3p	miRNA count	Term	Gene Count	% of Total Genes[1]	Raw *P* - Value	Genes	Fold Enrichment[2]	Bonferroni
											MAPK3, PPP3CA, PAK1, MAPK7, EGF, GADD45A, MYC *MAPK3, PPP3CA, PAK1, MAPK7, EGF, GADD45A, MYC*		
	x		x			2	mTOR signaling pathway	9	4.5	2.00E-05	*AKT1, EIF4EBP1, EIF4E, MAPK3, VEGFA, TSC2, IGF1, PIK3CA, RPS6KB1*	7.4584	0.002293
	x		x	x		3	Apoptosis	11	5.5	2.55E-05	*IRAK2, AKT1, TNFSF10, DFFA, BCL2, DFFB, TP53, PIK3CA, PPP3CA, BIRC2, ATM*	5.4486	0.002924
	x		x			2	VEGF signaling pathway	10	5	4.66E-05	*AKT1, CDC42, NRAS, KRAS, MAPK14, MAPK3, VEGFA, PIK3CA, PPP3CA, SRC*	5.7458	0.005347
	x		x			2	Tight junction	13	6.5	4.95E-05	*PPP2R1B, F11R, CDK4, PTEN, SRC, CTNNB1, AKT1, ACTG1, NRAS, CDC42, KRAS, MYH11, YES1*	4.1807	0.005672
	x		x			2	Aldosterone-regulated sodium reabsorption	7	3.5	3.04E-04	*IRS2, KRAS, MAPK3, HSD11B1, IGF1, PIK3CA, IRS1*	7.3574	0.034348

Total 6 17 2 17 8 1

[1]The list total was 118 genes

[2]The population total was 5085 genes