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The co-regulatory networks of tumor suppressor genes, oncogenes, and miRNAs in colorectal cancer

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Abstract

Tumor suppressor genes (TSGs) and oncogenes (OG) are involved in carcinogenesis. MiRNAs also contribute to cellular pathways leading to cancer. We use data from 217 colorectal cancer (CRC) cases to evaluate differences in TSGs and OGs expression between paired CRC and normal mucosa and evaluate how TSGs and OGs are associated with miRNAs. Gene expression data from RNA-Seq and miRNA expression data from Agilent Human miRNA Microarray V19.0 were used. We focus on genes most strongly associated with CRC (fold change (FC) of $\geq$1.5 or $\leq$0.67) that were statistically significant after adjustment for multiple comparisons. Of the 74 TSGs evaluated, 22 were associated with carcinoma/normal mucosa differential expression. Ten TSGs were up-regulated (*FAM123B*, *RB1*, *TP53*, *RUNX1*, *MSH2*, *BRCA1*, *BRCA2*, *SOX9*, *NPM1*, and *RNF43);* six TSGs were down-regulated (*PAX5*, *IZKF1*, *GATA3*, *PRDM1*, *TET2*, and *CYLD)*; four were associated with MSI tumors (*MLH1*, *PTCH1*, and *CEBPA* down-regulated and *MSH6* up-regulated); and two were associated with MSS tumors (*PHF6* and *ASXL1* up-regulated). Thirteen of these TSGs were associated with 44 miRNAs. Twenty-seven of the 59 OGs evaluated were dysregulated: 14 down-regulated (KLF4, BCL2, SSETBP1, FGFR2, TSHR, MPL, KIT, PDGFRA, GNA11, GATA2, FGFR3, AR, CSF1R, and JAK3), seven up-regulated (DNMT1, EZH2, PTPN11, SKP2, CCND1, MET, and MYC); three down-regulated for MSI (FLT3, CARD11, and ALK); two up-regulated for MSI (IDH2 and HRAS); and one up-regulated with MSS tumors (CTNNB1). These findings suggest possible co-regulatory function between TSGs, OGs, and miRNAs, involving both direct and indirect associations that operate through feedback and feedforward loops.

1 | INTRODUCTION

Tumor suppressor genes (TSGs) play a major role in the carcinogenic process by controlling cell growth and apoptosis, inhibiting the formation of tumors. Mutations in TSGs inactivate their inhibitory function, thereby contributing to the carcinogenic process. Proto-oncogenes likewise are involved in cell growth; when mutated, these oncogenes (OGs) promote cancer through proliferation of cells. Unlike TSGs which require a double hit to inactivate the gene, mutations to OGs are dominant with one copy of the gene needing to be mutated to promote cancer. Several TSGs have been linked to the colorectal cancer (CRC) carcinogenic process, with the adenomatous polyposis coli gene (*APC*) and *TP53* being two of the most commonly mutated TSGs in CRC.[1] Important OGs in CRC include the RAS genes (ie, *KRAS*, *HRAS*, and *NRAS)*, *BRAF*, *AKT1*, *EGFR*, *PIK3CA*, *MYC*, and *JAK*. Several of these oncogenes, including *KRAS*, *BRAF*, *MYC*, and *PIK3CA* have been shown to be mutated and/or have altered expression in colorectal cancer (CRC).[2–4] Genetic variation in the *JAK* genes also has been reported as increasing risk of developing CRC.[5] A balance of TSG function and regulation of OGs is needed to control cell growth.

MiRNAs are small, nonprotein-coding RNA molecules involved in the regulation of gene expression either by post-transcriptionally suppressing mRNA translation or by causing mRNA degradation.[6–11] While the function and importance of miRNAs in the carcinogenic process is not completely understood, it is thought that they help regulate cell proliferation and apoptosis and through the loss or gain-of-function

attributed to them, are likely part of the elaborate cellular pathways regulated by TSG and OGs.[12,13] MiRNA expression is frequently either down-regulated or up-regulated in CRC tissue when compared to normal mucosa,[14,15] supporting their relevance to neoplasia. Several miRNAs, including miR-21, miR-203, miR-155, miR-455–3p, and the miR-17–92 cluster interact with TSGs and OGs to influence cancer processes.[13,16–20] Groups of miRNAs, such as oncomiR1, are commonly up-regulated in tumor tissue; in turn these miRNAs along with *MYC* regulate expression of cell cycle transcription factor gene *ESF1*.[12,21] MiRNAs have been cited as being "critical effectors of several canonical oncogenic and tumor suppressor pathways".[22]

In this study we examine associations between gene expression of 74 TSGs and 59 OGs that have been previously identified as being associated with cancer[23] with miRNA expression levels. It is possible that, in addition to mutation, TSG and OG expression is indicative of dysregulated pathways involved in carcinogenesis and not mutated TSGs or OGs. We evaluate TSGs and OGs with a fold change (FC) between paired tumor and normal tissue $\geq$1.5 or $\leq$0.67 with miRNAs to have more meaningful levels of expression differences. We believe that insight into the co-regulator roles of TSG, OG, and miRNAs can further our understanding of the carcinogenic process.

2 | PATIENTS AND METHODS

2.1 | Study participants

Study participants come from two population-based case-control studies that included all incident colon and rectal cancer between 30 and 79 years of age in Utah or were members of Kaiser Permanente Medical Care Program (KPMCP) in Northern California. Participants were non-Hispanic white, Hispanic, or black for the colon cancer study; Asian race was included in the rectal cancer study.[24,25] Case diagnosis was verified by tumor registry data as a first primary adenocarcinoma of the colon and were diagnosed between October 1991 and September 1994 and for the rectal study were diagnosed between May 1997 and May 2001. Detailed study methods have been described.[15] The Institutional Review Boards at the University of Utah and at KPMCP approved the study.

2.2 | RNA processing

Formalin-fixed paraffin embedded tissue from the initial biopsy or surgery was used to extract RNA. RNA was extracted, isolated and purified as previously described[26] from carcinoma tissue and adjacent normal mucosa.

2.3 | mRNA: RNA-Seq sequencing library preparation and data processing

Total RNA from 245 colorectal carcinoma and normal mucosa pairs was chosen for sequencing based on availability of RNA and high quality miRNA data; 217 pairs passed quality control (QC) and are used in these analyses. RNA library construction was done with the Illumina TruSeq Stranded Total RNA Sample Preparation Kit with Ribo-Zero (Illumina, San Diego, California). 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 (Beckman Coulter, Indianapolis, Indiana). A more detailed description of the methods can be found in our previous work.[27] 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 disregarded genes that were not expressed in our RNA-Seq data or for which the expression was missing for the majority of samples.[27] We focused on expression of 74 TSGs and 59 OGs previously identified as being associated with cancer[23] (Supporting Information Table 1).

2.4 | miRNA

The Agilent Human miRNA Microarray V19.0 was used (Agilent, St Clara, California). 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. Samples failing to meet quality standards were re-labeled, hybridized to arrays, and re-scanned. If a sample failed QC assessment a second time, the sample was excluded from analysis. The repeatability associated with this microarray was extremely high ($r = 0.98$),[15] 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 the FCs were almost identical.[14] 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 which was the median of the 75th percentiles of all the samples divided by the individual 75th percentile of each sample.[28]

2.5 | Statistical methods

DESeq2 was used to identify TSGs and OGs that had a significant difference in expression between individual paired colorectal carcinoma and normal mucosa adjusting for age and sex. The Bioconductor package DESeq2, written for the *R* statistical programming environment, assumes the RNA-Seq counts are distributed according to negative binomial distributions.[29] It utilizes generalized linear modeling to test individual null hypotheses of zero log2 FCs between tumor and normal categories (ie, no differential expression) for each TSG and OG and it employs both an independent-filtering method and the Benjamini and Hochberg[30] procedure to improve power and control the false discovery rate (FDR). In identifying genes with significant differential expression, an FDR adjusted *P* value of 0.05 was used. We report the average

DESeq2-adjusted gene expression levels among individuals in the tumor and normal mucosa categories and include FC calculations associated with these genes. FC also was calculated as the ratio of a gene's mean expression among individuals in the tumor to its mean expression among normal; a FC greater than one indicates a positive differential expression (ie, up-regulated) while a FC between zero and one indicates a negative differential expression (ie, down-regulated).

We focus on those TSGs and OGs with FC of $\geq$1.5 or $\leq$0.67 for analysis with miRNAs to potentially have differences that were more biologically significant. There are 814 miRNAs expressed in greater than 20% of normal colorectal mucosa that were analyzed; differential expression was calculated as the expression in the carcinoma tissue minus the expression in the normal mucosa within each subject. In these analyses, we fit a least squares linear regression model to the reads per kilobase of transcript per million mapped reads (RPKMs) differential expression levels and miRNA differential expression levels. *P* values were generated using the bootstrap method by creating a distribution of 10,000 F statistics derived by resampling the residuals from the null hypothesis model of no association between miRNA and TSG or miRNA and OG differential expression using the boot package in R. Linear models were adjusted for age and sex. Multiplicity adjustments for gene/miRNA associations were made at the gene level using the FDR by Benjamini and Hochberg.[30] We transformed the RPKMs and miRNA to standard normal to standardize the regression slopes to compare the results across TSGs and OGs. We considered overall CRC as well as microsatellite unstable (MSI) and stable (MSS) tumors since MSI tumors are usually hyper-mutated.[31]

3 | RESULTS

The majority of cases were colon cancer (77.9%) while 22.1% were diagnosed with rectal cancer (Table 1). The population consisted of 54.4% men, 74.2% non-Hispanic white, and a mean age of 64.8 years. Based on the hot-spot locations sequenced for *TP53* 47.5% were mutated, 31.8% had a *KRAS* mutation, 10.1% had a *BRAF* mutation, 20.7% were CIMP high, and 13.4% were MSI.

Of the 74 TSGs evaluated, six (*PAX5*, *IZKF1*, *GATA3*, *PRDM1*, *TET2*, and *CYLD*) were significantly down-regulated with a FC of $\leq$0.67 after adjustment for multiple comparisons (Table 2). Five additional TSGs, (*ATM*, *SMAD4*, *APC*, *KDM6A*, and *FBXW7*), were significantly down-regulated when a FC of 0.75 or less was applied. Ten mRNAs were up-regulated with a FC $\geq$1.5 and an FDR of $<$0.05. These 10 TSGs were *FAM123B*, *RB1*, *TP53*, *RUNX1*, *MSH2*, *BRCA1*, *BRCA2*, *SOX9*, *NPM1*, and *RNF43*. *ASXL1*, *CDKN2A*, *MSH6*, and *PHF6* had a FC between 1.45 and 1.5. Other TSGs ($N = 30$) were statistically significantly up- or down-regulated after adjustment for multiple comparisons but with FCs closer to 1.0. Looking separately at MSI and MSS tumors showed some slight differences in magnitude of differential expression of TSGs. For MSI tumors (Supporting Information Table 2), three additional genes, (*MLH1*, *PTCH1*, and *CEBPA*) were significantly down-regulated and *MSH6* was significantly up-regulated (FCs: 0.48, 0.56, 0.40, and 1.51, respectively). For MSS tumors, *PHF6* and *ASXL1* (FCs: 1.57 and 1.50, respectively) were significantly up-regulated; APC was only slightly more downregulated in MSS tumors (0.72 vs. 0.74 overall) (Supporting information Table 3).

TABLE 1 Description of study population

		N	(%)
Site			
	Colon	169	77.9
	Rectal	48	22.1
Sex			
	Male	118	54.4
	Female	99	45.6
Age			
	Mean (SD)	64.8	10.1
Race			
	Non-Hispanic White	161	74.2
	Hispanic	14	6.5
	Non-Hispanic Black	8	3.7
	Unknown	34	15.7
AJCC Stage			
	1	58	27.1
	2	61	28.5
	3	72	33.6
	4	23	10.8
Tumor phenotype			
	TP53 mutated	103	47.5
	KRAS mutated	69	31.8
	BRAF-mutated	21	10.1
	CIMP High	45	20.7
	MSI	29	13.4
Vital status			
	Dead	92	42.6
	Alive	124	57.4

Further evaluation of the 22 TSGs that were significantly differentially expressed with a FC $\geq$1.5 or $\leq$0.67, either for overall CRC or MSI and MSS-specific tumors, showed that 13 TSGs were associated with miRNA expression (Table 3). Several miRNAs were associated with multiple TSGs. For instance, miR-150–5p was associated with five TSGs (*PRDM1*, *CYLD*, *GATA3*, *IKZF1*, and *PAX5*), miR-15a-5p with four TSGs (*RNF43*, *SOX9*, *RB1*, and *ASXL1*), miR-17–5p with six TSGs (*BRCA1*, *RNF43*, *SOX9*, *BRCA2*, *RB1*, and *ASXL1*), miR-203a with three TSGs (*RNF43*, *SOX9*, and *IKZF1*), miR-20a-5p with five TSGs (*RNF43*, *SOX9*, *BRCA2*, *RB1*, and *ASXL1*), miR-29a-3p with four TSGs (*RNF43*, *SOX9*, *RB1*, and *ASXL1*), miR-425–5p with four TSGs (*BRCA1*, *RNF43*, *SOX9*, and *ASXL1*), and miR-92a-3p with seven TSGs (*BRCA1*, *RNF43*, *SOX9*, *BRCA2*, *RB1*, *ASXL1*, and *FAM123B*). Interestingly, all of the TSGs associated with miR-150–5p were down-regulated as was miR-150–5p. Likewise, all TSGs associated with miR-17–5p, miR-20a-5p, miR-29a-3p, miR-425–5p, and miR-92a-3p were up-regulated as were the miRNAs themselves.

Evaluating CRC overall, 14 OGs were significantly down-regulated when a FC of $\leq$0.67 was applied (Table 4). Additionally, eight OGs were significantly down-regulated but with FC values above this level.

TABLE 2 Tumor suppressor genes (TSG) differentially expressed in colorectal cancer

Gene name	Mean expression		Fold change	Log2 ratio	Adjusted *P* value
	Tumor	Normal			
PAX5	7.39	31.89	0.23	−2.11	2.33E−44
IKZF1	39.20	102.32	0.38	−1.38	2.63E−66
GATA3	3.82	7.97	0.48	−1.06	2.77E−07
PRDM1	81.55	132.11	0.62	−0.70	4.15E−30
TET2	145.62	232.97	0.63	−0.68	4.65E−68
CYLD	88.07	133.85	0.66	−0.60	1.41E−34
ATM	266.98	362.05	0.74	−0.44	2.34E−25
SMAD4	102.47	138.70	0.74	−0.44	3.59E−27
APC	115.05	155.21	0.74	−0.43	3.59E−27
KDM6A	91.78	123.80	0.74	−0.43	3.03E−23
FBXW7	53.86	71.75	0.75	−0.41	2.80E−14
GATA1	0.70	0.93	0.75	−0.41	0.91
NCOR1	444.29	589.76	0.75	−0.41	0.95
ACVR1B	104.62	129.79	0.81	−0.31	3.11E−12
TSC1	127.06	157.25	0.81	−0.31	0.99
PTEN	143.07	174.86	0.82	−0.29	1.77E−13
SMAD2	186.11	223.36	0.83	−0.26	3.46E−16
CDKN2C	6.18	7.37	0.84	−0.25	0.44
EP300	326.24	387.06	0.84	−0.25	0.99
MLH1	37.54	43.60	0.86	−0.22	7.42E−03
ARID2	181.30	206.27	0.88	−0.19	0.99
MAP2K4	35.32	39.81	0.89	−0.17	0.02
ARID1A	259.52	291.77	0.89	−0.17	0.99
MAP3K1	83.61	93.65	0.89	−0.16	1.49E−04
MLL3	707.40	789.64	0.90	−0.16	0.99
PTCH1	149.31	165.98	0.90	−0.15	6.70E−03
BAP1	84.45	91.67	0.92	−0.12	7.17E−03
CIC	102.02	110.61	0.92	−0.12	0.06
SETD2	292.98	313.68	0.93	−0.10	0.99
CREBBP	294.85	313.68	0.94	−0.09	0.99
TNFAIP3	119.32	124.18	0.96	−0.06	0.20
MLL2	646.44	672.13	0.96	−0.06	0.99
ARID1B	246.14	255.23	0.96	−0.05	0.99
B2M	835.45	850.74	0.98	−0.03	0.99
NOTCH2	289.31	286.58	1.01	0.01	0.99
STK11	74.67	72.44	1.03	0.04	0.35
PIK3R1	174.64	168.38	1.04	0.05	0.99
FUBP1	205.57	196.55	1.05	0.06	0.99

(Continues)

TABLE 2 (Continued)

Gene name	Mean expression		Fold change	Log2 ratio	Adjusted *P* value
	Tumor	Normal			
PBRM1	176.09	166.12	1.06	0.08	0.99
MEN1	40.36	37.10	1.09	0.12	3.82E−03
CDC73	79.29	72.84	1.09	0.12	0.08
SOCS1	5.44	4.95	1.10	0.14	0.99
HNF1A	70.70	63.81	1.11	0.15	4.57E−04
NF2	91.86	81.64	1.13	0.17	1.98E−03
SMARCB1	42.07	37.28	1.13	0.17	1.62E−03
KDM5C	260.17	226.43	1.15	0.20	0.99
CDH1	591.70	512.34	1.15	0.21	5.32E−05
AXIN1	113.21	95.38	1.19	0.25	6.70E−06
CEBPA	59.18	49.57	1.19	0.26	0.08
CASP8	80.57	67.38	1.20	0.26	4.44E−07
BCOR	103.15	85.65	1.20	0.27	5.11E−11
VHL	102.14	84.59	1.21	0.27	1.77E−13
TRAF7	131.07	105.65	1.24	0.31	2.49E−10
DAXX	39.23	31.55	1.24	0.31	1.28E−08
NF1	418.54	329.17	1.27	0.35	0.99
SMARCA4	259.78	193.93	1.34	0.42	0.95
ATRX	316.31	230.97	1.37	0.45	0.95
NOTCH1	333.76	243.66	1.37	0.45	7.54E−19
STAG2	323.20	235.41	1.37	0.46	8.89E−27
ASXL1	243.04	168.14	1.45	0.53	6.68E−27
CDKN2A	9.33	6.41	1.46	0.54	5.25E−03
MSH6	83.43	56.02	1.49	0.57	1.07E−27
PHF6	78.99	52.85	1.49	0.58	1.02E−27
FAM123B	52.60	31.53	1.67	0.74	2.54E−26
RB1	118.98	69.86	1.70	0.77	1.82E−39
TP53	116.26	67.95	1.71	0.77	1.73E−23
RUNX1	285.66	155.93	1.83	0.87	4.94E−62
MSH2	54.53	29.60	1.84	0.88	2.30E−38
WT1	2.36	1.22	1.93	0.95	0.67
BRCA1	97.57	41.36	2.36	1.24	1.92E−56
BRCA2	95.40	39.33	2.43	1.28	7.21E−56
SOX9	297.74	122.07	2.44	1.29	4.90E−96
NPM1	242.28	90.51	2.68	1.42	2.76E−100
RNF43	641.08	179.11	3.58	1.84	3.06E−116

Seven OGs were up-regulated with FCs $\geq$1.5. An additional seven OGs were significantly up-regulated with FCs ranging from 1.1 to 1.38. Evaluation of tumors that had MSI specifically showed that three genes, (*FLT3*, *CARD11*, and *ALK*) were significantly down-regulated (FCs 0.30, 0.33, and 0.32, respectively) and two additional genes were significantly up-regulated (*IDH2* FC 1.69 and *HRAS* FC 1.85) (Supporting

TABLE 3 Significantly differentially expressed tumor suppressor genes (TSG) with ≥1.5 or ≤0.67 fold change and miRNA associations

TSG	TSG fold change	miRNA	Tumor mean	Normal mean	miRNA fold change	Beta between miRNA and TSG expression	FDR *P* value
BRCA1	2.36	hsa-miR-17–5p	61.04	16.38	3.73	0.27	0.041
		hsa-miR-425–5p	11.76	6.97	1.69	0.26	0.027
		hsa-miR-92a-3p	121.60	41.18	2.95	0.28	0.027
PRDM1	0.62	hsa-miR-146b-5p	4.46	2.67	1.67	0.28	0.023
		hsa-miR-150–5p	14.90	39.17	0.38	0.28	0.016
		hsa-miR-195–5p	3.59	12.18	0.29	0.23	0.041
		hsa-miR-199b-5p	4.69	1.53	3.07	0.26	0.016
		hsa-miR-650	4.51	16.60	0.27	0.30	0.016
CYLD	0.66	hsa-miR-150–5p	14.90	39.17	0.38	0.32	0.020
GATA3	0.48	hsa-miR-150–5p	14.90	39.17	0.38	0.34	0.041
RNF43	3.58	hsa-miR-106b-5p	15.90	5.19	3.06	0.22	0.017
		hsa-miR-1291	5.52	3.67	1.51	0.27	0.004
		hsa-miR-130b-3p	8.74	4.89	1.79	0.23	0.013
		hsa-miR-151a-3p	5.15	1.56	3.31	0.21	0.018
		hsa-miR-15a-5p	7.69	5.07	1.52	0.23	0.012
		hsa-miR-17–5p	61.04	16.38	3.73	0.29	0.004
		hsa-miR-196b-5p	17.89	5.53	3.24	0.19	0.035
		hsa-miR-199b-5p	4.69	1.53	3.07	0.18	0.049
		hsa-miR-19b-3p	29.80	10.42	2.86	0.21	0.015
		hsa-miR-203a	12.52	3.70	3.38	0.17	0.047
		hsa-miR-20a-5p	70.78	17.61	4.02	0.30	0.004
		hsa-miR-20b-5p	17.65	3.30	5.35	0.25	0.010
		hsa-miR-21–5p	463.11	167.37	2.77	0.18	0.042
		hsa-miR-221–3p	13.53	4.12	3.28	0.18	0.035
		hsa-miR-23a-3p	174.68	87.53	2.00	0.19	0.028
		hsa-miR-27a-3p	56.26	23.29	2.42	0.21	0.017
		hsa-miR-29a-3p	110.29	51.04	2.16	0.26	0.007
		hsa-miR-29b-3p	24.31	9.83	2.47	0.22	0.015
		hsa-miR-3191–3p	0.90	1.97	0.45	−0.18	0.042
		hsa-miR-361–5p	11.62	6.20	1.87	0.20	0.022
		hsa-miR-3651	58.66	25.92	2.26	0.24	0.007
		hsa-miR-378d	0.45	2.43	0.18	−0.19	0.033
		hsa-miR-3976	2.97	1.24	2.39	0.18	0.038
		hsa-miR-424–3p	39.81	25.37	1.57	0.26	0.007
		hsa-miR-425–5p	11.76	6.97	1.69	0.26	0.009
		hsa-miR-501–3p	7.07	2.95	2.39	0.25	0.007
		hsa-miR-513c-3p	2.15	3.50	0.62	−0.17	0.049
		hsa-miR-5685	1.28	2.78	0.46	−0.19	0.036

(Continues)

TABLE 3 (Continued)

TSG	TSG fold change	miRNA	Tumor mean	Normal mean	miRNA fold change	Beta between miRNA and TSG expression	FDR *P* value
		hsa-miR-663b	65.50	32.21	2.03	0.21	0.018
		hsa-miR-92a-3p	121.60	41.18	2.95	0.33	0.004
		hsa-miR-93–5p	41.72	15.20	2.74	0.21	0.017
SOX9	2.44	hsa-miR-1207–3p	1.18	1.93	0.61	−0.23	0.026
		hsa-miR-15a-5p	7.69	5.07	1.52	0.23	0.026
		hsa-miR-17–5p	61.04	16.38	3.73	0.24	0.026
		hsa-miR-1915–5p	1.04	1.77	0.59	−0.22	0.027
		hsa-miR-203a	12.52	3.70	3.38	0.21	0.038
		hsa-miR-20a-5p	70.78	17.61	4.02	0.23	0.025
		hsa-miR-21–5p	463.11	167.37	2.77	0.21	0.039
		hsa-miR-27a-3p	56.26	23.29	2.42	0.21	0.035
		hsa-miR-29a-3p	110.29	51.04	2.16	0.23	0.024
		hsa-miR-3651	58.66	25.92	2.26	0.20	0.038
		hsa-miR-425–5p	11.76	6.97	1.69	0.20	0.039
		hsa-miR-532–3p	2.74	1.67	1.64	0.20	0.050
		hsa-miR-92a-3p	121.60	41.18	2.95	0.25	0.018
		hsa-miR-93–5p	41.72	15.20	2.74	0.21	0.035
BRCA2	2.43	hsa-miR-17–5p	61.04	16.38	3.73	0.29	0.020
		hsa-miR-20a-5p	70.78	17.61	4.02	0.28	0.020
		hsa-miR-92a-3p	121.60	41.18	2.95	0.36	0.020
RB1	1.70	hsa-miR-1207–3p	1.18	1.93	0.61	−0.22	0.049
		hsa-miR-15a-5p	7.69	5.07	1.52	0.23	0.048
		hsa-miR-17–5p	61.04	16.38	3.73	0.22	0.049
		hsa-miR-1915–5p	1.04	1.77	0.59	−0.24	0.046
		hsa-miR-20a-5p	70.78	17.61	4.02	0.22	0.049
		hsa-miR-29a-3p	110.29	51.04	2.16	0.24	0.046
		hsa-miR-92a-3p	121.60	41.18	2.95	0.31	0.027
TET2	0.63	hsa-miR-375	20.50	54.53	0.38	0.32	0.041
		hsa-miR-663a	374.83	234.91	1.60	−0.31	0.041
ASXL1	1.50	hsa-miR-106b-5p	15.90	5.19	3.06	0.21	0.044
		hsa-miR-15a-5p	7.69	5.07	1.52	0.25	0.028
		hsa-miR-17–5p	61.04	16.38	3.73	0.26	0.021
		hsa-miR-20a-5p	70.78	17.61	4.02	0.27	0.016
		hsa-miR-25–3p	30.05	12.78	2.35	0.23	0.030
		hsa-miR-29a-3p	110.29	51.04	2.16	0.23	0.046
		hsa-miR-361–5p	11.62	6.20	1.87	0.22	0.038
		hsa-miR-424–3p	39.81	25.37	1.57	0.21	0.022
		hsa-miR-425–5p	11.76	6.97	1.69	0.24	0.026

(Continues)

TABLE 3 (Continued)

TSG	TSG fold change	miRNA	Tumor mean	Normal mean	miRNA fold change	Beta between miRNA and TSG expression	FDR *P* value
		hsa-miR-92a-3p	121.60	41.18	2.95	0.35	0.010
		hsa-miR-93–5p	41.72	15.20	2.74	0.22	0.038
FAM123B	1.67	hsa-miR-330–3p	2.81	5.59	0.50	−0.23	0.033
		hsa-miR-378d	0.45	2.43	0.18	−0.22	0.042
		hsa-miR-501–3p	7.07	2.95	2.39	0.21	0.045
		hsa-miR-532–3p	2.74	1.67	1.64	0.23	0.034
		hsa-miR-92a-3p	121.60	41.18	2.95	0.27	0.024
IKZF1	0.38	hsa-miR-146a-5p	10.73	6.93	1.55	0.28	0.031
		hsa-miR-150–5p	14.90	39.17	0.38	0.47	0.012
		hsa-miR-203a	12.52	3.70	3.38	−0.25	0.012
		hsa-miR-497–5p	1.77	7.12	0.25	0.24	0.041
		hsa-miR-650	4.51	16.60	0.27	0.36	0.012
PAX5	0.23	hsa-miR-150–5p	14.90	39.17	0.38	0.37	0.041

information Table 4). All other up- and down-regulated genes were similar except for *AR* which had a FC of 0.80 (adjusted $P = 0.04$) compared to CRC overall where *AR* had a FC of 0.6 (adjusted $P = 2.03E{-}13$). For MSS tumors, *CTNNB1*, which encodes β-catenin, was significantly up-regulated (Supporting information Table 5). *BRAF* and *KRAS* were not significantly differentially expressed in our data.

Of the 27 OGs that showed statistically significant FCs of ≥1.5 or ≤0.67, 12 were associated with miRNA differential expression (Table

TABLE 4 Oncogenes (OG) differentially expressed in colorectal cancer

Gene name	Mean expression		Fold change	Log2 ratio	Adjusted *P* value
	Tumor	Normal			
KLF4	75.45	324.72	0.23	−2.11	1.13E−149
ALK	1.68	6.64	0.25	−1.98	0.18
BCL2	26.44	73.52	0.36	−1.48	7.06E−72
SETBP1	40.32	106.95	0.38	−1.41	4.48E−62
FGFR2	31.67	81.23	0.39	−1.36	6.00E−49
TSHR	4.69	11.60	0.40	−1.31	8.41E−27
FLT3	2.55	5.92	0.43	−1.22	0.49
MPL	1.14	2.49	0.46	−1.13	1.95E−04
KIT	18.19	39.18	0.46	−1.11	2.11E−35
PDGFRA	98.04	195.40	0.50	−0.99	1.14E−38
GNA11	40.13	79.90	0.50	−0.99	8.87E−55
GATA2	10.61	20.64	0.51	−0.96	2.59E−17
FGFR3	44.59	85.93	0.52	−0.95	2.50E−35
AR	48.01	80.52	0.60	−0.75	2.03E−13
RET	5.62	9.04	0.62	−0.69	0.77
CSF1R	37.88	60.49	0.63	−0.68	4.02E−18
JAK3	53.42	82.50	0.65	−0.63	5.11E−12
GNAQ	139.24	197.36	0.71	−0.50	1.10E−34
EGFR	190.64	256.70	0.74	−0.43	0.91

(Continues)

TABLE 4 (Continued)

Gene name	Mean expression		Fold change	Log2 ratio	Adjusted *P* value
	Tumor	Normal			
MDM4	315.31	417.46	0.76	−0.40	0.91
SPOP	57.78	75.20	0.77	−0.38	6.69E−13
U2AF1	189.37	239.72	0.79	−0.34	0.92
ERBB2	246.04	307.97	0.80	−0.32	2.67E−11
JAK2	56.21	69.14	0.81	−0.30	1.41E−12
ABL1	181.26	212.55	0.85	−0.23	0.96
MYD88	69.45	79.03	0.88	−0.19	9.96E−04
SF3B1	480.83	537.63	0.89	−0.16	0.98
KRAS	127.67	139.54	0.91	−0.13	1.32E−06
JAK1	217.29	234.31	0.93	−0.11	0.98
AKT1	170.13	183.07	0.93	−0.11	0.98
H3F3A	53.68	57.59	0.93	−0.10	0.02
BRAF	60.66	63.92	0.95	−0.08	0.01
NFE2L2	142.43	144.43	0.99	−0.02	0.99
PPP2R1A	158.30	153.95	1.03	0.04	0.99
DNMT3A	78.42	75.20	1.04	0.06	0.47
MED12	137.54	130.81	1.05	0.07	0.55
CARD11	25.77	24.37	1.06	0.08	0.98
NCOA3	209.78	196.81	1.07	0.09	0.47
SMO	15.49	14.50	1.07	0.10	0.98
CBL	132.65	120.52	1.10	0.14	0.01
MAP2K1	35.34	32.02	1.10	0.14	0.07
SRSF2	166.29	139.17	1.19	0.26	0.96
MDM2	277.54	231.90	1.20	0.26	0.04
IDH1	92.17	75.94	1.21	0.28	1.21E−05
GNAS	632.89	490.95	1.29	0.37	0.92
NRAS	117.35	90.21	1.30	0.38	1.57E−09
MYCL1	22.87	17.34	1.32	0.40	1.44E−04
IDH2	102.70	75.07	1.37	0.45	2.82E−11
HRAS	21.15	15.30	1.38	0.47	7.11E−08
CTNNB1	630.77	417.97	1.51	0.59	0.81
DNMT1	140.56	87.48	1.61	0.68	1.52E−29
MYCN	3.22	1.91	1.69	0.76	0.77
EZH2	64.20	37.25	1.72	0.79	4.31E−30
NKX2−1	1.94	1.09	1.78	0.83	0.81
PTPN11	249.19	136.54	1.82	0.87	2.13E−72
SKP2	54.13	28.28	1.91	0.94	3.63E−36
CCND1	345.41	145.50	2.37	1.25	1.09E−102
MET	352.22	103.44	3.40	1.77	1.31E−128
MYC	207.70	60.72	3.42	1.77	6.94E−89

TABLE 5 Differentially expressed oncogenes (OG) associated with miRNA differential expression

Oncogene	Tumor mean	Normal mean	Fold change	MiRNA	Tumor mean	Normal mean	Fold change	Beta	Raw *P* value	FDR *P* value
FGFR2	31.67	81.23	0.39	hsa-miR-145–5p	132.97	223.14	0.60	0.27	0.0002	0.04
				hsa-miR-375	20.50	54.53	0.38	0.27	0.0002	0.04
				hsa-miR-663a	374.83	234.91	1.60	−0.26	0.0002	0.04
JAK3	53.42	82.50	0.65	hsa-let-7i-5p	62.16	39.97	1.56	0.23	0.001	0.04
				hsa-miR-146a-5p	10.73	6.93	1.55	0.27	<.0001	0.01
				hsa-miR-146b-5p	4.46	2.67	1.67	0.29	<.0001	0.01
				hsa-miR-150–5p	14.90	39.17	0.38	0.41	<.0001	0.01
				hsa-miR-650	4.51	16.60	0.27	0.33	<.0001	0.01
MET	352.22	103.44	3.40	hsa-let-7i-5p	62.16	39.97	1.56	0.20	0.004	0.03
				hsa-miR-106b-5p	15.90	5.19	3.06	0.24	0.001	0.01
				hsa-miR-1207–3p	1.18	1.93	0.61	−0.22	0.002	0.02
				hsa-miR-1246	629.21	412.81	1.52	0.24	0.0002	0.01
				hsa-miR-1258	1.82	3.73	0.49	−0.23	0.001	0.01
				hsa-miR-1291	5.52	3.67	1.51	0.19	0.007	0.04
				hsa-miR-151a-3p	5.15	1.56	3.31	0.21	0.003	0.02
				hsa-miR-17–5p	61.04	16.38	3.73	0.27	<.0001	0.004
				hsa-miR-1915–5p	1.04	1.77	0.59	−0.24	0.001	0.01
				hsa-miR-19b-3p	29.80	10.42	2.86	0.23	0.002	0.02
				hsa-miR-203a	12.52	3.70	3.38	0.28	<.0001	0.004
				hsa-miR-20a-5p	70.78	17.61	4.02	0.29	<.0001	0.004
				hsa-miR-20b-5p	17.65	3.30	5.35	0.19	0.007	0.04
				hsa-miR-2117	1.50	4.09	0.37	−0.20	0.003	0.02
				hsa-miR-21–5p	463.11	167.37	2.77	0.30	<.0001	0.004
				hsa-miR-221–3p	13.53	4.12	3.28	0.26	0.0002	0.01
				hsa-miR-222–3p	19.45	11.08	1.76	0.27	0.0003	0.01
				hsa-miR-23a-3p	174.68	87.53	2.00	0.31	<.0001	0.004
				hsa-miR-24–3p	106.75	62.39	1.71	0.28	<.0001	0.004
				hsa-miR-25–3p	30.05	12.78	2.35	0.20	0.006	0.04
				hsa-miR-27a-3p	56.26	23.29	2.42	0.34	<.0001	0.004
				hsa-miR-29a-3p	110.29	51.04	2.16	0.34	<.0001	0.004
				hsa-miR-29b-3p	24.31	9.83	2.47	0.30	<.0001	0.004
				hsa-miR-3181	2.11	3.71	0.57	−0.23	0.001	0.01
				hsa-miR-324–5p	5.20	2.27	2.29	0.21	0.003	0.03
				hsa-miR-330–3p	2.81	5.59	0.50	−0.22	0.001	0.02
				hsa-miR-34a-5p	25.15	12.32	2.04	0.19	0.005	0.04
				hsa-miR-3651	58.66	25.92	2.26	0.32	<.0001	0.004
				hsa-miR-424–3p	39.81	25.37	1.57	0.18	0.008	0.05
				hsa-miR-425–5p	11.76	6.97	1.69	0.22	0.002	0.02
				hsa-miR-4458	3.33	5.56	0.60	−0.23	0.001	0.01

(Continues)

TABLE 5 (Continued)

Oncogene	Tumor mean	Normal mean	Fold change	MiRNA	Tumor mean	Normal mean	Fold change	Beta	Raw *P* value	FDR *P* value
				hsa-miR-4469	1.11	2.41	0.46	−0.24	0.001	0.01
				hsa-miR-4520b-3p	1.96	3.17	0.62	−0.22	0.001	0.01
				hsa-miR-501–3p	7.07	2.95	2.39	0.20	0.004	0.03
				hsa-miR-513c-3p	2.15	3.50	0.62	−0.21	0.004	0.03
				hsa-miR-5685	1.28	2.78	0.46	−0.21	0.003	0.02
				hsa-miR-6071	0.97	1.70	0.57	−0.20	0.004	0.03
				hsa-miR-6515–5p	1.20	4.41	0.27	−0.24	0.001	0.01
				hsa-miR-92a-3p	121.60	41.18	2.95	0.32	<.0001	0.004
				hsa-miR-93–5p	41.72	15.20	2.74	0.28	<.0001	0.004
CCND1	345.41	145.50	2.37	hsa-miR-17–5p	61.04	16.38	3.73	0.27	0.0002	0.03
				hsa-miR-203a	12.52	3.70	3.38	0.28	<.0001	0.03
				hsa-miR-20a-5p	70.78	17.61	4.02	0.27	<.0001	0.03
				hsa-miR-21–5p	463.11	167.37	2.77	0.25	0.0004	0.04
				hsa-miR-27a-3p	56.26	23.29	2.42	0.28	0.0003	0.03
				hsa-miR-93–5p	41.72	15.20	2.74	0.26	0.0003	0.03
PDGFRA	98.04	195.40	0.50	hsa-miR-145–5p	132.97	223.14	0.60	0.28	0.0002	0.04
				hsa-miR-497–5p	1.77	7.12	0.25	0.24	0.0004	0.05
KLF4	75.45	324.72	0.23	hsa-miR-375	20.50	54.53	0.38	0.39	<.0001	0.03
				hsa-miR-6515–5p	1.20	4.41	0.27	0.27	0.0003	0.03
				hsa-miR-663a	374.83	234.91	1.60	−0.37	<.0001	0.03
				hsa-miR-663b	65.50	32.21	2.03	−0.31	<.0001	0.03
				hsa-miR-934	4.36	0.94	4.66	−0.26	0.0002	0.03
MYC	207.70	60.72	3.42	hsa-miR-1246	629.21	412.81	1.52	0.23	0.001	0.04
				hsa-miR-17–5p	61.04	16.38	3.73	0.35	<.0001	0.02
				hsa-miR-19b-3p	29.80	10.42	2.86	0.24	0.001	0.04
				hsa-miR-203a	12.52	3.70	3.38	0.23	0.001	0.04
				hsa-miR-20a-5p	70.78	17.61	4.02	0.34	<.0001	0.02
				hsa-miR-20b-5p	17.65	3.30	5.35	0.25	0.001	0.03
				hsa-miR-29a-3p	110.29	51.04	2.16	0.25	0.0003	0.02
				hsa-miR-29b-3p	24.31	9.83	2.47	0.23	0.001	0.04
				hsa-miR-330–3p	2.81	5.59	0.50	−0.24	0.001	0.03
				hsa-miR-3651	58.66	25.92	2.26	0.29	<.0001	0.02
				hsa-miR-501–3p	7.07	2.95	2.39	0.22	0.001	0.04
				hsa-miR-663b	65.50	32.21	2.03	0.25	0.0003	0.02
				hsa-miR-92a-3p	121.60	41.18	2.95	0.35	<.0001	0.02
				hsa-miR-93–5p	41.72	15.20	2.74	0.25	0.0003	0.02
SETBP1	40.32	106.95	0.38	hsa-miR-133b	1.71	6.94	0.25	0.30	<.0001	0.01
				hsa-miR-145–5p	132.97	223.14	0.60	0.38	<.0001	0.01
				hsa-miR-150–5p	14.90	39.17	0.38	0.32	<.0001	0.01

(Continues)

TABLE 5 (Continued)

Oncogene	Tumor mean	Normal mean	Fold change	MiRNA	Tumor mean	Normal mean	Fold change	Beta	Raw *P* value	FDR *P* value
				hsa-miR-195–5p	3.59	12.18	0.29	0.29	0.0002	0.01
				hsa-miR-30a-5p	2.38	4.61	0.52	0.28	<.0001	0.01
				hsa-miR-375	20.50	54.53	0.38	0.25	0.0003	0.01
				hsa-miR-497–5p	1.77	7.12	0.25	0.31	<.0001	0.01
				hsa-miR-650	4.51	16.60	0.27	0.23	0.001	0.04
				hsa-miR-663a	374.83	234.91	1.60	−0.26	0.0003	0.01
				hsa-miR-99a-5p	6.30	3.70	1.71	0.23	0.001	0.04
CTNNB1	690.35	441.80	1.56	hsa-miR-1915–5p	1.04	1.77	0.59	−0.28	<.0001	0.04
BCL2	26.44	73.52	0.36	hsa-miR-133b	1.71	6.94	0.25	0.22	0.002	0.04
				hsa-miR-145–5p	132.97	223.14	0.60	0.25	<.0001	0.01
				hsa-miR-150–5p	14.90	39.17	0.38	0.38	<.0001	0.01
				hsa-miR-195–5p	3.59	12.18	0.29	0.29	<.0001	0.01
				hsa-miR-30a-5p	2.38	4.61	0.52	0.26	0.0002	0.01
				hsa-miR-375	20.50	54.53	0.38	0.27	<.0001	0.01
				hsa-miR-497–5p	1.77	7.12	0.25	0.32	<.0001	0.01
				hsa-miR-583	6.61	3.22	2.05	−0.22	0.002	0.04
				hsa-miR-650	4.51	16.60	0.27	0.30	<.0001	0.01
				hsa-miR-663a	374.83	234.91	1.60	−0.32	<.0001	0.01
				hsa-miR-663b	65.50	32.21	2.03	−0.25	0.001	0.02
PTPN11	249.19	136.54	1.82	hsa-miR-106b-5p	15.90	5.19	3.06	0.23	0.001	0.04
				hsa-miR-1207–3p	1.18	1.93	0.61	−0.23	0.001	0.04
				hsa-miR-15a-5p	7.69	5.07	1.52	0.22	0.002	0.05
				hsa-miR-17–5p	61.04	16.38	3.73	0.24	0.0004	0.03
				hsa-miR-203a	12.52	3.70	3.38	0.22	0.002	0.04
				hsa-miR-20a-5p	70.78	17.61	4.02	0.25	0.001	0.03
				hsa-miR-23a-3p	174.68	87.53	2.00	0.22	0.002	0.05
				hsa-miR-27a-3p	56.26	23.29	2.42	0.24	0.0004	0.03
				hsa-miR-29a-3p	110.29	51.04	2.16	0.24	0.001	0.03
				hsa-miR-3651	58.66	25.92	2.26	0.24	0.001	0.03
				hsa-miR-425–5p	11.76	6.97	1.69	0.30	<.0001	0.03
				hsa-miR-92a-3p	121.60	41.18	2.95	0.28	<.0001	0.03
				hsa-miR-93–5p	41.72	15.20	2.74	0.24	0.001	0.03
CSF1R	37.88	60.49	0.63	hsa-miR-146b-5p	4.46	2.67	1.67	0.26	0.0003	0.04
				hsa-miR-150–5p	14.90	39.17	0.38	0.29	<.0001	0.03

5). *BCL2* was associated with 11 miRNAs, *CCND1* with six, *CSF1R* with two, *CTNNB1* with one, *FGFR2* with three, *JAK3* with five, *KLF4* with five, *MET* with 40, *MYC* with 14, *PDGFRA* with two, *PTPN11* with 13, and *SETBP1* with 10. Several miRNAs were associated with 2 OGs: let-7i-5p, miR-106b-5p, miR-1207–3p, miR-1246, miR-133b, miR-146b-5p, miR-1915–5p, miR-19b-3p, miR-195–5p, miR-20b-5p, miR-21–5p, miR-23a-3p, miR-29b-3p, miR-30a-5p, miR-330–3p, miR-425–5p, miR-501–3p, and miR-6515–5p. MiR-27a-3p, miR-29a-3p, miR-3651, miR-497–5p, miR-650, miR-663b, miR-92a-3p were associated with three OGs and miR-145–5p, miR-150–5p, miR-17–5p, miR-203a, miR-20a-

TABLE 6 Pathways and functions of tumor suppressor genes (TSG) and oncogenes (OG) significantly differentially expressed in colorectal tissue with fold change of $\geq$1.5 or $\leq$0.67

Overall	Up or down regulated	Major pathway	Major function
Tumor suppressor genes			
BRCA1	*Up-regulated*	DNA damage control	Genome maintenance
PRDM1	*Down-regulated*	NFkB-signaling; B cell development pathways; regulation of TP53 activity	A repressor of beta-interferon gene expression
CYLD	*Down-regulated*	TNF signaling; Immune System; NOD1/2 Signaling; RIG-1/MDA5 mediated induction of IFN-alpha/beta pathway; Wnt-signaling pathway	Ubiquitin-dependent protein catabolic process; regulation of tumor necrosis factor-mediated signaling pathway; cell cycle regulation
MSH2	*Up-regulated*	DNA damage control; mismatch repair	Mismatch repair gene; genome maintenance
GATA3	*Down-regulated*	IL-27 mediated signaling events; NFkB Signaling; IL-4 Signaling and their effects on immune response	Regulator of T-cell Development; Required for the T-helper 2 differentiation process following immune and inflammatory responses
RNF43	*Up-regulated*	Wnt-signaling	Inhibits Wnt-signaling; cell fate
SOX9	*Up-regulated*	Wnt-signaling; cAMP signaling	Normal skeletal development; acts as a transcription factor for other genes; cell survival
BRCA2	*Up-regulated*	DNA damage control	Genome maintenance
RB1	*Up-regulated*	Cellular senescence	Cell cycle regulator; transcription factor activity
TP53	*Up-regulated*	Apoptosis; DNA damage control	Cell survival; DNA repair
RUNX1	*Up-regulated*	Transport of glucose and other sugars, bile salts and organic acids; transcriptional misregulation in cancer	Transcription regulation; regulatory region DNA binding
TET2	*Down-regulated*	Activated PKN1 stimulates transcription of AR regulated genes; chromatin modification	Methylcytosine dioxygenase activity
NPM1	*Up-regulated*	BARD1 signaling; chromosome maintenance; apoptosis	Nucleic acid binding; cell survival
FAM123B (AMER1)	*Up-regulated*	Wnt-signaling	Regulates transcriptional activity several genes including APC; cell fate
IKZF1	*Down-regulated*	NFkB-signaling; transcription regulation	Cell fate
PAX5	*Down-regulated*	NFKB-signaling; C-MYB transcription factor network	Transcription factor activity; cell fate
MSI only			
MLH1	*Down-regulated*	DNA damage control; mismatch repair	Mismatch repair gene; genome maintenance
MSH6	*Up-regulated*	Mismatch repair; DNA damage control	Mismatch repair gene; genome maintenance
PTCH1	*Down-regulated*	Signaling by GPCR; Hedgehog pathway; PKA signaling	Protein complex binding; cell fate
CEBPA	*Down-regulated*	Adipogenesis; glucose energy metabolism; NF-KB signaling; PI3K; RAS	Transcription factor activity; cell survival
MSS only			
PHF6	*Up-regulated*	Transcriptional regulation	RNA binding and histone binding; cell fate
ASXL1	*Up-regulated*	Chromatin modification	Transcription co-activator activity; retinoic acid receptor binding; cell fate
Oncogenes			
AR	Down	Transcriptional regulation; regulation of nuclear SMAD2/3 signaling	Regulates gene expression; affects cellular proliferation
BCL2	Down	Cell cycle/apoptosis; TGF-beta pathway; TNFR1 pathway	Regulates cell death/cell survival
CCND1	Up	Cell cycle/apoptosis; Wnt pathway	Protein kinase activity; cell fate

(Continues)

TABLE 6 (Continued)

Overall	Up or down regulated	Major pathway	Major function
CSF1R	Down	PI3K; RAS; AKT1 signaling pathway	Mediates activation of MAP Kinase; Cell survival; promotes the release of pro-inflammatory chemokines in response to IL34 and CSF1; promotes cancer cell invasion
DNMT1	Up	Chromatin modification	Maintains methylation patterns following DNA replication; epigenetic gene regulation
EZH2	Up	Chromatin modification	Involved in maintaining the transcriptional repressive state of genes over successive cell generations; cell development
FGFR2	Down	PI3K; RAS; STAT; VEGF signaling pathway	Influences cell growth and differentiation; cell proliferation
FGFR3	Down	PI3K; RAS; STAT; VEGF signaling pathway	Influences cell growth and differentiation; cell proliferation
GATA2	Down	NOTCH, TGF-b; NF-κB signaling	Transcription factors
GNA11	Down	PI3K; RAS; STAT	Modulators or transducers in various transmembrane signaling
JAK3	Down	STAT; RET signaling; NK-κB signaling	Cytokine receptor-mediated intracellular signal transduction; predominately expressed in immune cells
KIT	Down	PI3K; RAS; STAT	Transmembrane receptor for mast cell growth factor (stem cell growth factor)
KLF4	Down	Transcriptional regulation; WNT; stem cell differentiation pathways	Transcription factors
MET	Up	PI3K; RAS	Cell survival, cell migration, and invasion
MPL	Down	JAK-STAT signaling; NF-κB signaling	Transmembrane signaling receptor activity; immune response
MYC	Up	Cell cycle/apoptosis; regulation of nuclear SMAD2/3 signaling	Cell cycle progression, apoptosis, cellular transformation; functions as a transcription factor; activates transcription of growth-related genes
PDGFRA	Down	PI3K; RAS	Plays a role in organ development, wound healing and tumor progression
PTPN11	Up	RAS; interferon gamma signaling; RET signaling	Signaling molecules that regulate cell growth, differentiation, mitotic, cycle and oncogenic transformation
SETBP1	Down	Chromatin modification; replication	DNA replication
SKP2	Up	Cell cycle/apoptosis	Protein binding; ubiquitin-protein transferase activity
TSHR	Down	PI3K; MAPK	Thyroid cell metabolism; cAMP signaling pathway
MSI only			
ALK	Down	PI3K; RAS; MAPK	Insulin receptor superfamily; cell proliferation induction; drives NF-κB activation
CARD11	Down	Cell cycle/apoptosis; immune response; RET signaling	Positive regulator of NF-κB activation;
FLT3	Down	PI3K; RAS; STAT	Involved in apoptosis, cell proliferation and differentiation
HRAS	Up	RAS; RET signaling; VEGF signaling	Signal transduction pathways
IDH2	Up	Chromatin modification; metabolism	Involved in intermediary metabolism and energy production'; NAP
MSS only			
CTNNB1	Up	Wnt-signaling; APC	Adherens junctions; regulate cell growth and adhesion between cells; transcription factor activity

5p, miR-375, miR-663a, and miR-93–5p were associated with four OGs. All but two OGs that were differentially expressed in CRC had a mixture of up- and down-regulated miRNAs associated with them. *CTNNB1*, which was up-regulated, was associated with one miRNA (miR-1915–5p) that was also up-regulated; *PDGFRA* which was down-regulated was associated with two miRNAs (miR-145–3p and miR-497–5p) which were also down-regulated.

4 | DISCUSSION

Of the 74 TSGs evaluated, 59 were significantly differentially expressed; 22 of these differentially expressed TSGs were more strongly associated with CRC either overall or for MSI and MSS tumors specifically as indicated by a FC $\geq$1.5 or $\leq$0.67. Of these 22 TSGs, 13 were up-regulated in carcinoma tissue compared to paired normal tissue. Evaluation of these 22 TSGs with differential expression of miRNAs showed that 13 TSGs were significantly associated with expression of 44 miRNA. Twenty-seven OGs were statistically significantly dysregulated when considering higher FC levels. Evaluation of MSI tumors showed that two additional OGs were statistically significantly up-regulated (*IDH2* FC 1.69 and *HRAS* FC 1.85) and three OGs were down-regulated (*FLT3* FC 0.30, *CARD11* FC 0.33, and *ALK* FC 0.32). *CTNNB1* was significantly up-regulated in MSS tumors. Twelve of the 27 OGs significantly differentially expressed were associated with 56 miRNAs. The majority of TSGs/OGs were associated with multiple miRNAs and miRNAs were associated with several TSGs/OGs.

Several factors need to be considered when evaluating TSG and OG differential expression. First, TSG and OG differential expression does not necessarily correlate with TSG and OG mutation. Our data suggest that in known *TP53*-mutated, *KRAS*-mutated, and *BRAF*-mutated samples there were no differences in gene expression between mutated and nonmutated samples (counts adjusted from DESeq2: *TP53*-mutated vs. not *TP53*-mutated 136 vs. 144 and TP53 expression in normal tissue of 63; *KRAS*-mutated 155 vs. not *KRAS*-mutated 155; *BRAF*-mutated 75 vs. not *BRAF*-mutated 70). We further evaluated TP53 expression based on loss of function (LOF) mutations such as frameshift, stop, and insertion/deletions which represented roughly 1/3 of *TP53* mutations. For LOF mutations the mean level of expression was 78.8 while for missense *TP53* mutations it was 153.1. This suggest that LOF mutations reduces expression to a level comparable to the normal level of expression, while TP53 expression is elevated in *TP53*-missense mutation mutated and non-TP53-mutated tumors. *APC*, another TSG, was down-regulated in our data (FC = 0.74); *APC* mutations are usually stop mutations and frame shifts, which would lead to loss of functional protein and possibly less stable mRNA through nonsense-mediated RNA decay;[32,33] these mutations occur in roughly 80% of the CRC cases and could affect gene expression and occurred in 35 of 40 individuals in this dataset for which we had *APC* mutational status. Down-regulation of *MLH1* would be expected in mismatch repair deficient tumors (as was seen in our data); *MLH1* promoter methylation and subsequent transcriptional silencing is the most common cause of sporadic mismatch repair deficiency.[34–36] In our data, tumors that had *MLH1* methylation had significantly lower levels of MLH1 expression than those that did not have *MLH1* methylation (19.9 vs. 50.0).

Several TSGs, including *TP53*, *RB1*, *BRCA1*, and *BRCA2*, were up-regulated, possibly in response to cell stress. Others have observed up-regulated expression of TSGs such as *CDKN2A* (p16) in CRC tumors.[37] In our data, *CDKN2A* was up-regulated with a fold change of 1.46. Romagosa and colleagues[37] offered several explanations for the up-regulation of *CDKN2A* in cancer. *CDKN2A* is part of a large pathway that includes RB, which is responsible for blocking S phase entry in the cell cycle; if the pathway is not functioning properly then the expected inactivation of cell proliferation may not occur. Romagosa et al.[37] interpreted their data to indicate that overexpression of *CDKN2A* in conjunction with expression of other genes, such as *COX2*, would impact the role of *RB* in the malignant lesion. Expression of *KRAS* was not significantly altered in our tumor samples although roughly 35% of our samples had a *KRAS* mutation. It has been shown that *KRAS* mutations can dysregulate genes associated with cell cycle and apoptosis,[38] supporting the hypothesis that mutations in genes can dysregulate pathways that may have clinical relevance to the carcinogenic process.

The gene expression patterns of differentially expressed TSGs and OGs in our data lend themselves to several distinct observations. First, the majority of significantly differentially expressed TSGs were up-regulated (19 TSG upregulated vs. 13 down-regulated). The second observed pattern was the unique functions and pathways associated with dysregulated TSGs. Five of the six of the TSGs most strongly down-regulated were linked to the NFκB-signaling pathway or immune response (Table 6). For instance, *CYLD* negatively regulates NFκB activation and is involved in other immune response mechanisms.[39] When TSGs such as *CYLD* are down-regulated, excessive inflammation occurs and tumorigenic factors can be promoted.[40] Conversely, TSGs that were up-regulated were more likely to be involved in cell cycle regulation, apoptosis, and cell growth, possibly as a response to cell stress in early stages of tumorigenesis. Several OGs that were significantly up-regulated, such as *DNMT1*, *EZH2*, and *IDH2*, are involved in chromatin modification and remodeling; *CCND1* (cyclin D1), *MYC*, and *SPK2* are important regulators of apoptosis, and *MET*, *PTPN11*, and *HRAS* are important signal transducers. Up-regulation of these OGs could promote cell growth. However, a larger number of OGs were down-regulated, possibly counteracting the carcinogenic process. These genes include *AR*, *BCL2*, *CSF1R*, *FGFR2*, *FGFR3*, *GATA2*, *GNA11*, *JAK3*, *KIT*, *KLF4*, *PDGFRA*, *SETBP1*, *TSHR*, *FLT3*, *ALK*, and *CARD11*, which mainly function as transcriptional regulators and are involved in regulation of major signaling pathways participating in inflammation or immune response: PI3K/AKT, JAK/STAT, RAS, TGFβ signaling, NFκB signaling, and VEGF signaling.

Increased inflammation, angiogenesis, and decreased immune response are hallmarks of many of the major pathways in which dysregulated TSGs and OGs operate. PI3K (*PIK3CA*) induces the activation of Akt1 (alias *PDK1*) and is recognized as an important regulator of cell proliferation and survival and links to inflammation.[41] Akt promotes tumorigenesis by inhibiting apoptosis by inactivating *BCL2*, by

stabilizing *MYC*, by inducing the degradation of cyclin-dependent kinase (*CDK1*), or by triggering activity of NFκB signaling.[42] Cytokine receptors utilize nonreceptor protein tyrosine kinases, such as JAK, to transmit their signals to the signal transducers and activators of transcription (STATs). A functional JAK/STAT pathway is also critical to an effective immune response.[43] *JAK3* and *JAK2* were down-regulated in our data; *JAK3* has been shown to be uniquely associated with intestinal epithelial cells. *JAK3* has been shown to interfere with *GATA3*, a TSG that was down-regulated in our data and is associated with NFκB-signaling.[43] Expression of *BCL2*, which is involved in apoptosis, has also been shown to be regulated by the JAK/STAT-signaling pathway and TGFβ-signaling;[44,45] *BCL2* was down-regulated in our data. Other protein tyrosine kinases, such as *FLT3*, *KIT*, and *EGFR*, are classified as receptor protein kinases. All of these OGs were down-regulated in our data and are involved in activation of multiple signaling pathways including cell proliferation, immune response, and angiogenesis.[46–48] *FLT3*, part of the VEGF-signaling pathway, is a key element in angiogenesis and ties into P13K/AKT signaling and requires *STAT3* for effective cell proliferation.[49]

Because MSI tumors are hyper-mutated, we thought that it was important to evaluate differential TSG/OG expression for MSI and MSS tumors separately. For the most part, the same genes were over or under-expressed in these specific tumor phenotypes. However, there was a difference in the FC of expression of several TSGs and OGs between MSI and MSS tumors. As might be expected, the difference in expression for mismatch repair genes in MSI tumors was greater. *MLH1* was strongly down-regulated while *MSH6* was strongly up-regulated in MSI tumors. *PTCH1*, involved in Hedgehog pathway and PKA signaling, and *CEBPA*, involved in NFκB-signaling and PI3K pathways, were down-regulated in MSI tumors. Two additional TSGs, *PHF6* and *ASXL1*, were strongly up-regulated in MSS tumors. Both of these genes are involved in transcription regulation and cell fate. Several OGs were significantly associated specifically with MSI tumors. *ALK*, *CARD11*, and *FLT3* were only significantly down-regulated in MSI tumors. Two other genes, *HRAS* and *IDH2*, were significantly up-regulated in overall colorectal tumors (*HRAS* FC 1.38; *IDH2* FC 1.37), but the FCs of these genes in MSI tumors was much stronger (*HRAS* FC 1.85; *IDH2* FC 1.69). These OGs are primarily involved in signal transduction, inflammation, or immune response pathways that include PI3K/AKT, MAPK, RAS, RET, and VEGF signaling.

The exact function of miRNAs is not clearly understood; however, our results indicate that they are part of regulatory networks through both direct and indirect effects on OGs and TSGs. It has been suggested that miRNAs work with OGs and TSGs.[13] A study in brain cancer has shown that miR-128 can activate gene expression by repressing nonsense-mediated RNA decay.[50] An example of the complexity of signaling and regulation networks is *MYC*, a frequently studied OG in cancer. In our data, *MYC* had a FC of 3.42. *MYC* has been shown to up-regulate oncomR1, which includes a cluster of six miRNAs, miR-17–5p, miR-18a, miR-19a, miR-20a, miR19b-1, and miR-92.[51] In our data, these miRNAs, except for miR-18a and miR-19a, were up-regulated and associated with *MYC* up-regulation. Three of the six miRNAs in miR-17–92 cluster also have been regulated in conjunction with the TSGs *RBL1*, *CDKN1A* (p21), *PTEN*, and *APC*.[18,19,22]

Studies have shown that some miRNAs, such as miR-16, restrict mediators needed to control inflammatory response; it has been suggested that other miRNAs might also work in similar manner to miR-16 to destabilize inflammatory response.[52,53] Studies have shown that miRNAs such as miR-320a directly target β catenin, a central component of the Wnt-signaling pathway, to suppress cell proliferation.[54] Several OGs, including *CTNNB1*, *CCND1*, and *KLF4*, were part of the Wnt-signaling pathway. In this pathway, associations are stronger for MSS tumor phenotype. Several miRNAs associated with *CCND1*, including miR-17–5p, miR-203a, miR-20a-5p, miR-21–5p, miR-27a-3p, miR-93–5p, were also associated with up-regulated TSGs in the Wnt-signaling pathway (ie, *RNF43* and *SOX9*).

Several miRNAs have been associated with the immune system, including miR-16, miR-142–3, miR-150, miR-125b, miR-21, miR-223, miR-9, miR-30, miR-181, miR-17–92 cluster, and miR-155.[53,55] MiR-150–5p was down-regulated in our data in conjunction with the five TSGs that were down-regulated and had immune and inflammation-related functions. Of these, *PRDM1* was previously cited as being down-regulated by several miRNAs including miR-30, miR-9 and miR-125b.[53] Five of the six TSGs that were down-regulated, were associated with miR-150–5p which is also down-regulated. All of these TSGs, including *GATA3*, were associated with inflammation-related pathways such as the NFκB-signaling pathway, suggesting a role in inflammation regulation. However, all of the OGs associated with miR-150–5p, namely *SETBP1*, *JAK3*, *BCL2*, and *CSF1R*, were also down-regulated. These OGs also are involved in inflammation-related pathways. MiR-150–5p expression may be reduced in response to less TSG protein production, as a reduction in target availability is related to miRNA down-regulation, resulting from dissociation of the miRNA-inducing silencing complex, which leaves miRNAs vulnerable to degradation.[56]

Some of the miRNAs and TSGs were inversely associated. Examples of these associations were miR-3191–3p (down-regulated) and *RNF43* (up-regulated); miR-378d (down) and *RNF43* and *FAM123B* (up); miR-1207–3p and 1915–5p (down) and *SOX9* and *RB1* (up); miR-663a (up) and *TET2* (down); and miR-146a-3p and miR-203-a (up) and *IKZF1* (down). However, often both the miRNA and TSG were either simultaneously up-regulated or down-regulated, which may imply indirect associations between the miRNA and the TSG or could be the result of modifying effects of either lifestyle or genetic factors.[57–62] Additionally, several TSGs also are transcription factors (TF), and as such may directly up-regulate miRNA transcription and co-regulate biological functions with miRNAs through feedback and feed forward loops.[63–65] In feedback loops, regulatory paths through TF and miRNAs can have either the same effect or opposite effects on target genes as well as on each other.[64] In feed-forward loops, a regulator such as a TF or miRNA, regulates the expression of a target via a direct as well as an indirect path. It has been suggested that regulatory paths involving

miRNAs and TF are prevalent mechanisms of gene expression.[64] *PAX5*, *IKZF1*, *GATA3*, and *PRDM1*, all TFs that were down-regulated TSGs in our CRC data, were simultaneously associated with down-regulated miRNAs. Studies have previously shown that *PAX5*, *PRDM1*, and *IKZF11* share a regulatory network with miR-150–5p via feed forward loops.[63] Similar mechanisms may be operating for other OGs in conjunction with miRNAs.

The study is uniquely suited to examine associations between differential TSG/OG expressions in CRC. Our large sample size offers power to determine significant associations; our use of RNA-Seq data as well as the Agilent miRNA platform allows us to take a discovery approach which enables us to better illuminate pathways of interest. We looked at TSG/OGs that had higher levels of differential expression, although the cut-points of $\geq$1.5 or $\leq$0.67 FC was arbitrary. Additionally, we were able to evaluate TSGs/OGs expression with miRNA expression. While we are able to identify numerous associations it is often difficult to determine if associations are direct or indirect in complex biological pathways. Other study strengths include our paired carcinoma and normal mucosa expression data. Having individuals paired data allows us to control for potential confounding effects of genetic and lifestyle factors that could influence both gene and miRNA expression.[57–59,62] Similarly, our tumor phenotype data allowed us to investigate differences in gene expression associated with MSS and MSI tumors, as well as *TP53*-mutated, *KRAS*-mutated, and *BRAF*-mutated tumors. Our expression data have been shown to have both high repeatability as well as reliability when compared to other ascertainment methods.[14,15] We encourage others with similar data to undertake replication of our findings in population-based studies as well as laboratory-based studies to better test the proposed functionality.

In summary, our data suggest that several TSG and OGs expression is dysregulated in CRC, suggesting a cellular response to stress. Our data suggest that miRNAs most likely have both direct and indirect effects on TSG and OGs. It is possible that they work as intermediary regulators between OGs and TSGs, and help to balance up- and down-regulation of these genes that can lead to, as well as counter, cell proliferation and apoptosis, which is the hallmark of carcinogenic processes.

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SUPPORTING INFORMATION

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

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