Research Article

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 > 1.5 or < 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, 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, PTH1, 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, SKP2, CCND1, MET, and MYC); three down-regulated for MSI (MLH1, PTH1, and CEBPA); seven down-regulated for MSI (MLH1, PTH1, and CEBPA); 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 (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

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attributed to them, are likely part of the elaborate cellular pathways regulated by TSG and OGs.\textsuperscript{12,13} MiRNA expression is frequently either down-regulated or up-regulated in CRC tissue when compared to normal mucosa,\textsuperscript{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.\textsuperscript{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.\textsuperscript{12,21} MiRNAs have been cited as being “critical effectors of several canonical oncogenic and tumor suppressor pathways.”\textsuperscript{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\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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 Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, Indiana). A more detailed description of the methods can be found in our previous work.\textsuperscript{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.\textsuperscript{27} We focused on expression of 74 TSGs and 59 OGs previously identified as being associated with cancer\textsuperscript{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 \((r = 0.98)\).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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 ≥1.5 or ≤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.21

### 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 ≤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 ≥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, PTTCH1, 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).

Further evaluation of the 22 TSGs that were significantly differentially expressed with a FC ≥1.5 or ≤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.

**Table 1** Description of study population

| Site      | N   | (%) |
|-----------|-----|-----|
| Colon     | 169 | 77.9|
| Rectal    | 48  | 22.1|

| Sex        | N   | (%) |
|------------|-----|-----|
| Male       | 118 | 54.4|
| Female     | 99  | 45.6|

| Age        | Mean (SD) | (%) |
|------------|-----------|-----|
|            | 64.8      | 10.1|

| Race       | N   | (%) |
|------------|-----|-----|
| Non-Hispanic White | 161 | 74.2|
| Hispanic   | 14  | 6.5 |
| Non-Hispanic Black  | 8   | 3.7 |
| Unknown    | 34  | 15.7|

| AJCC Stage | N   | (%) |
|------------|-----|-----|
| 1          | 58  | 27.1|
| 2          | 61  | 28.5|
| 3          | 72  | 33.6|
| 4          | 23  | 10.8|

| Tumor phenotype | N   | (%) |
|-----------------|-----|-----|
| 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 | N   | (%) |
|--------------|-----|-----|
| Dead         | 92  | 42.6|
| Alive        | 124 | 57.4|
| Gene name | Mean expression |
|-----------|-----------------|
|           | Tumor | Normal | Fold change | Log2 ratio | Adjusted P value |
| 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-25         |
| 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)
Seven OGs were up-regulated with FCs >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 $\geq 1.5$ or $\leq 0.67$ 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)
| TSG | TSG fold change | miRNA | Tumor mean | Normal mean | miRNA fold change | Beta between miRNA and TSG expression | FDR P value |
|-----|----------------|-------|------------|------------|------------------|-------------------------------------|------------|
| 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 | 6.04 | 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 |
|      |       | hsa-miR-93-5p | 41.72 | 15.20 | 2.74 | 0.21 | 0.035 |
| 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 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 4).

### Table 4 Oncogenes (OG) differentially expressed in colorectal cancer

| Gene name | Tumor | Normal | Fold change | Log2 ratio | Adjusted P value |
|-----------|-------|--------|-------------|------------|------------------|
| 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)
| 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        |
| 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        |
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-29a-3p, miR-29b-3p, miR-30a-5p, miR-330–3p, miR-425–5p, miR-501–3p, and miR-6515–3p. 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-

| Oncogene | Tumor mean | Normal mean | Fold change | MiRNA       | Tumor mean | Normal mean | Fold change | Beta | Raw P value | FDR P value |
|----------|------------|-------------|-------------|-------------|------------|-------------|-------------|------|-------------|-------------|
| **BCL2** | 26.44      | 73.52       | 0.36        | 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-20a-5p | 12.52      | 3.70        | 3.38        | 0.22 | 0.002       | 0.04        |
|          |            |             |             | 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        |
**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 |
| 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; PDGFRα 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 ≥1.5 or ≤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 TSGs 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-regulation of 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 colleagues37 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 DNM1, 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, FGF2, FGF3, GATA2, GNA11, JAK3, KIT, KLF4, PDGFRα, 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. 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. 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. Expression of BCL2, which is involved in apoptosis, has also been shown to be regulated by the JAK/STAT-signaling pathway and TGFβ-signaling. 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. FLT3, part of the VEGF-signaling pathway, is a key element in angiogenesis and ties into PI3K/AKT signaling and requires STAT3 for effective cell proliferation.

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. A study in brain cancer has shown that miR-128 can activate gene expression by repressing nonsense-mediated RNA decay. 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. 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. 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. 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. 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. 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.

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-203–3p 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. 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. 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. 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.\(^6\) 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 IKZF1 share a regulatory network with mir-150–5p via feed forward loops.\(^6\) 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 allowed 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.\(^57\)–\(^59\)\(^6\) 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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