

Exploration of the mechanism of colorectal cancer metastasis using microarray analysis

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Abstract. The aim of the present study was to investigate the mechanism of metastasis in colorectal cancer (CRC) using microRNA (miRNA) and mRNA expression profiles. The mRNA and miRNA expression profiles of the GSE2509 and GSE56350 datasets were obtained from the Gene Expression Omnibus database. The differentially expressed genes (DEGs) and differentially expressed miRNAs (DEMs) were identified using the limma software package. The Database for Annotation, Visualization and Integrated Discovery was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the DEGs. The predicted target genes associated with the DEMs were identified using the miRWalk database and the enrichment analysis was conducted using the clusterProfiler package. The miRNA-gene molecular interaction network was visualized using the Cytoscape software platform. A total of 544 DEGs and 42 DEMs were identified. DEGs were annotated in 320 GO terms and 11 KEGG pathways. Overall, 366 miRNA-gene pairs were identified and the miRNA-gene network was visualized. Furthermore, the predicted target genes were mainly classified in 12 pathways. The results of the present study suggest that fibronectin type III domain-containing 3B, cysteine rich transmembrane BMP regulator 1 and forkhead box J2 may be potential therapeutic and prognostic targets of metastatic CRC. In addition, pathways in cancer, the Wnt signaling pathway and extracellular matrix-receptor interaction may play a critical role in CRC metastasis.

Introduction

Colorectal cancer (CRC) is the third most common malignancy worldwide and the third leading cause of cancer-associated mortality in the United States (1,2). It is estimated that ~1.3 million new cases are diagnosed and ~0.7 million people succumb to the disease each year worldwide (3). According to recent statistics, it was estimated that 134,490 new cases and 49,190 fatalities would occur in America in 2016 (1). The incidence and mortality rates for men and women have improved in the last decades as a result of advances in screening and clinical treatment (4,5). Metastasis, the most common cause of cancer-associated mortality, is a multi-step process through which tumor cells spread from their primary site and form secondary growths at a distance (6). Metastasis is among the six initially described hallmarks of cancer and is a major cause of CRC-associated mortality (7,8). The 5-year survival rate of early-stage CRC ranges between 60 and 95%; however, for patients with metastatic tumors, the survival rate ranges from 10 to 35% (9-11). Despite improvements in diagnosis and treatment, ~90% of CRC-associated mortalities are due to metastases and it has been estimated that ~50% of all patients diagnosed with CRC eventually succumb to metastatic disease (12,13). Therefore, it is critical to investigate the mechanism of metastasis in CRC and identify novel molecular therapeutic and prognostic targets. To date, some progress has been made. Epithelial-mesenchymal transition was demonstrated to serve a pivotal and intricate role in promoting CRC metastasis (6,14). In addition, certain genes and proteins were found to be associated with CRC metastasis, including semaphoring 3F, stromal interaction molecule 1, forkhead box C2 and hes family BHLH transcription factor 1 (14-17), as well as Cyclin b1, Angiopoietin-like 4 and p21-activated kinase 1 and 4 (18-20). However, one study demonstrated that metastasis occurs through a multistep cascade of events, but that it was inefficient as a whole process (8). Even though mutations associated with metastasis have been investigated in the past, only a limited number of such genetic alterations have been identified and the underlying molecular mechanism remains unclear (21). The present study analyzed the microRNA (miR/miRNA) and mRNA expression profiles of CRC samples

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in order to investigate the mechanism of metastasis in CRC and identify novel molecular biomarkers.

Materials and methods

mRNA and miRNA expression data. The mRNA and miRNA expression profiles of the GSE2509 (22) and GSE56350 (23) datasets were obtained from the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/). GSE2509 contains the mRNA profile of 3 primary CRC samples and 3 samples with lymph node metastasis. These data were analyzed with the GPL96 [HG-U133A] Affymetrix Human Genome U133A Array platform version 2.0 (Affymetrix; Fisher Scientific, Inc., Waltham, MA, USA). Additionally, 46 primary CRC samples and 43 CRC samples with lymph node metastasis were obtained from the miRNA profile of GSE56350. Detection of the miRNA profile was performed using the PL16744 OSU-CCC Human and Mouse MicroRNA Microarray platform version 4.0 (Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA).

Data processing and differential expression analysis. The mRNA profile data were converted into recognizable format in R and then were normalized using the Robust Multi-Array Average algorithm from *Affy* version 1.40.0 package (<http://www.bioconductor.org/packages/2.13/bioc/html/affy.html>) (24). For the miRNA profile, the original expression value matrix was obtained and normalization was conducted using the *preprocessCore* function package version 3.5 (<http://www.bioconductor.org/packages/release/bioc/html/preprocessCore.html>) (25). Subsequently, the differentially expressed genes (DEGs) and differentially expressed miRNAs (DEMs) were identified in the CRC samples with lymph node metastasis compared with the primary CRC samples using the *limma* version 3.18.13 software package (<http://www.bioconductor.org/packages/2.13/bioc/html/limma.html>) (26). $P < 0.05$ and $\log_2(\text{fold-change}) > 0.2$ were used as threshold criteria.

Functional and pathway enrichment analysis of DEGs. The Database for Annotation, Visualization and Integrated Discovery version 6.8 (<https://david.ncicrf.gov/>) (27) was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs. GO terms and KEGG pathways with $P < 0.05$ were selected.

Construction of the miRNA-gene network. The miRWalk version 2.0 database (mirwalk.uni-hd.de/) is a publicly available comprehensive resource containing the predicted and the experimentally validated miRNA-target interaction pairs (28). The DEM-associated predicted target genes were selected when they were included in at least four out of five databases (miRanda-rel2010, miRDB version 4.0, miRWalk version 2.0, RNA22 version 2.0 and TargetScan version 6.2). Subsequently, the overlapping target genes were identified and the miRNA-gene pairs and DEMs were selected. The miRNA-gene network was formed and visualized using the Cytoscape version 3.5.1 (29) software (<http://www.cytoscape.org/download.php>).

Functional enrichment analysis of predicted target genes regulated by DEMs. The *clusterProfiler* version 3.5 (

Figure 1. Heat map of differentially expressed genes.

[bioconductor.org/packages/release/bioc/html/clusterProfiler.html](http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html)), an R package, was used to perform biological-term classification and enrichment analysis of gene clusters (30). Following the identification of the predicted target genes regulated by DEMs, enrichment analysis was performed and the enriched pathways with a P-value of < 0.05 were selected.

Results

Differentially expressed mRNAs and differentially expressed miRNAs. A total of 544 DEGs (227 upregulated and 317 downregulated) were identified, and the heat map of hierarchical clustering is presented in Fig. 1. Additionally, 42 DEMs (25 upregulated and 17 downregulated) were identified. The 20 most significant DEGs and the 20 most significant DEMs are presented in Tables I and II, respectively.

Enriched GO terms and KEGG pathways of differentially expressed mRNAs. DEGs were enriched in 320 GO terms and 11 KEGG pathways. The 10 most significant enriched GO terms and the enriched KEGG pathways are presented in Tables III and IV, respectively.

miRNA-gene pairs and miRNA-gene network. A total of 366 miRNA-gene pairs among the overlapped genes with the DEMs were selected, and the miRNA-gene network was generated and analyzed. The network is presented in Fig. 2 and the 20 highest degree nodes are presented in Table V. The term 'degree' represented connections of one node with other nodes.

Enriched pathways of predicted target genes. In total 271, 237, 192, 275 and 120 genes were respectively regulated by each of the 5 miRNAs *hsa-miR-106a*, *hsa-miR-15a*, *hsa-miR-16*,

Table I. 20 most significant differentially expressed genes in colorectal cancer samples with lymph node metastasis.

Gene	P-value	log ₂ FC
<i>RNF128</i>	4.07x10 ⁻¹⁵	4.435925
<i>CNN3</i>	6.05x10 ⁻¹⁵	4.696778
<i>WNT5A</i>	8.42x10 ⁻¹⁵	-5.04177
<i>TOX3</i>	9.65x10 ⁻¹⁵	5.640899
<i>FZD10</i>	1.13x10 ⁻¹⁴	3.438707
<i>IGFBP3</i>	3.26x10 ⁻¹⁴	-4.23336
<i>AKR1C3</i>	4.50x10 ⁻¹⁴	4.643379
<i>TRIP6</i>	5.04x10 ⁻¹⁴	-3.79486
<i>NPC2</i>	5.37x10 ⁻¹⁴	-3.86253
<i>KRT13</i>	8.49x10 ⁻¹⁴	-5.41489
<i>KRT23</i>	8.91x10 ⁻¹⁴	-4.84643
<i>NGFR</i>	9.23x10 ⁻¹⁴	-3.8684
<i>KRT81</i>	9.38x10 ⁻¹⁴	-3.54854
<i>CXCR4</i>	1.25x10 ⁻¹³	-3.41813
<i>ANXA6</i>	1.29x10 ⁻¹³	-3.29244
<i>MSX1</i>	1.64x10 ⁻¹³	-3.64506
<i>EFNB2</i>	1.76x10 ⁻¹³	3.675191
<i>SLC2A3</i>	1.77x10 ⁻¹³	-4.2354
<i>IGFBP7</i>	2.18x10 ⁻¹³	-3.27699
<i>GNG11</i>	2.77x10 ⁻¹³	-5.77814

FC, fold-change.

Table II. 20 most significant differentially expressed miRNAs in colorectal cancer samples with lymph node metastasis.

miRNA	P-value	log ₂ FC
<i>hsa-miR-342-3p</i>	5.2x10 ⁻¹⁰	1.3803
<i>hsa-miR-150</i>	1.31x10 ⁻⁹	1.5415
<i>hsa-miR-155</i>	2.73x10 ⁻⁷	1.5434
<i>hsa-miR-92b</i>	6.19x10 ⁻⁷	-1.6535
<i>hsa-miR-375</i>	4.04x10 ⁻⁶	-1.8421
<i>hsa-miR-142-5p</i>	3.72x10 ⁻⁶	1.752
<i>hsa-miR-453</i>	8.88x10 ⁻⁶	-1.1169
<i>hsa-miR-622</i>	1.47x10 ⁻⁵	-1.4782
<i>hsa-miR-595</i>	6.48x10 ⁻⁵	-1.0289
<i>hsa-miR-629</i>	8.46x10 ⁻⁵	-1.1146
<i>hsa-mir-621</i>	1.04x10 ⁻⁴	-1.2234
<i>hsa-miR-26a</i>	1.19x10 ⁻⁴	2.4369
<i>hsa-miR-146a</i>	1.51x10 ⁻⁴	2.1241
<i>hsa-miR-26b</i>	1.56x10 ⁻⁴	2.7188
<i>hsa-miR-200b</i>	2.05x10 ⁻⁴	-1.1071
<i>hsa-miR-146b-5p</i>	2.18x10 ⁻⁴	1.9222
<i>hsa-miR-107</i>	2.91x10 ⁻⁴	2.1441
<i>hsa-miR-560</i>	3.29x10 ⁻⁴	-1.4887
<i>hsa-mir-766</i>	3.33x10 ⁻⁴	-1.1362
<i>hsa-miR-103</i>	4.97x10 ⁻⁴	2.1098

miRNA/miR, microRNA; FC, fold-change; hsa, *Homo sapiens*.

Table III. 10 most significant enriched GO terms of differentially expressed microRNAs.

Category	GO ID	GO name	Count	P-value
BP	GO:0001944	Vasculature development	29	1.30x10 ⁻⁸
BP	GO:0001568	Blood vessel development	28	3.08x10 ⁻⁸
BP	GO:0016477	Cell migration	27	1.22x10 ⁻⁶
BP	GO:0048514	Blood vessel morphogenesis	22	5.54x10 ⁻⁶
BP	GO:0051674	Localization of cell	27	8.67x10 ⁻⁶
BP	GO:0048870	Cell motility	27	8.67x10 ⁻⁶
BP	GO:0042127	Regulation of cell proliferation	50	9.42x10 ⁻⁶
BP	GO:0051094	Positive regulation of developmental process	25	1.40x10 ⁻⁵
BP	GO:0006928	Cell motion	35	1.46x10 ⁻⁵
BP	GO:0045597	Positive regulation of cell differentiation	22	1.95x10 ⁻⁵

GO, Gene Ontology; BP, biological process.

hsa-miR-20a and *hsa-miR-29b*, respectively. Overall, 12 pathways were enriched and the results are presented in Fig. 3.

Discussion

In the present study, DEGs and DEMs were initially identified in colorectal cancer samples with lymph node metastasis compared with primary colorectal cancer samples. Subsequently, the functional and pathway enrichment analysis of DEGs and the predicted target genes regulated by DEMs was performed. The

over-represented pathways were associated with pathways in cancer, the Wnt signaling pathway and extracellular matrix (ECM)-receptor interaction. The major enriched pathway of DEMs was pathways in cancer characterized by the lowest P-values (Table IV). Overall, ~12% of predicted target genes regulated by *hsa-miR-15a*, *hsa-miR-16* and *hsa-miR-20a* were associated with this pathway; suggesting that it serves a critical role in CRC metastasis.

Wnt signaling is involved in embryonic development (31) and a number of studies demonstrated that aberrant Wnt

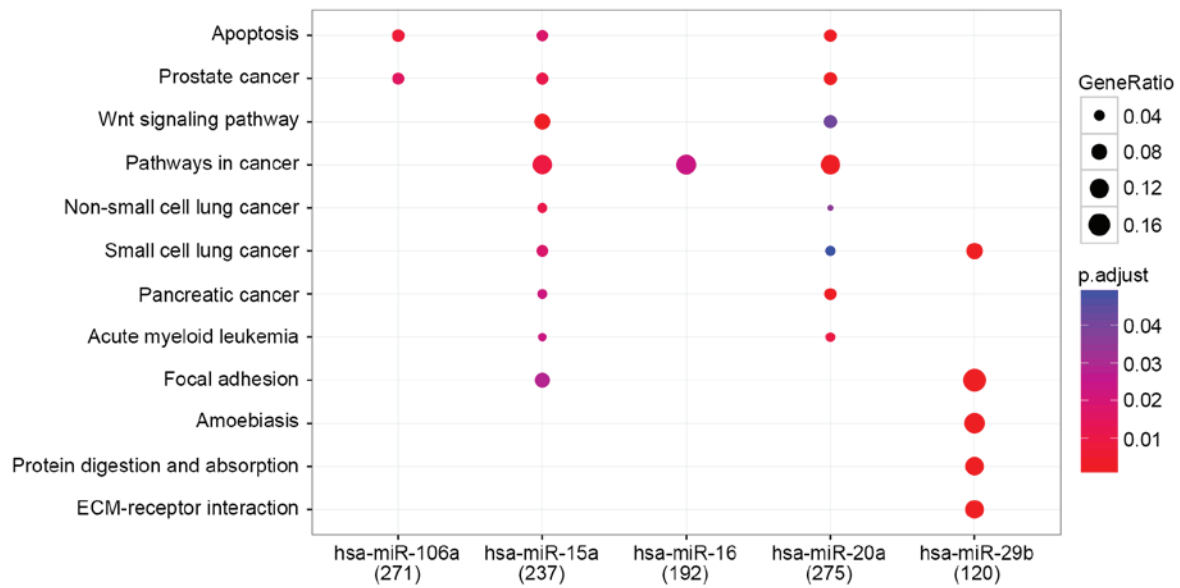


Figure 3. Enriched pathways of predicted target genes regulated by differentially expressed miRNAs. miRNA/miR, microRNA; ECM, extracellular matrix.

Table V. 20 highest degree nodes in the miRNA-gene network.

Node	Degree
<i>hsa-miR-20b</i>	38
<i>hsa-miR-20a</i>	36
<i>hsa-miR-106a</i>	33
<i>hsa-miR-23a</i>	32
<i>hsa-miR-200b</i>	30
<i>hsa-miR-17</i>	26
<i>hsa-miR-142-5p</i>	24
<i>hsa-miR-15a</i>	23
<i>hsa-miR-27a</i>	22
<i>hsa-miR-107</i>	17
<i>hsa-miR-103</i>	15
<i>hsa-miR-16</i>	15
<i>hsa-miR-29b</i>	14
<i>hsa-let-7g</i>	11
<i>hsa-miR-150</i>	9
<i>FNDC3B</i>	8
<i>CRIM1</i>	7
<i>FOXJ2</i>	7
<i>hsa-miR-146a</i>	7
<i>hsa-miR-155</i>	7

hsa, *Homo sapiens*; miRNA/miR, microRNA; *FNDC3B*, fibronectin type III domain-containing 3B; *CRIM1*, cysteine rich transmembrane BMP regulator 1; *FOXJ2*, forkhead box J2.

The ECM regulates tissue architecture and adipogenesis, which involves a complex mixture of structural and functional macromolecules, including glycosaminoglycans and fibrous proteins (36). One recent study revealed that twist-related protein 2 (Twist2) regulates the expression of integrin α -4 and CD44 antigen, two major proteins in the ECM-receptor

interaction pathway (37). Furthermore, it was also demonstrated that the overexpression of Twist2 may be involved in cell growth regulation, apoptosis and motility, and that Twist2 may serve as a potential therapeutic target for the treatment of kidney cancer (37). Additionally, twist family BHLH transcription factor 2 was significantly overexpressed in several solid tumors and contributed to tumor progression (38). The results of the present study suggest that ECM-receptor interaction may be associated with CRC metastasis, however, further research is required to validate this association.

miRNA-gene network analysis revealed that fibronectin type III domain-containing 3B (*FNDC3B*), cysteine rich transmembrane BMP regulator 1 (*CRIM1*) and forkhead box J2 (*FOXJ2*) were the genes with the highest degree (Table V). It has been demonstrated that *FNDC3B* is a positive regulator of adipocyte differentiation, and that it suppresses the invasion and metastasis of melanoma cells (39). *FNDC3B* mutations were associated with rapid postnatal death and the inhibition of cellular proliferation, adhesion and migration (40). *FNDC3B* has been associated with the activation of several cancer pathways and tumor progression (41). *CRIM1* encodes the cysteine-rich motor neuron 1 protein (CRIM1), which has been characterized as a potential cancer biomarker (42). It has been reported that increased CRIM1 inhibits the proliferation and migration of vascular endothelial cells (43). Additionally, increased *CRIM1* expression has been reported in drug-resistant myeloid leukemia cells compared with drug-sensitive cells (44). *FOXJ2* serves an important role in the migration of glioma cells (45) and *FOXJ2* overexpression decreases the migration of breast cancer cells (46). Furthermore, abnormal expression of *FOXJ2* suppressed migration and invasion in extrahepatic cholangiocarcinoma, which was associated with an improved prognosis (47). *FNDC3B*, *CRIM1* and *FOXJ2* have been associated with tumor migration and prognosis. The findings of the present study suggest that they may also be associated with CRC metastasis.

In conclusion, the present study demonstrated that DEGs and predicted target genes of the DEMs are enriched

in pathways in cancer, the Wnt signaling pathway and ECM-receptor interaction, which may serve a critical role in the metastatic mechanism of CRC. Furthermore, *FNDC3B*, *CRIMI* and *FOXJ2* are proposed as potential biomarkers for metastatic CRC.

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