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# Identification of Molecular Targets for Predicting Colon Adenocarcinoma

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**Background:** Colon adenocarcinoma mostly happens at the junction of the rectum and is a common gastrointestinal malignancy. Accumulated evidence has indicated that colon adenocarcinoma develops by genetic alterations and is a complicated disease. The aim of this study was to screen differentially expressed miRNAs (DEMs) and genes with diagnostic and prognostic potentials in colon adenocarcinoma.



**Material/Methods:** In this study we screened DEMs and their target genes (DEGs) between 100 colon adenocarcinoma and normal samples in The Cancer Genome Atlas (TCGA) database by using the DEseq toolkit in Bioconductor. Then Go enrichment and KEGG pathway analysis were performed on the selected differential genes by use of the DAVID online tool. A regulation network of miRNA-gene was constructed and analyzed by Cytoscape. Finally, we performed ROC analysis of 8 miRNAs and ROC curves were drawn.

**Results:** A total of 159 DEMs and 1921 DEGs were screened, and 1881 pairs of miRNA-target genes with significant negative correlations were also obtained. A regulatory network of miRNA-gene, including 60 cancer-related genes and 47 miRNAs, was successfully constructed. In addition, 5 clusters with several miRNAs regulating a set of target genes simultaneously were identified through cluster analysis. There were 8 miRNAs involved in these 5 clusters, and these miRNAs could serve as molecular biomarkers to distinguish colon adenocarcinoma and normal samples indicated by ROC analysis.

**Conclusions:** The identified 8 miRNAs were closely associated with colon adenocarcinoma, which may have great clinical value as diagnostic and prognostic biomarkers and provide new ideas for targeted therapy.

**MeSH Keywords:** **Adenocarcinoma • Gene Expression Profiling • Gene Regulatory Networks**

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## Background

Colon adenocarcinoma ranks the third leading cause of cancer mortality worldwide and the annually death number is increasing. Although the current adjuvant treatment modalities may improve survival for some colon adenocarcinoma patients to a certain extent, others benefit little [1]. Moreover, recently accumulated evidence indicates that colon adenocarcinoma is a genetically heterogeneous and complicated disease caused by abnormalities in gene structure and/or expression [2]. Therefore, it is important to identify novel biomarkers with diagnostic and prognostic potentials in colon adenocarcinoma and to develop new targeted therapy.

Recently, MicroRNAs (miRNAs) have become a focus in cancer research. miRNAs are a class of endogenous, small, noncoding RNA molecules (18–25 nucleotides), able to repress protein translation by binding to the target mRNAs [3]. miRNAs have critical regulatory roles in several basic cellular processes, such as differentiation and proliferation, which may affect some major biological systems, like cancer [4]. In addition, miRNAs can target up to hundreds of mRNAs, which makes them very powerful regulators. Aberrant miRNA expression can disturb a multitude of cell signaling pathways and profoundly influence cancer onset and progression. Changes in the expression of miRNAs have been observed in a variety of human tumors, including colon adenocarcinoma [5]. Currently, there are 2 major approaches applied to explore the relationships between miRNAs and colon adenocarcinoma. One is that miRNAs can regulate many known oncogenic and tumor suppressor pathways involved in the pathogenesis of colon adenocarcinoma. For example, p53, a well-known tumor suppressor gene, has been found to be mutated in about 40–50% of colon adenocarcinoma [6]. Recent studies have proved there are important connections between miRNAs and p53 [7]. The other is that expression profiles of various miRNAs may show potentials as biomarkers for prediction of prognosis and a distinction of certain diseases, including colon adenocarcinoma. It was reported that patients with overexpressed miR-21 have worse survival prognosis for stage II or stage III colon adenocarcinoma [5,8].

In this study, we analyzed the expression data of miRNAs of colon adenocarcinoma in The Cancer Genome Atlas (TCGA) database and screened the differentially expressed miRNAs (DEMs) and their target genes (DEGs). Through constructing a regulation network of miRNA-gene by Cytoscape, we hoped to discover new biomarkers with diagnostic and prognostic potentials and contribute to the diagnosis and therapy of colon adenocarcinoma.

## Material and Methods

### miRNA data

The colorectal adenocarcinoma miRNASeq and level 3 RNAseq data were downloaded from the TCGA database. There were a total of 228 samples, including 220 colon adenocarcinoma and 8 normal samples; these data were classified into 2 cohorts. Each sample consisted of the corresponding miRNA-seq and RNA-seq data. The sequenced data generated from IlluminaHiSeq\_miRNASeq and IlluminaHiSeq\_RNASeq sequencing platforms were all publicly available data; therefore, no ethical issues were involved. The study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of Kunming Medical University.

### Data preprocessing

Because the data came from a public platform, sample source, development degree of cancer, and cancer subtypes may affect the subsequent analysis. Therefore, the samples were firstly analyzed by Hierarchical Clustering algorithm and then grouped based on gene expression (RSEM value) level [9]. Tumor samples with similar gene molecular type were selected and miRNA data corresponding to samples were further screened.

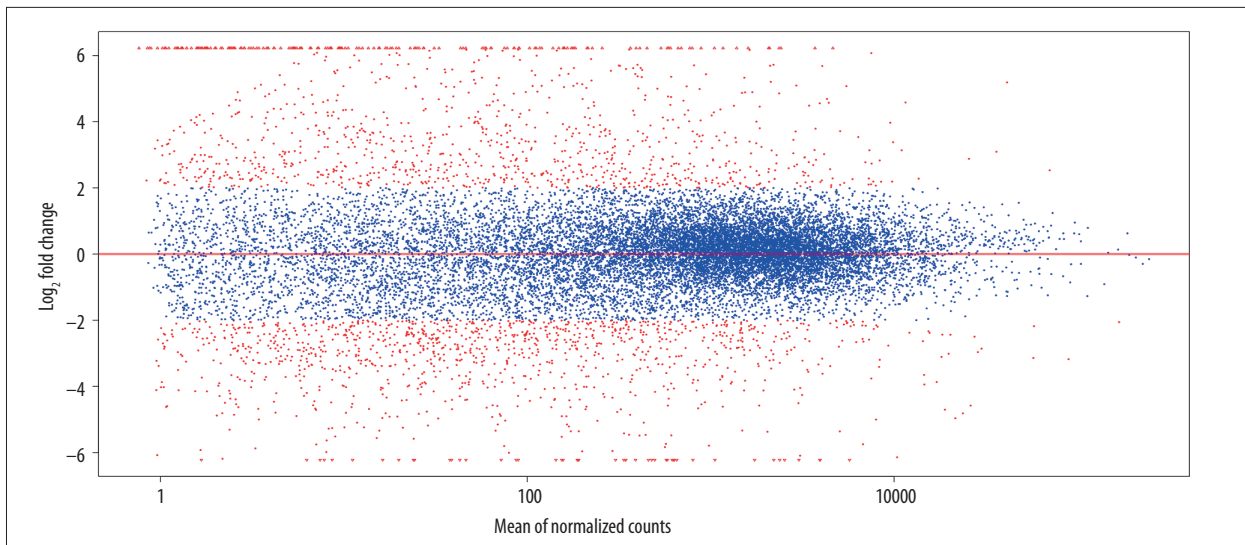
After tumor sample and normal sample data were combined, the miRNA data and gene data with no expression were filtered, so as to ensure there was at least 1 count average in each sample for every participated miRNA and gene.

### Differential expression analysis

The cancer samples and normal samples in each group were all subjected to differential expression analysis using DESeq toolkits in Bioconductor [10]. Then we carried out differential expression analysis after standardization and variance estimation. The standards for screening the expression differences of miRNA and gene were  $P < 0.001$ ,  $FDR < 0.001$ , and  $|\log_2 FC| > 2$ .

### Screening of target genes

We predicted and screened the candidate target genes of DEMs using v6.2 TargetScan Human Database [11]. For the predicted “miRNA-target gene” relationships, we calculated the Pearson correlation coefficient between their gene expression levels according to the expression data [12]. Due to the inhibition of miRNA on the expression of its target genes, the “miRNA-target gene” relationship that the expression of target gene was significantly negatively associated with miRNA should have biological significance. The screening criterion was correlation coefficient  $r < -0.3$ ,  $P < 0.05$ .



**Figure 1.** The expression and multiple relationships of differentially expressed genes between tumor samples and normal samples. The ordinate shows the multiple relationships of gene expression changes after  $\log_2$  conversion, while the abscissa shows gene count value. Red dots represent genes with  $|\log_2 FC| > 2$ .

### Annotation of DEGs

The DAVID tool was utilized to execute GO enrichment, KEGG pathway analysis, annotation and analysis of related diseases on the DEGs. Benjamini method was used to conduct multiple testing correction, and  $P < 0.05$  was considered as the significantly enriched threshold [13].

### Construction of a miRNA-gene interaction network

As the interactions among differentially expressed cancer genes came from IntAct and reactome database, the data from 2 databases were merged according to the set of node and edge [14,15]. The relationships between target genes of the DEMs were integrated into the interaction network. Finally, the network topology was generated by Cytoscape 3.2.0 [16,17].

### Analysis of miRNA-gene interaction network

Some basic properties of the network, including Edge Count In degree, Out degree, Betweenness Centrality, Clustering Coefficient, and Closeness Centrality parameters, were analyzed by built-in tools in Cytoscape. The key nodes with different biological significance in the network were filtered based on these parameters.

Network clustering is an important means to identify functional modules, which not only helps understand the organizational structure of biological systems, but also contributes to predicting biomolecule functions. CytoCluster plugin can be used to identify gene clusters in the sub-network constructed by miRNA and its target gene (<http://apps.cytoscape.org/apps/>

*cytocluster*). IPCA algorithm was used in cluster identification with default parameters. The identified clusters were further filtered, and only the network clusters of the same target genes commonly regulated by more than 2 miRNAs were retained.

### Analysis of the receiver operating characteristic

We performed ROC analysis on 8 miRNAs in the commonly controlled network cluster, based on the miRNA expression data of patients by pROC packet in R language. The corresponding miRNA expression was arranged sequentially and patients' diseased states were marked (status, tumor sample was 1, normal sample was 0) one by one, the area under the ROC curve (AUC) was calculated and the ROC curve was drawn.

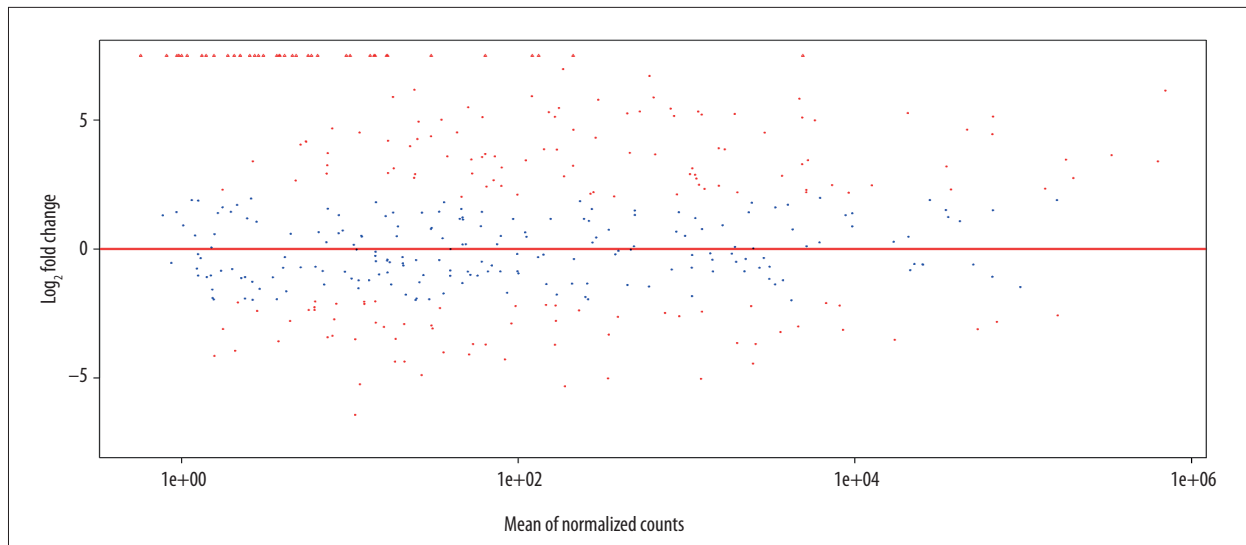
### Statistical analysis

Statistical analysis was done using SPSS software (version 17.0, SPSS, New York, NY, USA). All the data are presented as mean  $\pm$  standard deviation (SD), and comparison among the groups were done using a single-factor analysis of variance (one-way ANOVA).  $P < 0.05$  was considered as statistically significant.

## Results

### Data preprocessing

We conducted clustering analysis of the gene expression profile of 220 colon adenocarcinoma samples, and then selected a total of 100 samples consisting of a group of 92 tumor samples with similar molecular type and 8 normal samples, which



**Figure 2.** The expression and multiple relationships of differentially expressed miRNAs between tumor samples and normal samples. The ordinate shows the multiple relationships of miRNA expression changes after  $\log_2$  conversion, while the abscissa shows miRNA count value. Red dots represent miRNAs with  $|\log_2 FC| > 2$ .

contained 20 531 genes. We selected 705 miRNA data corresponding to the 100 samples for further analysis.

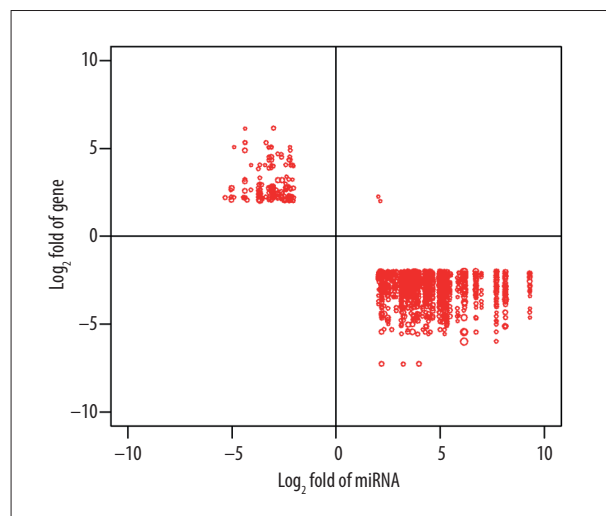
### Differential expression analysis

A total of 1921 genes showed significant expression differences ( $P < 0.01$ ,  $FDR < 0.01$ ). Compared to normal samples, 802 genes were up-regulated, while 1119 genes were down-regulated in cancer samples (Figure 1).

Differential expression analysis of the miRNA expression profiles showed there were 159 miRNAs exhibited significant differences ( $P < 0.01$ ,  $FDR < 0.01$ ). Comparing to normal samples, 96 miRNA were up-regulated while 63 down-regulated in cancer samples (Figure 2).

### Screening of miRNAs and target gene

We obtained 9146 pairs of “miRNA-target gene” interaction relationships through prediction of the target relations between DEMs and genes by TargetScan software. We screened 1881 pairs of “miRNA- target gene” interaction relationships with significantly negative correlations ( $r < -0.3$ ,  $P < 0.01$ ), with a total of 413 genes and 126 miRNAs involved. The differential relationships and correlation strength between miRNA and target gene are shown in Figure 3. The results indicate the “miRNA-target genes” relationships with significant correlations in tumor samples occurred between the up-regulated miRNAs and down-regulated genes.



**Figure 3.** The expression variations between miRNA and its target genes and their correlations. The ordinate and abscissa show the fold variation of target genes and miRNA, respectively. Dot size indicates the correlation coefficient between miRNA expression and its target genes.

### GO, KEGG and disease annotation

We used 1978 DEGs to perform disease annotation, among which 105 DEGs were associated with cancer, and 44 with colon adenocarcinoma or colorectal cancer; while in 413 target genes of miRNA, 26 were associated with cancer and 10 with colon adenocarcinoma or colorectal cancer (Table 1).

**Table 1.** Genes associated with miRNA regulated colorectal adenocarcinoma or colorectal cancer.

Genes	Change fold	Targeted miRNAs
ADRB2	-2.386	hsa-mir-98
CASR	-3.841	hsa-mir-15a, hsa-mir-16-1, hsa-mir-429
CXCL12	-2.356	hsa-mir-101-1, hsa-mir-135b, hsa-mir-19a, hsa-mir-19b-1, hsa-mir-19b-2, hsa-mir-454
CDKN2B	-3.868	hsa-mir-582
DPYD	-2.269	hsa-mir-32
ESR1	-2.005	hsa-mir-148a, hsa-mir-188, hsa-mir-18a, hsa-mir-190, hsa-mir-19a, hsa-mir-19b-1, hsa-mir-19b-2, hsa-mir-26b, hsa-mir-301a, hsa-mir-337, hsa-mir-454, hsa-mir-590, hsa-mir-660
IGF1	-2.203	hsa-mir-148a, hsa-mir-152, hsa-mir-203, hsa-mir-590
MMRN1	-2.763	hsa-mir-374a
MRC1	-2.061	hsa-mir-450b
PTGS1	-2.252	hsa-mir-148a, hsa-mir-152, hsa-mir-203, hsa-mir-590

Through KEGG annotation and enrichment analysis of the 413 target genes, we obtained 3 significantly enriched pathways, including calcium signaling pathway, neuroactive ligand-receptor interaction pathway, and axon guidance pathway.

Through GO annotation and enrichment analysis of the 413 target genes, we revealed the roles of gene products from biological processes, cellular localization, and molecular function). The most significant comment in enrichment analysis results showed the main aspect of biological processes was closely associated with ion transport; intracellular localization showed the gene products were mainly located on the membrane; molecular function also showed that the main function of the gene product was related to metal ion and calcium ion transportation. All these comments results coincided with that of the KEGG pathway.

#### Analysis of miRNA-Gene regulatory network for colon adenocarcinoma

A miRNA-Gene regulatory interaction network (Figure 4) consisting of differentially expressed genes was eventually constructed

after the data in IntAct and Reactome database were merged. The network contained a total of 107 nodes and 153 pairs of interaction relationships. The nodes contained a total of 47 miRNAs and 60 cancer-related genes, wherein 32 cancer genes were associated with colon adenocarcinoma or colorectal cancer. In this regulatory interaction network, 63 nodes were up-regulated (red) and 44 were down-regulated (green).

In a network, the nodes often connected to many peripheral nodes were referred to as hub nodes. In the miRNA-gene network of colon adenocarcinoma, sorted according to the descending number of connections, the top 10% of nodes were IGF1, ESR1, NR3C1, PGR, MMP1, F2, CXCL12, MMP3, hsa-mir-590, FGFR2, IGF2, and GFRA1. IGF1, ESR1, MMP1, F2, CXCL12, MMP3, IGF2 had already been identified as colon adenocarcinoma-related genes. Although NR3C1 and PGR were not annotated to be colon adenocarcinoma-related genes, they were also associated with other cancers, and their statuses in connection with other genes were second only to IGF1 and ESR1. Therefore, we speculated that these 2 genes had significant relationships with colon adenocarcinoma.

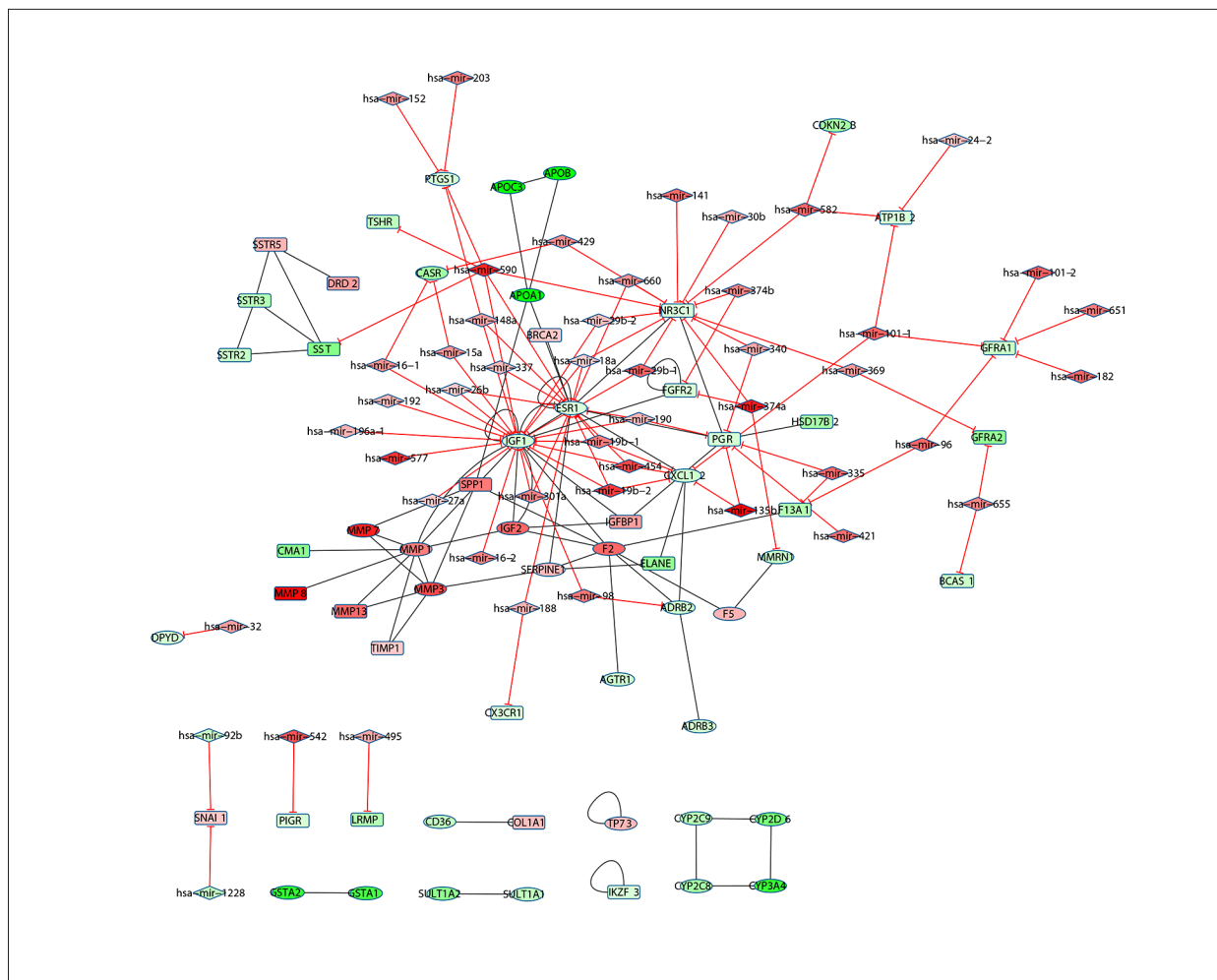
Five network clusters with many miRNAs regulating the target genes collectively were obtained by network cluster analysis (Figure 5), involving a total of 8 miRNAs, including hsa-mir-454, hsa-mir-19b-1, hsa-mir-19b-2, hsa-mir-590, hsa-mir-18a, hsa-mir-148a, hsa-mir-301a, and hsa-mir-26b. NR3C1 combined with IGF1 and ESR1 were regulated by hsa-mir-590 and hsa-mir-18a collectively, indicating that NR3C1 may be also associated with colon adenocarcinoma.

#### ROC analysis

The ROC analysis of 8 miRNAs that synergistically regulated colon adenocarcinoma-related genes showed they were suitable to be used as bio-molecular markers to distinguish tumor and normal samples. Sorted according to the descending size of AUC, they were hsa-mir-19b-2 (1.000), hsa-mir-454 (0.997), hsa-mir-19b-1 (0.995), hsa-mir-590 (0.995), hsa-mir-148a (0.991), hsa-mir-26b (0.980), hsa-mir-301a (0.962), and hsa-mir-18a (0.794).

#### Discussion

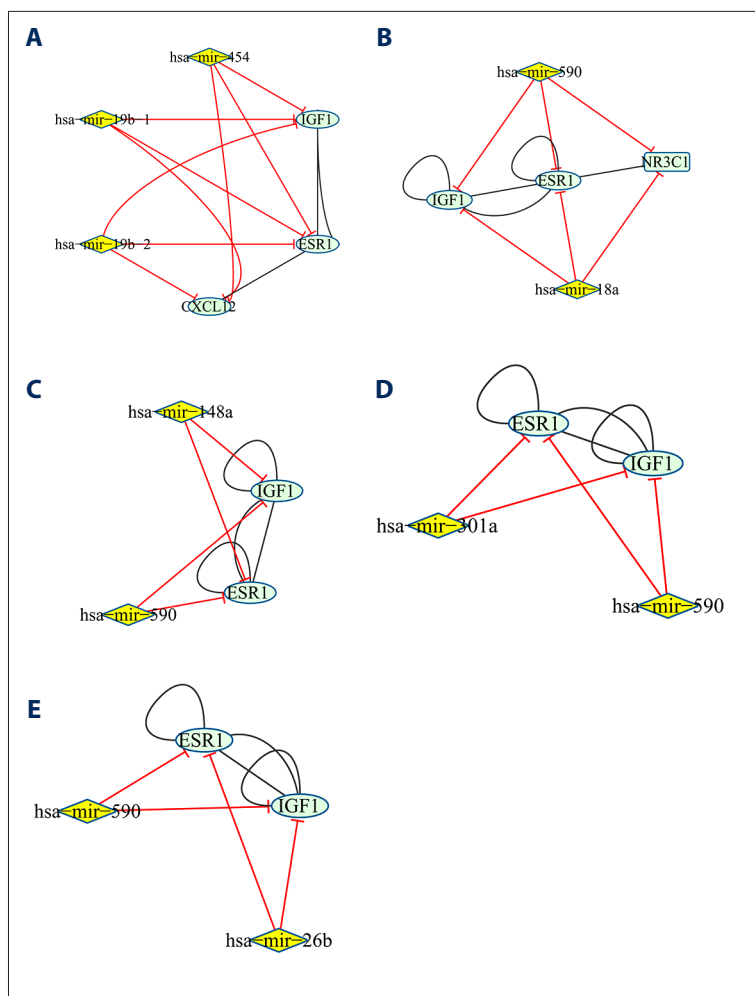
In this study, we constructed a miRNA-gene regulatory network through analysis of the differentially expressed miRNAs and their target genes between colon adenocarcinoma and normal samples. GO annotation and KEGG pathway analysis showed these target genes were mainly enriched in ion, especially calcium ion (Ca<sup>2+</sup>) transportation. Ca<sup>2+</sup> can regulate various cellular processes through activating or inhibiting cellular signaling pathways and many Ca<sup>2+</sup>-mediated signaling pathways



**Figure 4.** miRNA-Gene interaction network of colon adenocarcinoma. The diamond nodes represent colon adenocarcinoma-related miRNAs and the rounded rectangle nodes represents other cancer-related miRNAs. The side with the red T-shaped arrow indicates the regulation relationship between miRNA and gene, and the black edge indicates the interaction between gene products. The colors of nodes indicate the fold change of miRNA or gene expression; green means down-regulated and red means up-regulated.

are involved in tumorigenesis, such as invasion and metastasis [18]. It was reported the aberrant expression of some  $Ca^{2+}$  channels or pumps may be associated with tumorigenesis. For example, TRPM8 is over-expressed in prostate cancer while SERCA3 is down-regulated in colon adenocarcinoma [19]. Considering the ability of  $Ca^{2+}$  signaling to regulate pathways like proliferation or apoptosis, alteration of  $Ca^{2+}$  channels or pumps in cancers may be considered as therapeutic targets. Furthermore, many pharmacological agents can modulate  $Ca^{2+}$  channels and pumps; thus, they are regarded as druggable. Previous studies also found that changes in the ER  $Ca^{2+}$  pump SERCA3 were extensively characterized in colon adenocarcinoma [20,21], which suggests that  $Ca^{2+}$  pump or the genes regulating them could serve as anticancer therapeutic targets for colon adenocarcinoma.

In the constructed miRNA-gene network, several genes, including IGF1, ESR1, MMP1, and F2, were already known as colon adenocarcinoma-related genes. For example, insulin-like growth factor-1 (IGF-1) is one of the factors contributing to the increased risk in colon cancer [22]. IGF-1 can activate many signaling pathways involved in carcinogenesis, such as PI3K/Akt, MAPK, and STAT3 [23]. Inhibition of these pathways may have preventive and therapeutic potential. Estrogen receptor-alpha (ESR1) was also shown to increase colon cancer cell proliferation and increase cancer incidence. Although NR3C1 and PGR genes were not annotated to be colon adenocarcinoma-related, they were associated with other cancers. The nuclear receptor subfamily 3, group C, member 1 (NR3C1) is a gene that exists in the cytoplasm in a multiprotein complex, and it can encode glucocorticoid receptor. The expression level of NR3C1 was an important factor for glucocorticoid

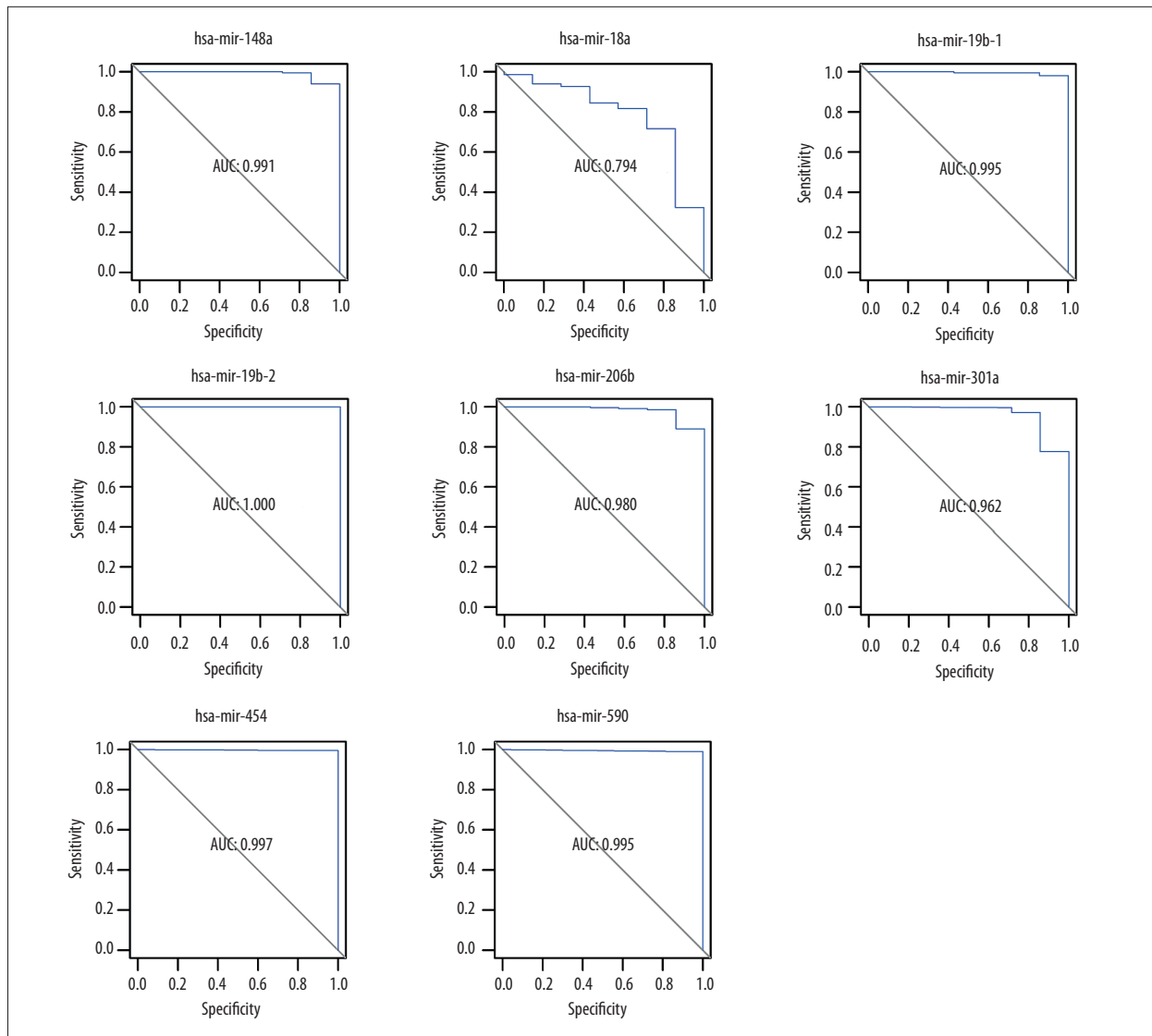


**Figure 5.** The clusters of target genes jointly regulated by miRNA in miRNA-Gene network. Yellow node and blue green node represent miRNA and target gene, respectively. The red edge represents the relationship between miRNA and gene, while the black edge represents the interaction relationships of gene products.

sensitivity [24]. Evidence shows that NR3C1 displayed methylation in carcinomas and some adenomas, and it was identified as a novel target gene inactivated by promoter hypermethylation during colorectal cancer development, indicating it may appear occasionally in the early events in colorectal cancer development [25]. PGR (progesterone receptor) status was also shown to exert a significant impact on prognosis of ER+/HER2- breast cancer [26]. It was found that PGR positivity correlated with response to tamoxifen therapy, both in the adjuvant setting and metastatic setting. Thus, the PGR status could serve as an independent predictive factor for survival [27–29]. In addition, NR3C1 and PGR statuses in connection to other genes were second only to IGF1 and ESR1. Therefore, we speculated that these 2 genes may also have significant relationships with colon adenocarcinoma.

Finally, we obtained 8 miRNAs (hsa-mir-454, hsa-mir-19b-1, hsa-mir-19b-2, hsa-mir-590, hsa-mir-18a, hsa-mir-148a, hsa-mir-301a, and hsa-mir-26b) regulating colon adenocarcinoma-related genes and they may be considered as biomarkers for distinguishing colon tumor samples and normal samples.

miR-590 was found to be a complement region of RhoB gene [30]. RhoB is known to show tumor suppressor activity, and its down-regulation was related to many changes, such as migration and adhesion in aggressive tumors. Studies indicated that RhoB could be considered as an important target for miR-590 in regulating cell proliferation and invasion in colorectal cancer [31]. He et al. reported that in clinical colorectal cancers, the expression level of miR-17-92 was up-regulated in 15% of tested samples and showed 4-fold up-regulation of the corresponding normal tissues [32]. Moreover, it was demonstrated that patients with high expression of miR-18a tended to have a poorer prognosis than patients with expression group [33]. Therefore, the expression of miR-18a was believed to be a prognostic factor for predicting survival of colorectal cancer patients. As in the expression of miR-18a, metastatic lymph node ratio is also an important prognostic factor of colorectal cancer patients. Expression of miR-18a at metastatic lymph nodes may decrease the prognosis [34]. Moreover, NR3C1 combined with IGF1 and ESR1 were all regulated by hsa-mir-590 and hsa-mir-18a, further indicating that NR3C1 was related to colon adenocarcinoma.



**Figure 6.** ROC curve analysis results of the miRNA that synergistically regulated colon adenocarcinoma-related genes. The ordinate and abscissa show specificity and sensitivity, respectively. AUC was the area under the ROC curve, and the ability of miRNA as a molecular marker showed positive correlation to AUC size.

## Conclusions

We successfully constructed a miRNA-Gene regulatory network, which involved 60 cancer-related genes and 47 miRNAs. Eight

miRNAs were found to play important roles in regulating hub nodes in the network and these 8 miRNAs could serve as biomarkers to identify colon adenocarcinoma.

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