Mechanism analysis of colorectal cancer according to the microRNA expression profile

HONG LI, HUICHAO ZHANG, GANG LU, QINGJING LI, JIFENG GU,
YUAN SONG, SHEJUN GAO and YAWEN DING

Department of Clinical Laboratory, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei 050035, P.R. China

Abstract. The present study aimed to identify specific microRNAs (miRs) and their predicted target genes to clarify the molecular mechanisms of colorectal cancer (CRC). An miR expression profile (array ID, GSE39833), which consisted of 88 CRC samples with various tumor-necrosis-metastasis stages and 11 healthy controls, was downloaded from the Gene Expression Omnibus database. Subsequently, the differentially expressed miRs and their target genes were screened. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathways of target genes were analyzed using the Database for Annotation Visualization and Integrated Discovery. A protein-protein interaction (PPI) network of the target genes was constructed using the Search Tool for the Retrieval of Interacting Genes database. The present study identified a total of 18 differentially expressed miRs (upregulated, 8; downregulated, 10) in the sera of the CRC patients compared with the healthy controls. Of these, 3 upregulated (let-7b, miR-1290 and miR-126) and 2 downregulated (miR-16 and miR-760) differentially expressed miRs and their target genes, including cyclin D1 (CCND1), v-myc avian myelocytomatosis viral oncogene homolog (MYC), phosphoinositide-3-kinase, regulatory subunit 2 (beta) (PIK3R2) and SMAD family member 3 (SMAD3), were significantly enriched in the CRC developmental pathway. All these target genes had higher node degrees in the PPI network. In conclusion, let-7b, miR-1290, miR-126, miR-16 and miR-760 and their target genes, CCND1, MYC, PIK3R2 and SMAD3, may be important in the molecular mechanisms for the progression of CRC.

Introduction

Colorectal cancer (CRC) is a global health challenge with >1.2 million novel cases and 600,000 mortalities every year (1).

Survival rates of CRC greatly depend on the stage at primary diagnosis, and the 5-year survival rate is 93 and 8% for stage I and IV, respectively (2). Systematic methods for diagnosis, including flexible sigmoidoscopy and implementation of the fecal occult blood test, may contribute to a high detection rate of patients in the early stages of CRC, thus reducing mortality rates (3). There are numerous screening methods, drugs and chemotherapy regimens, including irinotecan and oxaliplatin, available; however, the majority of these have low sensitivity and treatment-limiting side effects (4-6). Therefore, the development of highly sensitive and CRC-specific diagnostic and prognostic biomarkers is required for the diagnosis and treatment of early stage CRC.

MicroRNAs (miRs) have emerged as important molecules involved in tumorigenesis and disease progression and metastasis in various cancers, including CRC (7). miRs are endogenous, short non-coding RNAs that effectively inhibit target gene activity by binding to target mRNAs and hindering their translation (8). Each miR achieves functional specificity by targeting a core network of genes and has potential effects on almost every genetic pathway (9). Increasing evidence suggests that aberrant miR expression is clearly associated with the initiation and progression of certain cancers, and miRs may act as oncogenes and/or tumor suppressor genes (10). miR-21 downregulates tumor suppressor programmed cell death 4 at a post-transcriptional level and promotes the invasion, intravasation and metastasis of CRC (11). miR-335 exhibits anti-tumoral activity in various tumors, including gastric cancer (12), ovarian cancer (13) and CRC (14). Therefore, pivotal miRs may be informative biomarkers for detection, diagnosis and prognosis of various types of cancer. Furthermore, miRs have emerged as highly tissue-specific biomarkers (15,16), and previous studies have paved the way for miR-based cancer tissue classification (17,18). However, miRs associated with CRC and their roles in the molecular pathogenesis of CRC remain unclear.

To date, one of the most interesting findings is that secreted miRs potentially influence target cell function via exosomes (19). Exosomes are small (30-90 nm) membrane vesicles that carry mRNAs, proteins, lipids and miRs, depending on the origin of the secreting cells (20,21). Previous studies have demonstrated that exosomes are the newest family members of 'bioactive vesicles' that promote intercellular communication and immunoregulatory processes via

Correspondence to: Dr Shejun Gao, Department of Clinical Laboratory, The Fourth Hospital of Hebei Medical University, 169 Tianshan Street, Shijiazhuang, Hebei 050035, P.R. China
E-mail: shejungaosjg@163.com

shuttling molecules between cells (22,23). miRs carried in exosomes are secreted from cancer cells into body fluids, such as blood, urine, breast milk and saliva (21). Therefore, exosomal miRs in body fluids may be useful diagnostic and prognostic biomarkers of cancers (24). However, research on the association between exosomal miR profiles in blood and the pathological condition of cancer patients are limited.

In a previous study, the exosomal miR profile (GSE39833) (25) has been used to highlight the most abundant miRs in the blood of patients with CRC using an arbitrary boundary. *In silico* analysis of these miRs was performed to explore the function of miRs as tumor surveillance mechanisms exerting continuous inhibition on tumor formation. There was no systematic and comprehensive analysis for the miR functions in CRC. Therefore, the present study used microarray analysis to screen the differentially expressed miRs in the sera of patients with CRC. Additionally, in order to improve the understanding of miR functions, the predicted target genes of miRs were identified. Comprehensive bioinformatics methods were used to investigate the function and pathways of target genes of miRs and construct a protein-protein interaction (PPI) network to identify the hub target genes and miRs associated with CRC. The present study aimed to investigate the key role of potentially important miRs and their target genes in the initiation and progression of CRC. The present study may provide a basis for screening novel biomarkers of CRC for therapeutic interventions.

Materials and methods

Microarray data of miRs. The miR expression profile was downloaded from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/) using the series accession number GSE39833, which was deposited by Ogata-Kawata *et al* (3). The GSE39833 dataset consisted of 88 CRC patients with various tumor-necrosis-metastasis stages (stage I, 20 patients; II, 20 patients; IIIa, 20 patients; IIIb, 16 patients; IV, 12 patients) between the ages of 45-65 years and 11 healthy controls. An Agilent Human miRNA Microarray platform was used (product no., G4470C; design ID, 021827; Agilent Technologies, Inc., Santa Clara, CA, USA).

Data preprocessing and screening of differentially expressed miRs. Limma (26) (version 2.7.10; bioconductor.org/packages/release/bioc/html/limma.html) package in R (version 2.3.1; www.r-project.org/) was used for data preprocessing and normalization. A T-test (26) in Limma package was used to identify the significantly differentially expressed miRs in the CRC group compared with the healthy control group. P<0.05 and |log2 fold change|>1.5 were considered to indicate a statistically significant difference.

Prediction of the target genes for miRs. Identification of the predicted target genes of miRs is conducive to improve the understanding of miR functions. miRecords (version 3; c1.accurascience.com/miRecords/) and miRWalk (verison 2.0; zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) were used to retrieve the target genes of the differentially expressed miRs. miRecords (27), an integrated database for miR-target interactions (MTIs), hosts a large, high-quality, manually curated

Table I. Differentially expressed miRs between colorectal cancer and healthy control samples.

A, Upregulated differentially expressed miRs

miR	Log2, FC	P-value
hsa-let-7b	0.58796649	2.89×10^{-3}
hsa-miR-1246	2.59391919	7.14×10^{-10}
hsa-miR-126	3.16477901	6.27×10^{-9}
hsa-miR-1290	1.32767795	3.49×10^{-5}
hsa-miR-181b	1.08748377	3.88×10^{-2}
hsa-miR-181d	1.41294860	2.45×10^{-2}
hsa-miR-23a	1.87225376	6.94×10^{-8}
hsa-miR-654-5p	1.35980450	1.25×10^{-3}

B, Downregulated differentially expressed miRs

miR	Log2, FC	P-value
hsa-miR-1225-5p	-0.81943754	9.73×10^{-4}
hsa-miR-1287	-0.72720715	2.84×10^{-3}
hsa-miR-1295	-1.44266151	5.07×10^{-5}
hsa-miR-1299	-1.22393055	4.15×10^{-3}
hsa-miR-144	-2.48669140	1.47×10^{-9}
hsa-miR-16	-1.35722402	2.42×10^{-5}
hsa-miR-513a-5p	-0.90185107	3.77×10^{-4}
hsa-miR-575	-0.86229919	5.94×10^{-10}
hsa-miR-652	-1.77364130	8.49×10^{-12}
hsa-miR-760	-0.91368658	1.36×10^{-2}

miR, microRNA; FC, fold change.

database of experimentally validated MTIs. miRWalk (28), a comprehensive database on miRs, hosts predicted and validated miR binding sites and information on all known genes of human, mouse and rat. Only miR-target interactions present in the two databases were used, since target genes shared by the two databases were considered to be more reliable.

Function and pathway enrichment analysis. Functional analysis of large gene lists from high-throughput genomic, proteomic and bioinformatics scanning approaches increases the likelihood for researchers to identify biological processes most associated with their study (29). Gene Ontology (GO) analysis is increasingly applied for functional studies of microarray data (30). Kyoto Encyclopedia of Genes and Genomes (KEGG) is the major public pathway-associated database (31), and Database for Annotation Visualization and Integrated Discovery (DAVID) is a tool for providing functional annotation behind large-scale genomic or transcriptomic data (32). DAVID online analytical tool (version 6.7; david.ncifcrf.gov/) was utilized to analyze the significantly enriched GO terms (geneontology.org/) and KEGG pathways (www.genome.jp/kegg/) for target genes of miRs. Each GO term or KEGG pathway included at least two genes, and P<0.05 was considered to indicate a statistically significant difference.
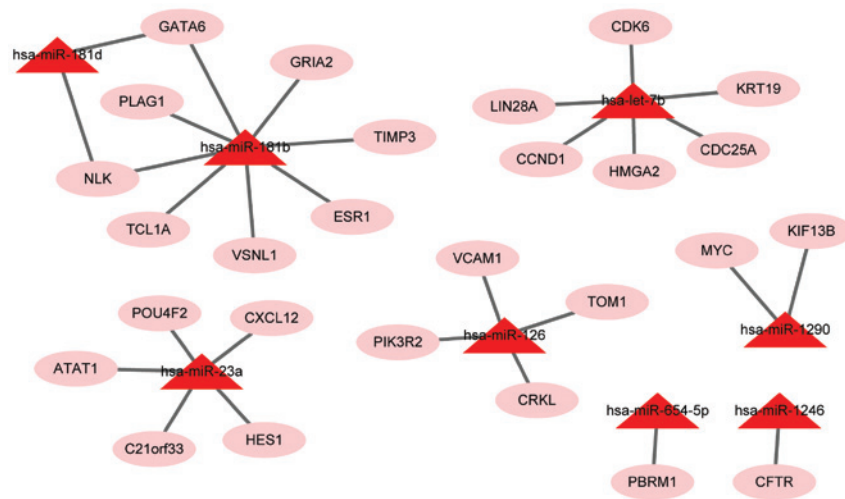
Figure 1. Upregulated miR-target gene interactions. Red triangles represent miRs and pink ovals represent their target genes. The lines demonstrate the associations between the miRs and the target genes. miR, microRNA.
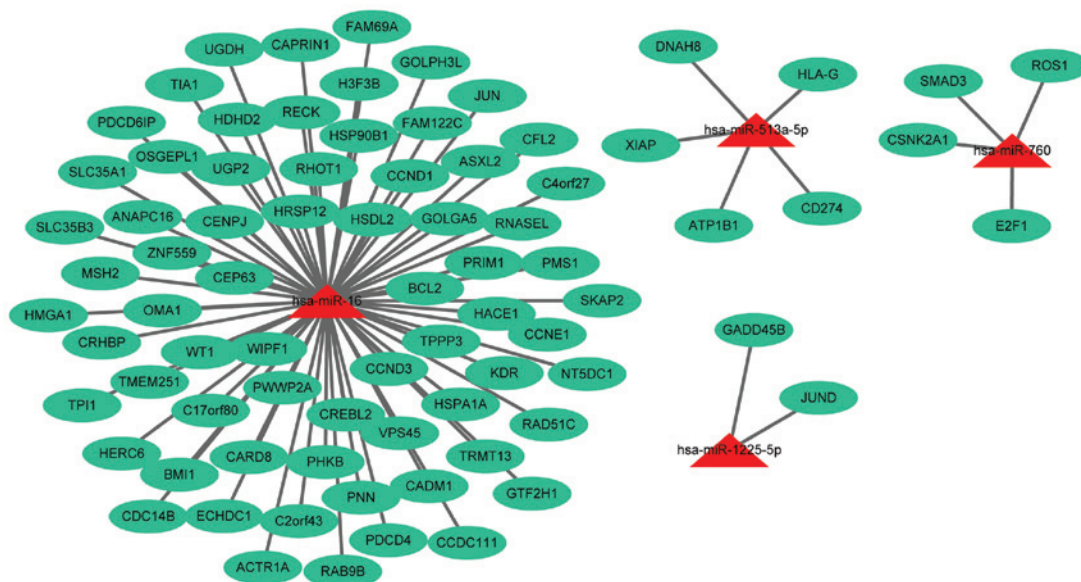

Figure 2. Downregulated miR-target gene interactions. Red triangles represent miRs and green ovals represent their target genes. The lines demonstrate the associations between the miRs and the target genes. miR, microRNA.

Construction of a PPI network. Search Tool for the Retrieval of Interacting Genes (STRING) (33) is a database providing the predicted protein interaction information in a given cell context. PPI networks are increasingly becoming the focus of research in the identification of the cellular function of proteins in various organisms (34). Based on the information of the STRING database (version 8.3; string-db.org/), a protein interaction network was constructed by mapping the target genes of miRs. The interaction pairs with a combined score of 0.4 were selected in this network.

Results

Screening of differentially expressed miRs. In the present study, the expression levels of 851 miRs were acquired following normalization. Following a comparison of the expression between the CRC and healthy control samples

using a t-test, 18 differentially expressed miRs were identified (Table I). Among them, 8 miRs were upregulated (Table IA) and 10 were downregulated (Table IB).

Prediction of the target genes for miRs. Based on the information of miRecords and miRTarBase database, 27 target genes corresponding to the 8 upregulated miRs (Fig. 1) and 79 target genes corresponding to 4 out of 10 downregulated miRs (Fig. 2) were obtained. In total, 6 out of 10 downregulated miRs, miR-1287, miR-1295, miR-1299, miR-144, miR-575 and miR-652, did not have their target genes identified.

Function and pathway enrichment analysis. In the present study, 15 KEGG pathways corresponding to the upregulated miRs and 9 KEGG pathways corresponding to the down-regulated miRs were enriched, respectively, using a cutoff criteria of $P<0.05$ (Table II). The present results revealed that

Table II. Enriched KEGG pathways of target genes in colorectal cancer samples.

A, Enriched KEGG pathways of upregulated target genes

KEGG ID	Description	Count	P-value	Genes
hsa05220	Chronic myeloid leukemia	5	1.33×10^{-5}	CCND1, CRKL, CDK6, MYC, PIK3R2
hsa05200	Pathways in cancer	5	3.90×10^{-3}	CCND1, CRKL, CDK6, MYC, PIK3R2
hsa05222	Small cell lung cancer	4	6.52×10^{-4}	CCND1, CDK6, MYC, PIK3R2
hsa04110	Cell cycle	4	2.07×10^{-3}	CCND1, CDK6, MYC, CDC25A
hsa05213	Endometrial cancer	3	5.32×10^{-3}	CCND1, MYC, PIK3R2
hsa05223	Non-small cell lung cancer	3	5.73×10^{-3}	CCND1, CDK6, PIK3R2
hsa05221	Acute myeloid leukemia	3	6.58×10^{-3}	CCND1, MYC, PIK3R2
hsa05214	Glioma	3	7.73×10^{-3}	CCND1, CDK6, PIK3R2
hsa05218	Melanoma	3	9.75×10^{-3}	CCND1, CDK6, PIK3R2
hsa05212	Pancreatic cancer	3	1.00×10^{-2}	CCND1, CDK6, PIK3R2
hsa05210	Colorectal cancer	3	1.35×10^{-2}	CCND1, MYC, PIK3R2
hsa04012	ErbB signaling pathway	3	1.44×10^{-2}	CRKL, MYC, PIK3R2
hsa04670	Leukocyte transendothelial migration	3	2.56×10^{-2}	VCAM1, CXCL12, PIK3R2
hsa04310	Wnt signaling pathway	3	4.04×10^{-2}	CCND1, NLK, MYC
hsa04630	Jak-STAT signaling pathway	3	4.24×10^{-2}	CCND1, MYC, PIK3R2

B, Enriched KEGG pathways of downregulated target genes

KEGG ID	Description	Count	P-value	Genes
hsa05200	Pathways in cancer	9	5.85×10^{-4}	E2F1, CCNE1, CCND1, HSP90B1, XIAP, MSH2, JUN, BCL2, SMAD3
hsa04110	Cell cycle	7	8.70×10^{-5}	E2F1, CCNE1, CCND1, CCND3, CDC14B, SMAD3, GADD45B
hsa04510	Focal adhesion	6	6.74×10^{-3}	CCND1, CCND3, XIAP, JUN, BCL2, KDR
hsa05210	Colorectal cancer	5	1.55×10^{-3}	CCND1, MSH2, JUN, BCL2, SMAD3
hsa05222	Small cell lung cancer	5	1.55×10^{-3}	E2F1, CCNE1, CCND1, XIAP, BCL2
hsa05215	Prostate cancer	5	1.92×10^{-3}	E2F1, CCNE1, CCND1, HSP90B1, BCL2
hsa04310	Wnt signaling pathway	5	1.26×10^{-2}	CCND1, CSNK2A1, CCND3, JUN, SMAD3
hsa04144	Endocytosis	5	2.43×10^{-2}	VPS45, HSPA1A, PDCD6IP, HLA-G, KDR
hsa04115	p53 signaling pathway	4	7.87×10^{-3}	CCNE1, CCND1, CCND3, GADD45B

Count, number of up/downregulated target genes enriched in KEGG pathway; KEGG, Kyoto Encyclopedia of Genes and Genomes.

the pathways enriched by differentially expressed miRs were not only associated with various types of cancer, including chronic myeloid leukemia, small cell lung cancer, endometrial cancer and CRC, but also with important signaling pathways, including the ErbB, Wnt, Jak-STAT and p53 signaling pathways (Table II). Notably, the present results demonstrated that the target genes of upregulated miRs, such as cyclin D1 (CCND1), v-myc avian myelocytomatosis viral oncogene homolog (MYC) and phosphoinositide-3-kinase, regulatory subunit 2 (beta) (PIK3R2) (Table IIA), and target genes of downregulated miRs, such as CCND1, mutS homolog 2 (MSH2), jun proto-oncogene (JUN), B-cell CLL/lymphoma 2

(BCL2) and SMAD family member 3 (SMAD3) (Table IIB), were all enriched in CRC pathways; therefore, these target genes may be closely associated with CRC. Additionally, the upregulated miRs of these target genes were let-7b (CCND1), miR-1290 (MYC) and miR-126 (PIK3R2). The downregulated miRs of these target genes were miR-16 (CCND1, MSH2, JUN, BCL2) and miR-760 (SMAD3).

Furthermore, based on GO enrichment analysis associated with the biological processes of target genes, 35 and 96 GO terms were identified, which were enriched corresponding to the up/downregulated miRs, respectively (Table III). The results revealed that the target genes of differentially expressed

Table III. Ten of the most enriched GO terms associated with the biological process of target genes in colorectal cancer samples.

A, Enriched GO terms of upregulated target genes

GO ID	Description	Count	P-value
GO:0045449	Regulation of transcription	10	2.14×10^{-2}
GO:0010033	Response to organic substance	7	1.04×10^{-3}
GO:0022402	Cell cycle	6	2.25×10^{-3}
GO:0007049	Cell cycle	6	8.69×10^{-3}
GO:0010604	Positive regulation of macromolecule metabolic process	6	1.30×10^{-2}
GO:0043627	Response to estrogen stimulus	5	2.71×10^{-5}
GO:0048545	Response to steroid hormone stimulus	5	2.82×10^{-4}
GO:0045596	Negative regulation of cell differentiation	5	4.41×10^{-4}
GO:0009725	Response to hormone stimulus	5	3.14×10^{-3}
GO:0043627	Response to estrogen stimulus	5	3.23×10^{-3}

B, Enriched GO terms of downregulated target genes

GO ID	Description	Count	P-value
GO:0006915	Apoptosis	13	1.80×10^{-5}
GO:0012501	Programmed cell death	13	2.08×10^{-5}
GO:0008219	Cell death	13	1.01×10^{-4}
GO:0016265	Cell death	13	1.08×10^{-4}
GO:0007049	Cell cycle	12	8.20×10^{-4}
GO:0051726	Regulation of cell cycle	11	2.71×10^{-6}
GO:0006793	Phosphorus metabolic process	11	1.38×10^{-2}
GO:0006796	Phosphate metabolic process	11	1.38×10^{-2}
GO:0016310	Phosphorylation	10	1.13×10^{-2}
GO:0042981	Regulation of apoptosis	10	1.17×10^{-2}

GO ID, identification number of enriched GO term associated with a biological process; Count, number of up/downregulated target genes; GO, Gene Ontology.

miRs were significantly associated with cell cycle processes and response to hormone stimulus.

PPI network construction of differentially expressed miR target genes. According to the information from STRING database, a PPI network of differentially expressed miR target genes was constructed (Fig. 3). The results demonstrated that the top 6 target genes, from high to low expression based on node degree, were estrogen receptor 1 (degree=19), MYC (degree=18), JUN (degree=17), CCND1 (degree=15), cell division cycle 25A (degree=10) and H3 histone, family 3B (degree=10). Notably, all the target genes enriched in the CRC pathway, including CCND1, MYC, PIK3R2, MSH2, JUN, BCL2 and SMAD3, had higher node degrees in the PPI network and the node degrees of these genes was >4.

Discussion

CRC is a complex disease with genetic and epigenetic alterations in numerous key oncogenes and tumor suppressor genes (35). However, the precise molecular mechanisms leading to anticancer activities in CRC remain unclear. miR biology has increasingly become a focus in the field of cancer research, and secreted miRs embedded in exosomes have emerged as diagnostic biomarkers for cancer detection (24). The present study analyzed the exosomal miR profile in the sera of CRC patients, derived from a GEO database, to screen differentially expressed miRs between CRC patients and healthy controls, and their target genes for therapeutic intervention.

A total of 18 differentially expressed miRs were identified in the sera of CRC patients compared to healthy controls. Furthermore, the present results revealed that 3 upregulated (let-7b, miR-1290 and miR-126) and 2 downregulated (miR-16 and miR-760) differentially expressed miRs and their target genes, including CCND1, MYC, PIK3R2 and SMAD3, were significantly enriched in the CRC pathway. All these target genes had higher node degrees in a PPI network constructed in the present study.

CCND1 was the identified crucial target gene of let-7b and miR-16 in the network of miRs and target genes. let-7b is a member of the let-7 family, which may inhibit cancer cell growth and promote cell apoptosis via post-transcriptional repression
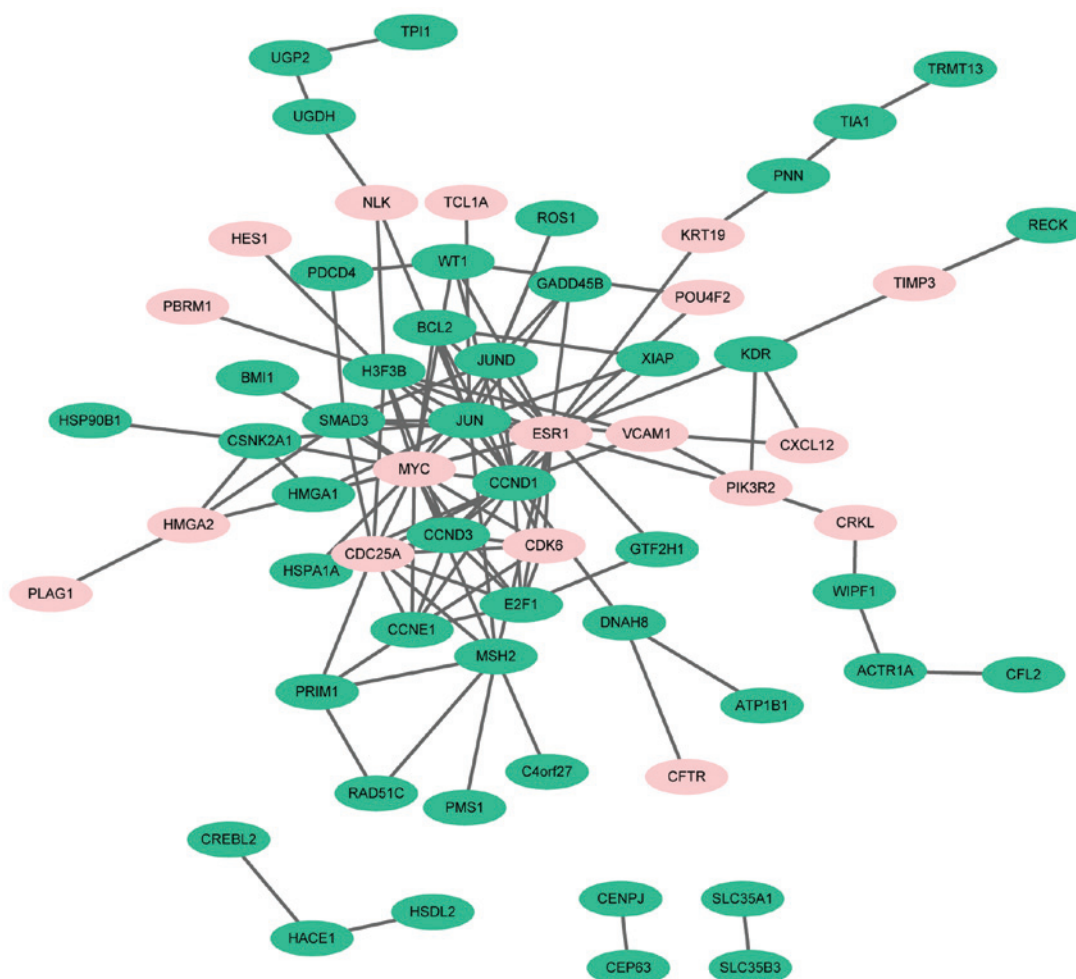
Figure 3. Protein to protein interaction network of differentially expressed miR target genes. Pink ovals represent target genes corresponding to upregulated miRs and green ovals represent target genes corresponding to downregulated miRs. The lines demonstrate the interaction between target genes. miR, microRNA.

of cytochrome P450 epoxygenase 2J2 (CYP2J2) (36). CYP2J2 has been observed to have a tumor-promotion function in human cancer (36). In addition, miR-16 is a regulator of microtubule-associated protein 7 in modulating the proliferation in several types of cancer cell (37). *In vitro* experiments reveal that overexpression of miR-16 represses the proliferation and induces apoptosis of CRC cells by regulating the p53/survivin signaling pathway (38). Furthermore, CCND1 is widely recognized to be involved in cancer cell cycle progression and is vital in controlling G1/S cell cycle transition (39). CCND1 regulates the switch between proliferation and growth arrest, and the CCND1 G870A polymorphism contributes to CRC susceptibility (40). Additionally, CCND1 is regulated by the miR-16 family, and the miR-16 family induces G1 arrest by regulating the expression of CCND1 (41). let-7b inhibits cell cycle progression and anchorage-independent growth of melanoma cells via the downregulation of CCND1 (42). The results of the present study are similar to those of previous studies, as let-7b and miR-16 could directly target CCND1. The present results additionally suggest that let7b and miR-16 inhibit CRC progression by targeting CCND1.

miR-1290 was also demonstrated to be upregulated in the present study. miR-1290 and its target gene MYC are considered to be crucial in the regulation of CRC. Upregulation of

miR-1290 in colon cancer cells impairs cytokinesis, which is the final step of cell division (43). Furthermore, overexpression of miR-1290 activates the Wnt pathway (43), which has been demonstrated in several types of cancer, including CRC (44), and is important in tumorigenesis (45). The MYC proto-oncogene encodes a prototypical oncogenic transcription factor that is crucial in the genesis of various cancers (46). MYC is part of the Mad-Max network that regulates the cell cycle, and cell differentiation and apoptosis (47,48). Previous experimental data indicates that even a slight inhibition of c-MYC expression may permanently inhibit tumor growth and enhance the regression of tumors (49). A previous study by Wu *et al* (43) demonstrated that miR-1290 was involved in the reprogramming of colon cancer cells by increasing the expression of c-MYC. Thus, miR-1290 may be a putative oncogenic miR in promoting CRC progression.

Similarly, the upregulated miR-126 and target gene PIK3R2 were also identified in the network of miRs and target genes. miR-126 is upregulated in primary CRC tissues and cell lines; a previous study verified that epigenetic silencing of miR-126 in CRC cells effectively leads to cell growth, migration and invasion (50,51). PIK3R2 is a key regulator in the phosphoinositide 3-kinase (PI3K) pathway, which is a key signal transduction system that associates oncogenes with numerous

cellular functions and has a role in human cancer (52). In addition, miR-126 may be important in tumorigenesis and metastasis by targeting PIK3R2 to regulate the vascular endothelial growth factor/PI3K/protein kinase B signaling pathway in human breast cancer (53). On the basis of the present results, the present authors hypothesise that miR-126 may be an important molecule in the pathogenesis of CRC via the regulation of PIK3R2.

In the present study miR-760 was downregulated, and was demonstrated to interact with SMAD3. SMAD3 is a downstream mediator of the transforming growth factor-β (TGF-β) signaling pathway (54). TGF-β signaling regulates tumorigenesis, and its signaling pathway is crucial in tumor progression in human cancer (55). Additionally, SMAD3 expression decreases susceptibility to tumorigenicity in the early stages of gastric carcinogenesis (56). In the present study, miR-760 was downregulated, which was similar to a previous finding that miR-760 had a lower expression in CRC tissues and might be a biomarker for the early detection of CRC (57). Therefore, miR-760 may indirectly inhibit tumorigenesis and progression of CRC by targeting SMAD3. However, few functional studies have been performed on miR-760; therefore, additional experimental validation and high throughput data analysis are required.

In conclusion, the present study has identified differentially expressed miRs in the sera of CRC patients compared with healthy controls. let-7b, miR-16, miR-1290, miR-126 and miR-760, and their target genes, CCND1, MYC, PIK3R2 and SMAD3, may be important in the initiation and progression of CRC. let-7b and miR-16 may regulate cell cycle processes by interacting with CCND1, thus inhibiting cancer cell proliferation and growth and induce cell apoptosis. miR-1290 and miR-126 may function in promoting the tumorigenesis and metastasis of CRC cells via the regulation of MYC and PIK3R2, respectively, while miR-760 may indirectly prevent the progression of CRC by targeting SMAD3. Therefore, these 5 miRs and their target genes may be candidate biomarkers for the diagnosis and treatment of CRC. In addition, the present study provides intriguing clues as to the identification of potential candidate biomarkers. The target genes of 6 downregulated miRs, including miR-1287, miR-1295, miR-1299, miR-144, miR-575 and miR-652, were not identified in the present study; therefore, whether they are associated with CRC requires addition study. Overall, the present study may aid additional clinical molecular target therapy experiments concerning CRC.

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