



# Comprehensive analysis of competitive endogenous RNA network in colorectal cancer

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**Background:** Colorectal cancer (CRC) is a leading cause of cancer-related deaths worldwide. Growing evidence supports a role for noncoding RNAs (ncRNAs) in CRC. In particular, they form competitive endogenous RNA (ceRNA) networks involved in the regulation of mRNA expression. However, the role of these networks in the pathogenesis of CRC is not fully understood. The aim of this study was to elucidate the role of circRNA/lncRNA-miRNA-mRNA systems in CRC pathogenesis based on the construction of a ceRNA network.

**Methods:** RNA expression profiles were obtained from public datasets in the Gene Expression Omnibus (GEO) database and used for further analysis by online databases and tools.

**Results:** In total, 245 circRNAs, 1,666 lncRNAs, 5 miRNAs, and 934 mRNAs were differentially expressed in CRC samples. Functional enrichment analysis identified altered biological functions related to the mRNAs in the ceRNA network, and it was found that the oxytocin signaling pathway was significantly enriched ( $P < 0.05$ ) in genes with differential expression in CRC. Additionally, we established a protein-protein interaction (PPI) network and identified 10 hub genes for the construction of circRNA/lncRNA-miRNA-hub gene regulatory modules.

**Conclusions:** We identified several ncRNAs with a possible pathogenetic role in CRC and built a CRC-specific ceRNA network. The results of our study provide novel insights into the molecular events implicated in CRC.

**Keywords:** Chinese population; circRNA; colorectal cancer (CRC); long noncoding RNA; microRNA

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## Introduction

Colorectal cancer (CRC), also known as bowel or colon cancer or rectal cancer, is an aggressive human tumor (1,2). According to the statistics, more than 51,020 people will die from this disease in 2019, accounting for about 8% of all cancer deaths. Moreover, it has been estimated that, as of January 1, 2019, more than 1.5 million people in the United States received a diagnosis of CRC, and 145,600 new cases

are predicted to be diagnosed in 2019 (3,4). With the rise of the Human Development Index (HDI), the incidence of CRC has gradually increased worldwide. Consistently, the incidence and mortality of CRC in China have grown in the past decade (5). Therefore, understanding CRC carcinogenesis at the molecular level is a pivotal step for improving early diagnosis and prognosis, as well as for developing effective therapeutics.

Although transcriptionally inactive, noncoding RNAs

(ncRNAs) regulate the expression of target genes (6,7). Long noncoding RNAs (lncRNAs) are a class of ncRNAs with a length of >200 nucleotides, affecting many cellular processes. Recent studies demonstrated that many lncRNAs play an essential role in cancer pathogenesis, including tumor formation and progression (8). Unlike linear RNAs, circular RNAs (circRNAs) have a unique circular covalently bonded structure, which confers them a high resistance to exonucleases. Due to their conservation, abundance, and tissue specificity, accumulating evidence indicates that circRNAs may serve as specific molecular markers in multiple cancers, including gastric cancer, hepatocellular carcinoma, and lung cancer (9-11). MicroRNAs (miRNAs) are a variety of small ncRNA molecules ranging in size between 15 and 21 nucleotides and exerting post-transcriptional regulation of gene expression and RNA silencing by binding to the 3'UTR regions of target genes. In the past few decades, significant achievements have been made about the correlations between miRNAs and cancers (12-14). A *competitive endogenous RNA (ceRNA) hypothesis* was first proposed by Salmena *et al.* to describe the interactions between different types of ncRNAs and postulated that such networks regulate the expression of downstream target genes in multiple malignancies (15). Numerous studies support this hypothesis, confirming the involvement of lncRNA-miRNA-messenger RNA (mRNA) and circRNA-miRNA-mRNA ceRNA networks in various cancers, including CRC (16-18). However, the mechanisms by which these networks may affect CRC progression and prognosis are as yet unclear.

In light of the ceRNA theory, we reconstructed a molecular network potentially implicated in CRC using online data from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI-GEO), which contained 44 circRNAs, 2 lncRNAs, 5 miRNAs, and 36 mRNAs. Following this, based on the ceRNA network, we performed functional enrichment analysis and core gene speculation, for the further construction of a core gene-centered RNA interaction network. Indeed, several differentially expressed RNAs identified have been reported in CRC previously, but the majority have mainly focused on lncRNA or circRNA mediated networks. In our research, we considered the interaction between lncRNAs and circRNAs and constructed a multi-molecule co-mediated ceRNA network. Besides, to specifically explore the molecular mechanisms associated with CRC in the Chinese population, only tumor specimens from Chinese patients were analyzed in all datasets. Our data may inspire new approaches to treatment and prevention.

## Methods

### Data source

Gene expression profiles of CRC patients and healthy individuals (GSE108153, GSE126093, GSE126094, GSE126092, and GSE41657) were obtained from NCBI-GEO, a free database of microarray/gene profile and next-generation sequencing data. The GSE108153 and GSE126093 datasets contained miRNA microarray data of 21 and 10 paired samples from patients with CRC and healthy individuals, respectively. GSE126094 contained circRNA microarray data of 10 paired samples from CRC patients and 10 healthy individuals. GSE126092 contained data of 10 paired samples from CRC patients and 10 healthy individuals and was used to analyze the lncRNAs. Finally, mRNA profiles were analyzed in the GSE41657 dataset, comprising 25 paired CRC patients and 12 healthy individuals.

### Screening of DE miRNAs, circRNAs, lncRNAs, and mRNAs

R software (version 3.5.2) was utilized to select DE miRNAs from the GSE108153 and GSE126093 datasets separately. The two series of data were merged, and miRNAs with the same seed sequence and expression pattern were selected as co-expressed miRNAs. The R software was also used to identify DE circRNAs, lncRNAs, and mRNAs in the GSE126094, GSE126092, and GSE41657 datasets, respectively. For all analyses, we applied a  $\log_2FC > 2$ , and an adjusted P value  $< 0.05$  was the cut-off for statistical significance. Volcano maps were generated based on the DE circRNAs, lncRNAs, and mRNAs using the ggplots packages in the R platform.

### Construction of the ceRNA network

The Starbase database (<http://starbase.sysu.edu.cn/>) was used to predict interactions between lncRNA/circRNA and miRNAs. Starbase is an open-source platform for studying miRNA-ncRNA or miRNA-mRNA interaction and contains gene expression data of 32 types of cancers from 10,882 RNA-Seq and 10,546 miRNA-Seq datasets. Moreover, Starbase includes 10 different programs to predict miRNA targets (PITA, RNA22, miRmap, microT, miRanda, PicTar, TargetScan, AgoExpNum, CleaveExpNum, and Pan-Cancer) and was used to identify mRNA targets of the identified miRNAs. The genes that were recognized as targets of the selected miRNAs by more than five prediction programs



**Figure 1** Identification of 5 differentially expressed microRNAs (DEmiRNAs) in common between two public microarray datasets (GSE108153 and GSE126093). (A) Up-regulated miRNAs. (B) Down-regulated miRNAs. Color areas represent different datasets. The overlapping areas indicate frequent changes. Cut-off criteria for statistically significant variations were adjusted to P value <0.05 and  $\log_2FC > 2$ .

were considered for further analysis.

### Functional enrichment analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) is an online resource platform (available at <https://david.ncifcrf.gov/>), which allows for systematic and comprehensive analysis of large gene lists. To assess the functional enrichment of the DEmRNAs, GO terms, and KEGG pathways were analyzed using DAVID. P values <0.05 were considered indicative of statistically significant DEmRNA enrichment in KEGG pathways or GO terms.

### Construction and analysis of a PPI network

The STRING database (<http://stringdb.org>) was used to predict potential interactions between DEmRNAs. The results were visualized by Cytoscape 3.7.1. In addition, the CytoHubba tool was used to identify hub genes belonging to the obtained PPI network. Finally, we analyzed PPI subnetworks based on the identified hub-genes.

### Data availability

The datasets analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository, <http://www.ncbi.nlm.nih.gov/geo/>.

## Results

### Differentially expressed ncRNAs in CRC

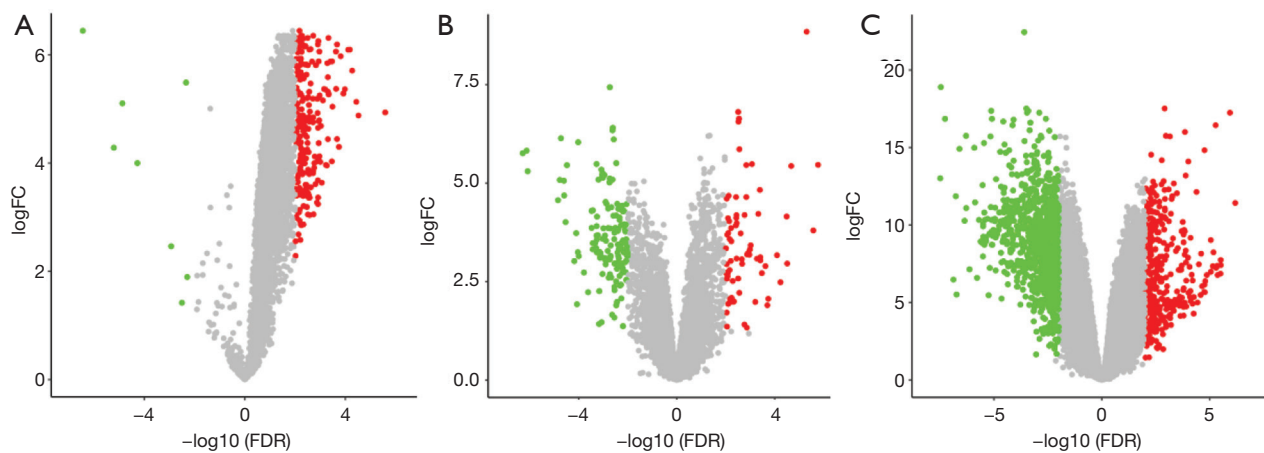
DEmiRNAs in CRC patients and paired healthy controls

from all datasets were selected, using a P value <0.05 and a  $\log_2FC > 2$  as cut-off criteria. Eight and 103 DEmiRNAs were extracted from the GSE108153 and GSE126093 microarray datasets, respectively. Five of these DEmiRNAs, 3 up-regulated and 2 downregulated in CRC compared to healthy subjects, were common to both datasets (Figure 1).

In the GSE126094 dataset, 245 differentially expressed circRNAs (DEcircRNAs) were found, of which 237 were up-regulated and 8 downregulated in CRC patients compared to healthy individuals. Then, GSE126092 microarray data were used to identify differentially expressed lncRNAs (DElncRNAs). As a result, 555 up-regulated and 1,111 downregulated lncRNAs were selected. Further, the GSE41657 microarray dataset was used to identify differentially expressed mRNAs (DEmRNAs), revealing 260 up-regulated and 674 downregulated mRNAs in CRC patients. The selected variations had a  $\log_2FC > 2$  and an adjusted P value <0.05 (Figure 2). At the same time, the differentially expressed RNAs were listed in supplementary (Figure S1).

### Construction of a ceRNA regulatory network in CRC

To explore pathogenetically relevant interactions between DEcircRNAs, DElncRNAs, DEmiRNAs, and DEmRNAs, we constructed a lncRNA/circRNA-miRNA-mRNA ceRNA regulatory network for CRC. First, we predicted the interaction pairs between the five selected DEmiRNAs and the DEcircRNAs, lncRNAs, and mRNAs using the StarBase database. By merging the three datasets, 44 overlapping DEcircRNAs (43 up-regulated and 1 downregulated), 2 overlapping DElncRNAs, and 36 overlapping DEmRNAs (13 up-regulated and 23 downregulated) were obtained



**Figure 2** Volcano plot of differentially expressed circRNAs (DEcircRNAs), lncRNAs (DElncRNAs), and mRNAs (DEmRNAs). Red spots represent up-regulated genes, and green spots represent down-regulated genes.

and imported into Cytoscape for analysis. The constructed network is shown in *Figure 3*.

### Functional analysis of DEmRNAs

To investigate the biological function of the 36 DEmRNAs, data were clustered by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses using the DAVID software. P values <0.05 were considered to be statistically significant (*Figure 4*). DEmRNAs were classified based on three functional criteria: biological process (BP), molecular function (MF), and cellular component (CC). According to BP ontology, DEmRNAs were mainly involved in the repression of transcription from the RNA polymerase II promoter. Based on CC ontology, DEmRNAs were mainly enriched in the nuclear compartment. According to MF ontology, DEmRNAs were enriched in sequence-specific DNA binding and RNA polymerase II core promoter proximal region sequence-specific binding. Furthermore, according to the KEGG pathway enrichment analysis, DEmRNAs were implicated in the oxytocin signaling pathway.

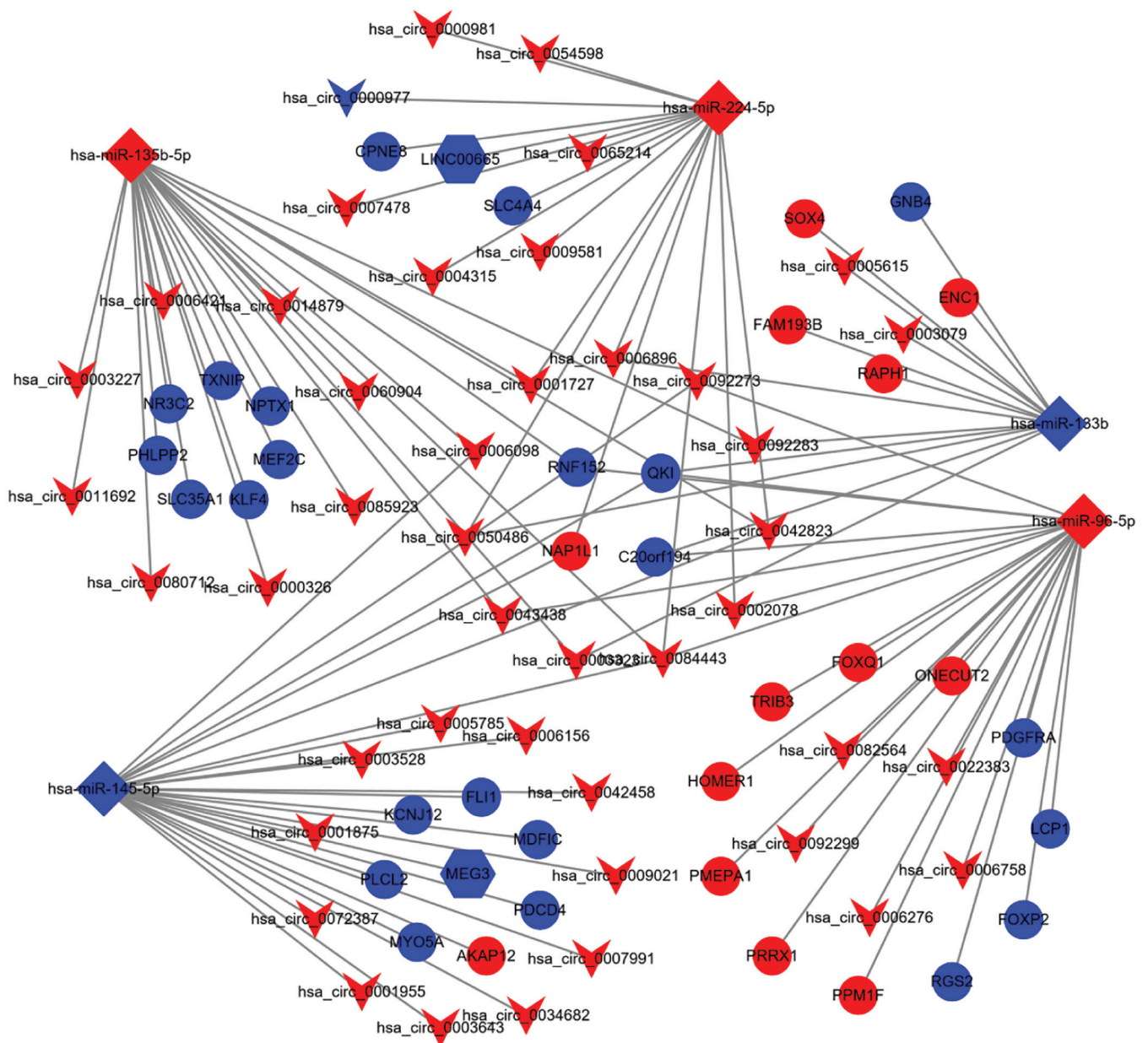
### Construction of a protein-protein interaction (PPI) network based on DEmRNAs

To discover the core molecules of cellular processing at the protein level, we used the Search Tool for the Retrieval of Interacting Genes (STRING), a database of known and predicted protein interactions, to identify functional PPIs based on the 36 DEmRNAs. A network consisting

of 32 nodes and 76 edges, with an enrichment P value of  $1.3 \times 10^{-6}$ , was obtained. Proteins with essential roles in the network were identified based on the scores assigned by Cytoscape to the nodes and edges (<https://cytoscape.org/>) (*Figure 5*). Both the size and color shade of nodes reflect the degree of centrality. In particular, the larger and darker a node, the higher the number of interactions. The thickness of edges between nodes represents the statistical significance of the interactions. In our network, several node proteins, including KLF4, SOX4, PDGFRA, MEF2C, FOXQ1, FOXQ2, TRIB3, NAPAL1, and QKI, showed a strong association with other node proteins, reflecting high hub degrees. Therefore, they may participate in the core role of PPIs.

An in-depth analysis of the PPI network was performed using the Cytoscape plugin CytoHubba, which provides 12 topological methods for ranking the properties of nodes in the network. As a result, potential functionally relevant proteins can be accurately selected based on the scores assigned to node proteins using different *algorithms* (*Figure 6*). The proteins assigned high scores with all algorithms were considered to be potentially relevant in CRC. Ten genes (*KLF4*, *SOX4*, *PDGFRA*, *MEF2C*, *FOXQ1*, *FOXQ2*, *TRIB3*, *PRRX1*, *PPM1F*, and *HOMER1*) were found to be crucial for PPIs and were named “hub genes”. Based on these results, we established a circRNA/lncRNA-miRNA-hub gene subnetwork, including 28 ceRNA regulatory modules (*Figure 7*). This network highlighted hub genes with likely involvement in CRC pathogenesis, which may serve as a useful guide for further targeted research into identifying new therapeutic targets





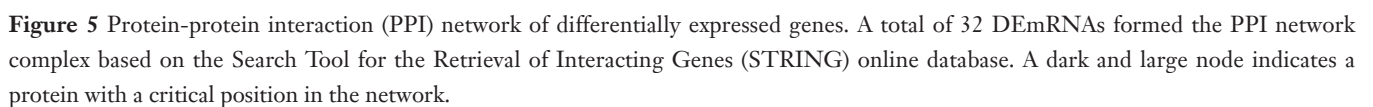
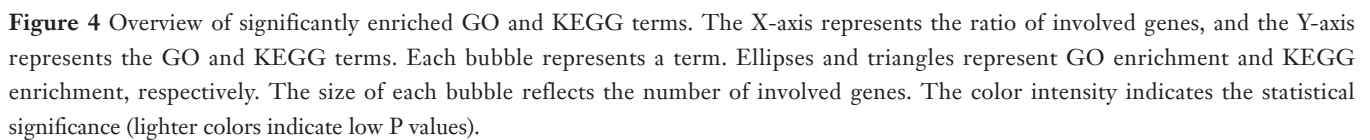
**Figure 3** The ceRNA regulatory network in CRC. The nodes highlighted in red and blue indicate upregulation and downregulation, respectively. circRNA, lncRNAs, miRNAs, and mRNAs are represented by V-shapes, hexagons, diamonds, and ellipses, respectively.

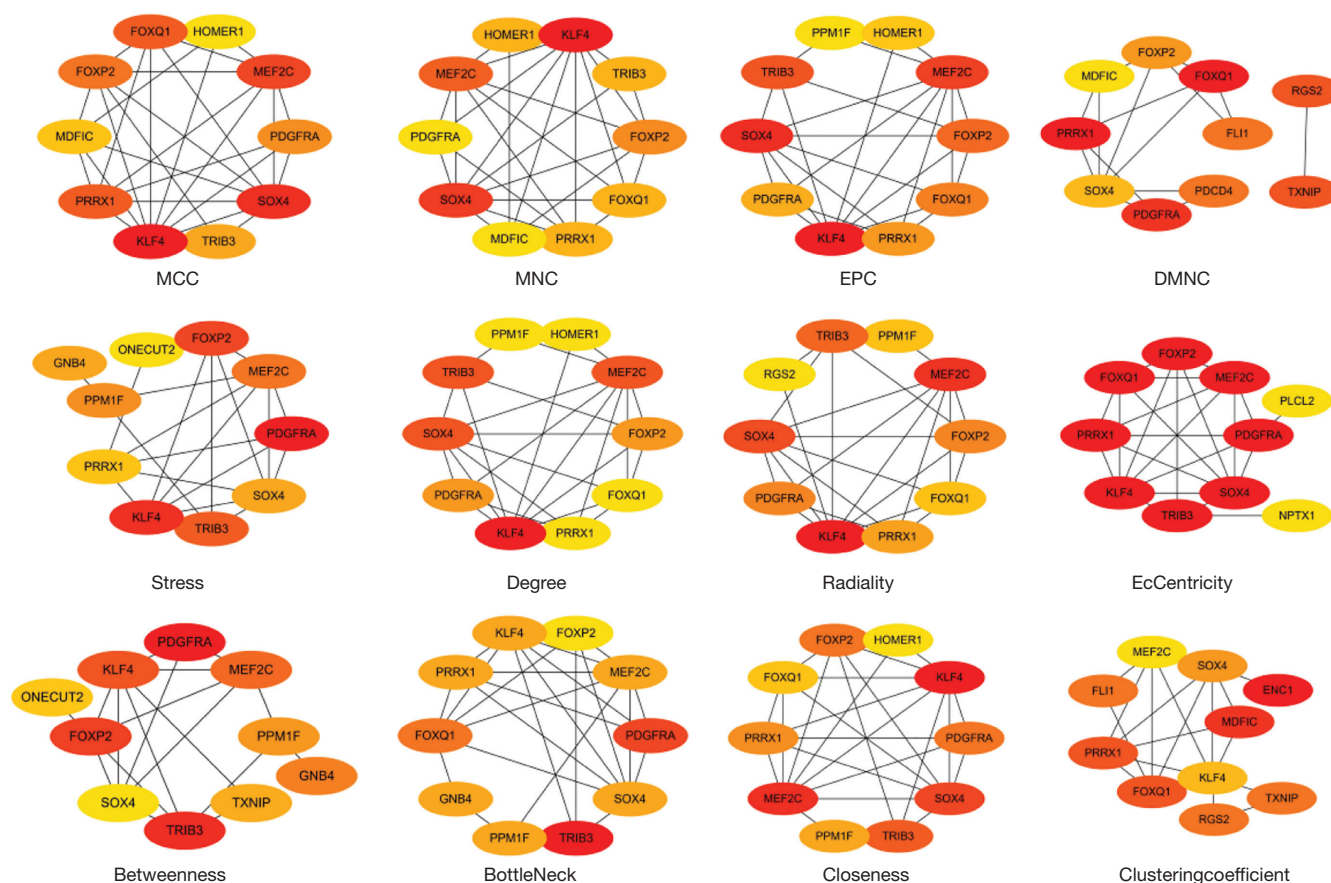
or biomarkers. At the same time, these highlighted genes will also become the target genes for further experimental verification.

## Discussion

In recent years, owing to the ceRNA theory and the use of

TCGA or NCBI-GEO databases, growing knowledge has been gained on the interactions between mRNAs, lncRNAs, circRNAs, and miRNAs with differential expression in tumor and adjacent non-tumor tissues. These studies mainly focused on lncRNA- or circRNA-based ceRNA networks. The mechanism by which lncRNAs and circRNAs affect miRNA action on downstream target genes in CRC is



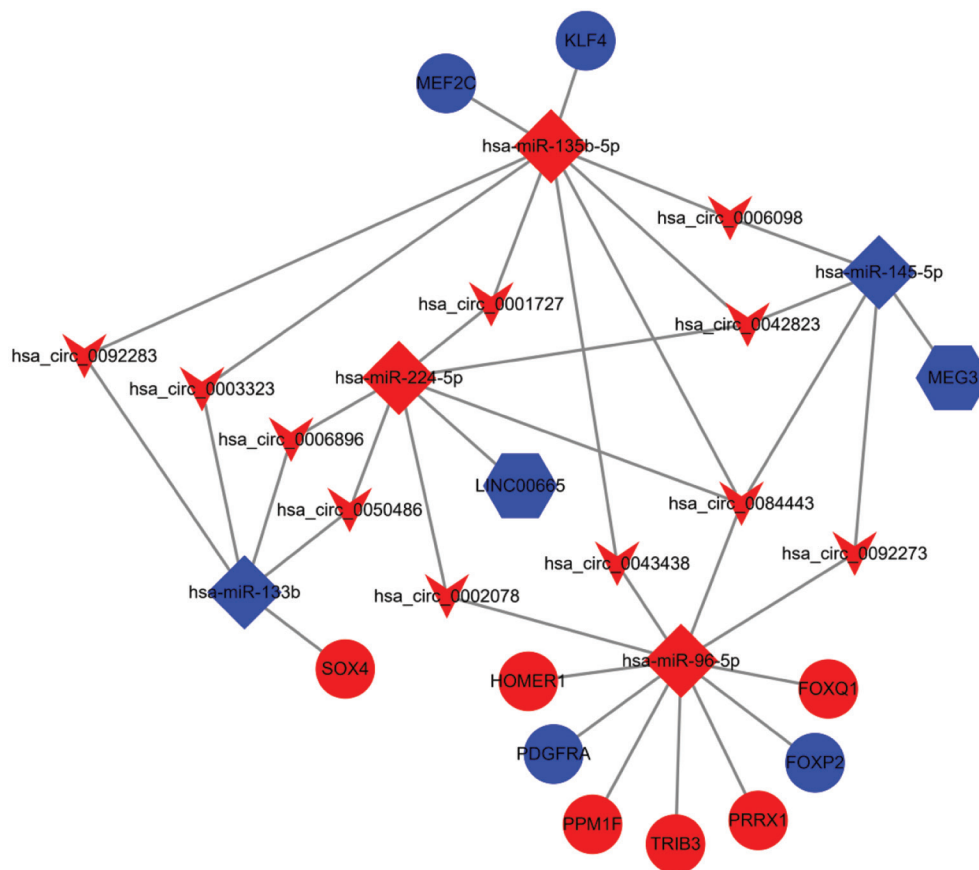


**Figure 6** Analysis of PPI using 12 CytoHubba algorithms. The color intensity of the nodes reflects the score assigned by each algorithm to the corresponding proteins; red indicates the highest score, followed by orange and yellow.

not clear. In this study, we identified distinct circRNAs, lncRNAs, mRNAs, and miRNAs implicated in CRC, providing novel insights into tumor prognosis. Moreover, a circRNA/lncRNA-miRNA-mRNA regulatory network was constructed by integrated bioinformatics analysis.

circRNAs are a new class of endogenous ncRNAs, extracted initially from RNA viruses in 1976 and initially considered as the result of splicing errors (19). Later on, owing to deep RNA sequencing technology, as well as advancements in bioinformatic analysis, circRNAs have become a research hotspot. Multiple functions have been attributed to circRNAs, including the regulation of transcription (20), competition with pre-mRNA splicing, miRNA sponging (21-23), and formation of proteins involved in various BPs (24). Recent studies have revealed that some circRNAs are associated with tumor initiation, progression, and metastasis (25,26). In particular, increasing evidence indicates a role of circRNAs in the initiation and

progression of CRC. Tian *et al.* analyzed 192 pairs of CRC tissues and blood samples and found that hsa\_circ\_0004585 is significantly up-regulated in both tumors and peripheral blood of CRC patients (27). Moreover, the latter study showed that hsa\_circ\_0004585 is positively correlated with tumor size, indicating a role of this circRNA in CRC carcinogenesis and metastasis. Furthermore, Li *et al.* found that the expression of hsa\_circ\_102958 significantly increased in CRC tissues, compared to adjacent control tissues. Moreover, CCK8, colony formation, and Transwell assays revealed that hsa\_circ\_102958 promotes CDC25B expression by sponging miR-585 in CRC (28). In our study, we analyzed data from CRC and normal colorectal tissue samples and found that 44 circRNAs were involved in the ceRNA network, based on differential expression of circRNAs and on the identification of circRNA-miRNA interaction pairs. In addition, 11 circRNAs were selected after establishing a circRNA/lncRNA-miRNA-hub gene



**Figure 7** circRNA/lncRNA-miRNA-hub gene network. The network consists of 11 circRNAs, 2 lncRNAs, 5 miRNAs, and 10 hub genes. Red and blue nodes indicate upregulation and downregulation, respectively. circRNAs, lncRNAs, miRNAs, and mRNAs are represented by V-shapes, hexagons, diamonds, and ellipses, respectively.

network, and it was found that none of them was previously described to be associated with CRC.

Recently, lncRNAs have received attention in relation to various cancers, including CRC. For example, lncRNA-MEG3, a maternally imprinted gene containing ten exons and encoding an approximately 1.6-Kb long ncRNA (29), is abundantly expressed in many tissues but is downregulated in a variety of primary human cancers (30). Another study found that MEG3 levels are decreased in CRC tissues and evidently associated with histological grade, tumor invasion depth, and TNM stage (31). Our data on differential lncRNA expression are consistent with that of the above study. In addition, Wang *et al.* found that MEG3 expression was downregulated in CRC tissues and cell lines, especially in oxaliplatin-resistant tissues and cells. Moreover, the latter study revealed a novel MEG3/miR-141/PDCD4 regulatory axis that could restrain oxaliplatin resistance in CRC (32).

In this study, we identified a possible regulatory interaction between MEG3 and hsa-miR-145-5p. However, due to a lack of experimental validation and regulation of circRNA upstream, the target mRNA is temporarily undetermined. A previous study had identified another lncRNA, LINC00665, as a regulator of viability, apoptosis, and autophagy via the miR-186-5p/MAP4K3 axis in hepatocellular carcinoma (33). We reasoned that LINC00665 could also affect the expression of target genes in CRC by interacting with miR-224-5p. On the other hand, the mechanism by which lncRNAs and circRNAs together affect downstream target genes has not been clarified.

In the ceRNA network constructed in this study, 5 miRNAs were differentially expressed in CRC patients compared to healthy individuals. Previous research showed that aberrantly expressed miRNAs regulate the expression of multiple oncogenes and tumor suppressors and have



been widely associated with cancer development (34). Sun *et al.* demonstrated that lincRNA-SNHG15 promotes the development of colorectal carcinoma by functioning as a ceRNA controlling the miR-141/SIRT1/Wnt/ $\beta$ -catenin axis (35). Another study found that LINC00858 promotes tumor cell proliferation, migration, and invasion by acting as a ceRNA for miR-22-3p in CRC (36). Of the five selected DE miRNAs involved in our ceRNA network, four have been reported to play important roles in the initiation and development of CRC, namely hsa-miR-135b-5p, hsa-miR-145-5p, hsa-miR-224-5p, and hsa-miR-133b (37-40). However, to date, a role for hsa-miR-96-5p in CRC has not been reported. One study showed that hsa-miR-96-5p may serve as a marker to distinguish human ovarian cancer tissues from their normal counterparts, with a 97% sensitivity and a 92% specificity, suggesting that this miRNA is involved in ovarian cancer development (41). In addition, Piotto *et al.* indicated that hsa-miR-96-5p directly regulates the expression of target genes involved in homologous recombination and the non-homologous end-joining pathway (i.e., *RAD51*, *BRCA2*, *PRKDC*, *XRCC5*, and *LIG1*), thus affecting the efficacy of radiotherapy in non-small cell lung cancer (42). This evidence highlights the importance of hsa-miR-96-5p in pan-cancer events and indicates that it is inextricably linked to CRC initiation and development.

To further explore the core genes participating in the regulatory network, functional enrichment analyses were performed, and a PPI network was established. Finally, a circRNA/lncRNA-miRNA-hub gene network, including 28 ceRNA regulatory modules, was constructed, and 10 hub genes were identified. The results showed that the selected DE miRNAs were significantly enriched in the oxytocin signaling pathway. Deiner *et al.* found that mice lacking the netrin-1 receptor, which is deleted in CRC, exhibited abnormalities in hypothalamic-controlled neuroendocrine pathways, such as oxytocin secretion, compared to normal mice (43). Also, Cassoni *et al.* showed that oxytocin and the oxytocin-analog F314 inhibit tumor cell proliferation and growth in colon and mammary carcinomas in mice (44). Therefore, the identified DE miRNAs are involved in many important CRC-associated biological functions and metabolic pathways. Moreover, except for *HOMER1*, *PPM1F*, and *TRIB3*, on which no evidence has been previously published, many reports have shown that the hub genes identified in this study are abnormally expressed in CRC, due to upstream lncRNA- or circRNA-mediated miRNA changes, ultimately affecting cell proliferation,

tumor metastasis, and drug resistance (45-49).

However, our study presents several limitations. First, as we analyzed pre-existing data, our conclusions may be affected by certain errors and uncontrollable factors, such as sample age classification and tumor staging, and need to be experimentally verified, with particular regard to previously unreported hub genes. Second, it is still unclear how lncRNAs and circRNAs may act in combination to control miRNA expression within the constructed lncRNA/circRNA-miRNA-hub gene network. To validate these findings, we will perform *in vitro* and *in vivo* experiments focusing on a larger number of clinical samples. Finally, our experimental data can be combined with the clinical data of patients with CRC to explore further the effects of hub genes on the prognosis and treatment of patients. All data analyzed in this study were from Chinese subjects and may inspire future research on the mechanism underlying CRC development in the Chinese population.

## Conclusions

We identified several noncoding RNAs with a possible pathogenetic role in CRC and built a CRC-specific ceRNA network based on our bioinformatics analysis. Our study provides novel insights into the molecular events implicated in CRC.

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## Footnote

*Conflicts of Interest:* Both authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr-19-2973>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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## Supplementary

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**Figure S1** Differentially expressed circRNAs, lncRNAs, miRNAs, and mRNAs in CRC