ORIGINAL RESEARCH

Integrated Analysis of mRNAs, lncRNAs, and circRNAs Expression Profile in Human Colorectal Cancer

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ABSTRACT

Background • Colorectal cancer is the third most common cancer worldwide and one of the leading causes of cancer-related death. However, current treatment options are not ideal.

Methods • In this study, mRNAs, lncRNAs, and circRNAs expression profiles in four pairs of HCT116 and FHC cells were detected by microarray technology. The potential functions and enriched pathways of the differentially expressed RNAs were predicted using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analyses. Bioinformatics software was used to construct co-expression networks of lncRNA-miRNA-mRNA and circRNA-miRNA to reveal the potential mutual regulatory mechanisms of ceRNA.

Results • The result showed that 6681 mRNAs, 12784 lncRNAs, and 14301 CircRNAs were changed in HCT116 and FHC groups. Meanwhile, the differentially expressed RNAs were confirmed by RT-qPCR and then focused on the up-regulated genes of hsa_circ_0025288, ENSG00000233429.9, and NT5E by Maximum difference expression level.

Conclusion • This study provides a theoretical basis for the regulatory mechanism of non-coding RNAs in the pathogenesis and development of colorectal cancer cells. Therefore, this study pointed to ENSG00000233429.9 and hsa_circ_0025288 as potential molecular targets for colorectal cancer treatment in the future. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

Colorectal cancer (CRC) is one of the most recognized malignancies in the world. According to the latest research report, an estimated 1.9 million new cases and over 900 000 deaths globally have occurred in 2020.[1] Metastatic progression is the overwhelming cause of CRC death as is the case for most solid tumors.[2] Research over the years shows great progress in CRC diagnosis and treatment, but precise treatment still faces great challenges. Therefore, research for more promising therapeutic targets and biomarkers in CRC is urgently needed.

It is well known that the structure of long non-coding RNA (lncRNA) consists of more than 200 nucleotides.[3,4] They were initially thought to have no biological function, merely the byproducts of RNA polymerase II transcription, and the noise of genome transcription. However, several studies have shown that the expression or dysfunction of lncRNA plays a crucial role in most human diseases.[5-9] Circular RNA (circRNA) is a circular non-coding RNA, with stable expression and is not easily degraded.[10-12] In recent years, several circRNAs have been identified.[13,14] Recent studies have shown that circRNA acts as a sponge to adsorb miRNAs, thereby increasing the expression level of target proteins.[15-17] Despite many reports on IncRNAs and circRNAs, their regulatory mechanisms in CRC remain unclear.

In this study, the expression level of RNAs in HCT116 and FHC cells was detected using microarray. Meanwhile, these differentially expressed RNAs were analyzed by GO and KEGG pathways. Next, some differentially expressed RNAs in the microarray were confirmed by RT-qPCR. In addition, regulatory network modes of lncRNA-miRNA-mRNA and circRNA-miRNA were predicted using bioinformatics software. Thus, the study offers a theoretical basis for further exploring the role of non-coding RNA (ncRNA) on the development and progression and potential molecular targets for treating colorectal cancer.

METHODS

Cell Culture

Human colorectal carcinoma HCT116 (American Type Culture Collection [ATCC]), FHC colorectal epithelial cells were cultured *in vitro*. HCT116 and FHC were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin plus 100 mg/ml streptomycin. They were incubated at 37°C with 5% CO_2.

Microarray Information

A microarray scan was performed by Agilent ceRNA Microarray 2019 and OE Biotechnology Co., Ltd., (Shanghai, China) performed the data analysis.

Data Analysis

Total RNA was quantified by the NanoDrop ND-2000 (Thermo Scientific) and the RNA integrity (RIN) was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies). The sample labeling, microarray hybridization, and washing were performed based on the manufacturer's standard protocols. Briefly, total RNA was dephosphorylated, denatured, and labeled with Cyanine-3-CTP. After purification, the labeled RNAs were hybridized onto the microarray. After washing, the arrays were scanned with the Agilent Scanner G2505C (Agilent Technologies).

Feature Extraction software (version 10.7.1.1, Agilent Technologies) was used to analyze array images to get raw data. Next, the raw data was normalized with the quantile algorithm. The probes that at least 100.0 percent of samples in any 1 condition out of 2 conditions have flags in "Detected" were chosen for further data analysis.

The statistical significance of differentially expressed RNAs between HCT116 and FHC was identified by selecting fold change ≥2.0, $P < .05$, and false discovery rate (FDR) < .05.

Bioinformatics Analysis

CircRNA-miRNA was constructed by TargetScanHuman7.2, RNAhybrid, and miRanda, based on the seed sequence complementary matching principle. Then, the circRNA-miRNA networks were built using Cytoscape.

The potential functions of ncRNA host genes and mRNAs were explored through GO (gene ontology) analysis. Besides, the involvement of parental genes in the biological pathways was detected by KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis.

Validation

Total RNA was extracted by TRIzol Reagent (Invitrogen). Then, cDNA was synthesized with FastKing RT Kit (TIANGEN). RT-qPCR was performed using indicated primers and ChamQ SYBR qPCR Master Mix (Vazyme).

Statistical Analysis

All data are presented as the mean ± SEM (standard error of the mean). Statistical analyses were performed using GraphPad Prism 9.0. Significant differences were assessed using Student's *t* test. Significance was set as $P < .05$.

RESULTS

Differentially Expressed lncRNA and mRNA Profiles in HCT116

Microarray results for HCT116 and FHC cells were obtained from Agilent ceRNA Microarray. The result showed that a total of 12784 significantly dysregulated lncRNAs (fold change ≥2.0, $P < .05$) (8664 up-regulated and 4120 down-regulated) and 6681 mRNAs (3134 up-regulated and 3547 down-regulated) were screened out to be differentially expressed (Figure 1A-F). LncRNA and mRNA expression patterns between HCT116 and FHC cells were distinguished via hierarchical clustering heat maps (Figure 1A-B). Additionally, the differences in lncRNA and mRNA expression between HCT116 and FHC cells have been presented using volcano and scatter plots (Figure 1CF). Meanwhile, this study found that differentially expressed lncRNAs tend to be located on chromosomes 1, 2, 6, and 5 (Figure 1G).

GO and KEGG Pathway for Differentially Expressed lncRNAs

GO and KEGG pathway analyses were performed for the differentially expressed lncRNAs. The result showed that the highest enriched GO targeted by up-regulated transcripts were cell adhesion (biological process), DNA binding transcription factor activity (molecular function), and transcriptional repressor complex (cellular component) (Figure S1). Meanwhile, regulation of transcription, DNA templated (biological process), nucleoplasm (cellular component), and DNA binding (molecular function) were the highest enriched GOs targeted by down-regulated transcripts (Figure S1). Next, a KEGG pathway analysis was performed to predict potential gene function. The result showed that the hippo signaling pathway, Wnt signaling pathway, cGMP-PKG signaling pathway, ferroptosis, apoptosis, relaxin signaling pathway, NF-kappa B signaling pathway, NOD-like receptor signaling pathway, Jak-STAT signaling pathway, cell cycle, and TNF signaling pathway, which are associated with the occurrence and development of cancer, are significantly enriched in the HCT116 (Figure S1).

Construction of the lncRNA-mRNA

To identify interactions between differentially expressed mRNAs and lncRNAs, the lncRNA-mRNA regulatory network was constructed. First, 32 differentially expressed mRNAs (HSPA1A, CDX1, CST1, LYZ, FGG, CD24, PMCH, PAGE4, GALC, TUBB6, SPAG16, CDH3, ASS1, HIST3H2A,

Figure 1. Bioinformatics Analysis of Differential Expression of mRNAs and lncRNAs Between HCT116 and FHC. (A-B) Heat-Map Clustering Shows All Dysregulated lncRNAs and mRNAs. (C-D) The Scatter Plot Exhibits Variation in lncRNAs and mRNAs Expression. (E-F) Volcano Plots Display the Differences of lncRNAs and mRNAs Expression. (G) Histogram Plots Indicate the Distribution of Differentially Expressed lncRNAs.

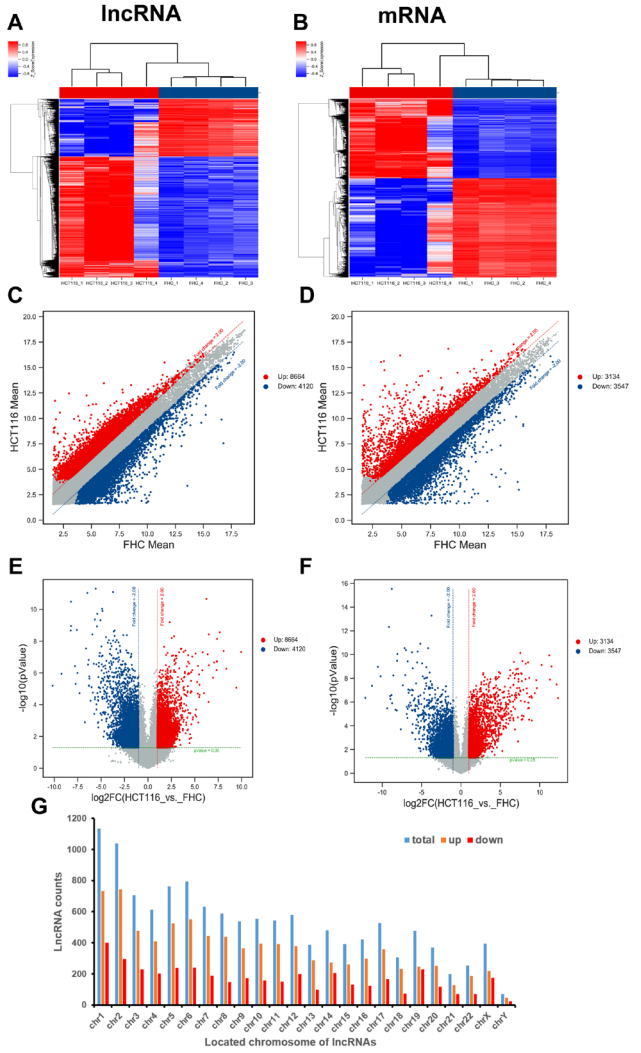
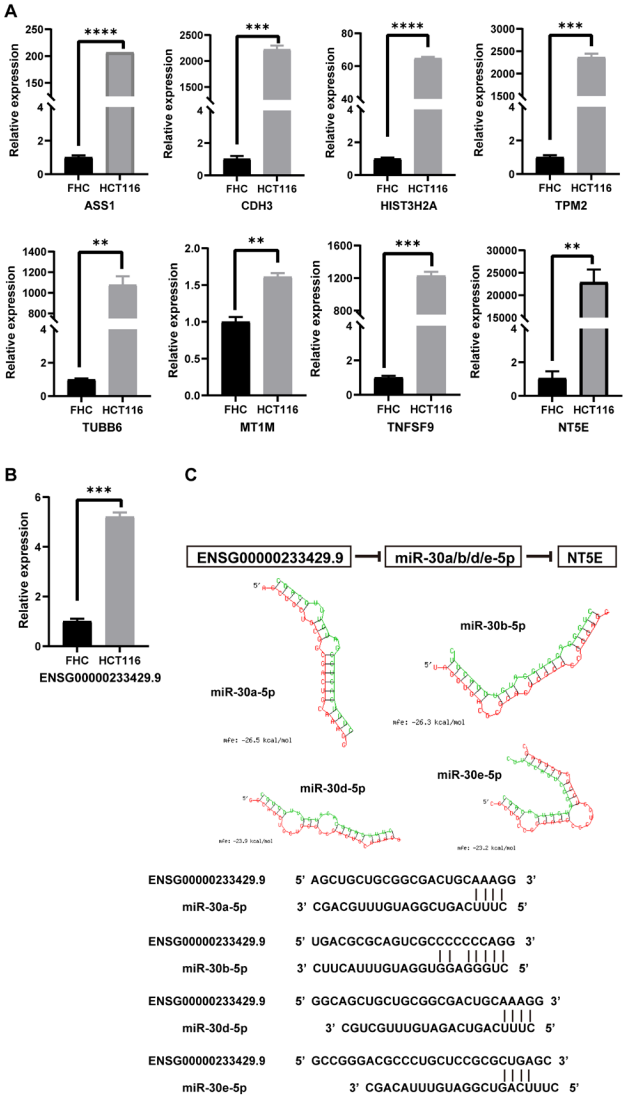


Figure 2. (A) The Eight Key Differentially Expressed mRNAs Identified by qRT-PCR in HCT116 Compared to FHC. (B) Expression of ENSG00000233429.9 Between the HCT116 and FHC. (C) Schematic Cartoon of ENSG00000233429.9 Regulates Expression of NT5E by Sponging miRNAs in CRC.



*P < .05, **P < .01, ***P < .001.

KLHDC8B, PPIC, KHDC1, GATM, TNFSF9, TPM2, NKD2, CST2, CST4, NT5E, EEF1A2, FSCN1, MT1M, DUSP23, ZNF595, MT1E, GAL, DMKN) were selected based on highly abnormal expression. To determine the relationship between these differential RNAs, a ceRNA network was constructed between differential mRNAs and lncRNAs. In this experiment, a total of 500 pairs of mRNA and lncRNA regulatory relationships were found by correlation coefficient analysis (Figure S2). It was hypothesized that these RNA interactions may play a major role in colorectal cancer genesis.

Validation of the Vital Differentially Expressed mRNAs and lncRNAs

32 mRNAs were chosen for verification of the microarray results by RT-qPCR. The results showed that ASS1, CDH3, HIST3H2A, TPM2, TUBB6, MT1M, NT5E, and TNFSF9 were found to be significantly up-regulated in the HCT116 which were consistent with the microarray data. Among them, the NT5E expression was the most significantly up-regulated (Fold Change (FC) = 6795) (Figure 2A). Similarly, four abnormal lncRNAs were selected for verification of the microarray results by RT-qPCR. The results showed that ENSG00000233429.9 expression was consistent with the microarray data, the most significantly up-regulated (FC = 5.21) (Figure 2B). Finally, the binding sequence between ENSG00000233429.9 and NT5E was predicted by RNAhybrid, to obtain the potential miRNAs (miR-30a/b/d/e-5p) (Figure 2C).

Figure 3. Bioinformatics Analysis of Differential Expression of circRNAs Between HCT116 and FHC. (A) Heat-Map Clustering Shows All Dysregulated circRNAs in HCT116 and FHC Cells. (B) Scatter Plot Exhibits Variation in circRNAs Expression. (C) Volcano Plots Dislay the Differences in circRNAs Expression. (D) Histogram Plots Show the Distribution of Differentially Expressed circRNAs on the Location of Human Chromosomes (E) Histogram Plots Show the Distribution of Differentially Expressed circRNAs on the Length of Nucleic Acids.

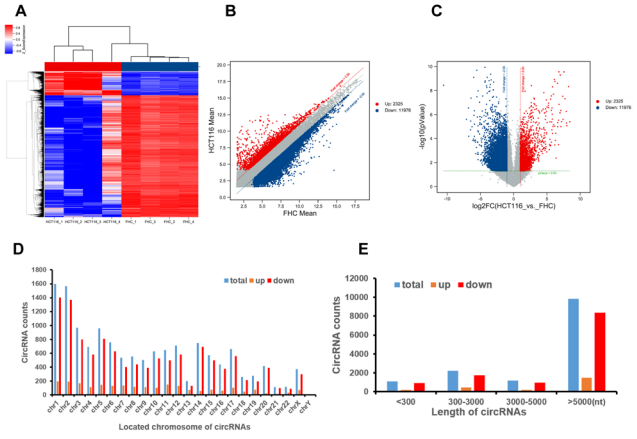
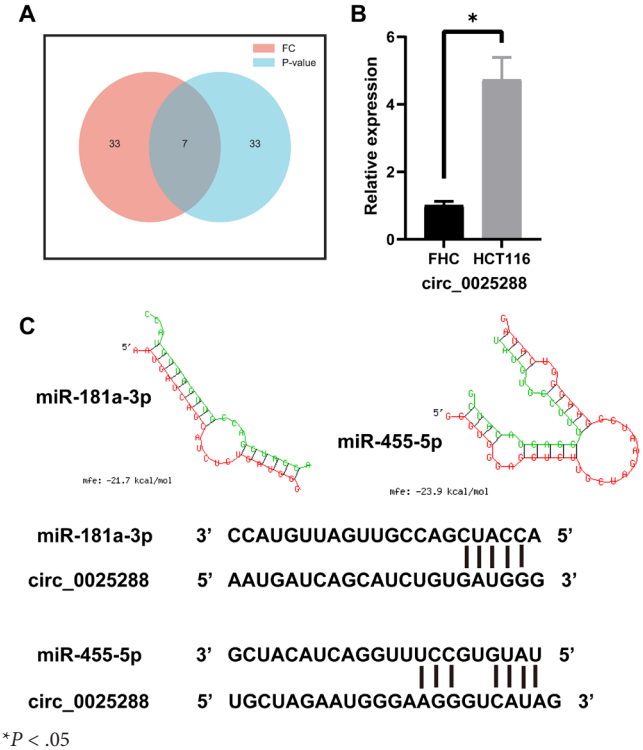


Figure 4. (A) Venn Diagram Shows Differentially Expressed circRNAs Based on Fold Change (Pink Circle) and the Value of *P* (Blue Circle). (B) Expression of circ_0025288 in HCT116 Compared to FHC. (C) The Interaction of circ_0025288 with Potential miRNA Binding Sites as Predicted Through RNAhybrid.



*$P < .05$

Identification and Characteristics of the Crucial Differentially Expressed circRNAs

In this experiment, the differentially expressed circRNAs between HCT116 and FHC were detected by microarray. The result showed that a total of 14301 significantly dysregulated circRNAs (fold change ≥2.0, $P < .05$) (2325 up-regulated and 11976 down-regulated) were screened out to be differentially expressed (Figure 3A-C). CircRNA expression patterns between HCT116 and FHC cells were distinguished via hierarchical clustering heat maps (Figure 3A). Meanwhile, differences in circRNA expression between HCT116 and FHC cells are presented using volcano plots and scatter plots (Figure 3B-C). In addition, this study found that differentially expressed circRNAs tend to be located on chromosomes 1, 2, 3, and 5 (Figure 3D). By analyzing circRNA lengths, it was also confirmed that most differentially expressed circRNAs were more than 5000 nucleotides (Figure 3E).

GO and KEGG Pathway for Differentially Expressed circRNAs

The functional role of target host genes was predicted by GO. The results showed that the viral process was most significantly enriched for biological processes (GO: 0016032, P =1.31E-11); the most significantly enriched for cellular components was cytosol (GO: 0005829, P = 7.51E-62); and, the most significant enriched for molecular functions was ATP binding (GO: 0005524, P = 9.04E-28) (Figure S3). Meanwhile, KEGG analysis was used to predict pathways related to gene function. The results showed that circRNAs in the network were enriched in the ErbB signaling pathway. Numerous studies have verified that the signaling pathways exert their effects in various cancer genesis (Figure S3).

Construction of a circRNA-miRNA Interaction Network

It is well known that circRNA has miRNA binding sites, which act as miRNA sponges and competitively bind miRNA. 20 differentially expressed circRNAs were selected based on fold change for bioinformatics analysis and generated circRNA-miRNA networks by Cytoscape (Figure S4). The result showed that circRNAs are bound to miRNAs via a one-to-many binding way and one miRNA could be regulated by multiple circRNAs. This form of binding and regulatory characteristics are similar to the action mode of miRNA. For instance, circ_0032846 was ceRNA of miR-423-3p, miR-193a-5p, and miR-146b-5p. On the other hand, miR-625-5p was correlated with circ_0075336, circ_0069996, circ_0083771, and circ_0048718.

Validation of the Key Differentially Expressed circRNAs

For further study, the key circRNAs were identified based on the fold change and P value in the microarray and by Venn diagram intersection (Figure 4A). Therefore, this study focused on seven key circRNAs including circ_0025289, circ_0027510, circ_0027511, circ_0075196, circ_0025288, circ_0020275, and circ_0025285. Then, their expression levels were verified by qPCR in HCT116 and FHC. The experimental results showed that circ_0025288 had the greatest fold change in HCT116 compared to FHC and its expression pattern was consistent

with the microarray data (Figure 4B). Next, the candidate genes of circ_0025288 were studied. Through bioinformatics analysis, it was confirmed that circ_0025288 had multiple matching sequences to bind various miRNAs, such as miR-181a-3p and miR-455-5p (Figure 4C).

DISCUSSION

Recently, numerous studies confirmed that ncRNAs play a key role in various cancer genesis.[18-21] However, so far, the expression profiles of differentially expressed RNAs in HCT116 and FHC have not been reported by microarray. Therefore, this study explored the expression profiles and mutual regulatory networks of mRNAs, lncRNAs, and circRNAs in HCT116 by microarray and bioinformatics analysis, and predicted their potential functions.

In this study, the result showed that a total of 12784 significantly dysregulated lncRNAs (8664 up-regulated and 4120 down-regulated), 6681 mRNAs (3134 up-regulated and 3547 down-regulated), and 14301 circRNAs (2325 up-regulated and 11976 down-regulated) were differentially expressed. For further study, 32 mRNAs were selected based on high differential expression fold in the microarray results and a Coding-Non-Coding (CNC) network was constructed using bioinformatics software. This helped to reveal the key lncRNAs that may be closely related to the occurrence and development of CRC. Finally, 500 pairs of mRNA-lncRNA were obtained. To identify the key genes, differentially expressed genes in the microarray results were confirmed by RT-qPCR, and the abnormal genes were verified. Thus, the mRNA expression of NT5E was most significantly up-regulated (FC = 6795). NT5E (CD73) is a cell surface protein anchored by glycosyl-phosphatidylinositol.[22,23] It is well known that the main signaling molecules of the purinergic signaling pathway include extracellular adenosine, AMP, and ATP. Multiple studies have confirmed that the purinergic signaling pathway comes into play in various cancer genesis. Moreover, recent studies have demonstrated that CD73 plays an important role in promoting metastasis and proliferation in many types of cancer.[24-28] Meanwhile, using bioinformatics analysis and qRT-PCR, the study confirms that ENSG00000233429.9 may be associated with NT5E to affect CRC progression. Thus, it is crucial to reveal the inherent regulatory relationship between them in the occurrence and development of CRC.

CircRNAs are emerging as critical regulators in various diseases, such as cardiovascular diseases,[29] diabetes,[30] neurological dysfunction,[31] and cancer.[32,33] In this study, several differentially expressed circRNAs were detected in HCT116 and FHC. Thereafter, GO and KEGG pathway analysis were used to predict that these circRNAs were functionally related to gene expression and metabolic pathways. The results showed that the most significant enriched GO for biological processes was the viral process; in cellular components, it was cytosol; and in molecular functions, it was ATP binding. Meanwhile, circRNAs in the network were enriched in ErbB signaling pathway by KEGG pathway analysis. According to ceRNA theory, a circRNA-miRNA regulatory network was constructed by the bioinformatics method. First, the key circRNAs were identified based on the fold change and *P* value in the microarray and by Venn diagram intersection. Then, the expression levels of selected 7 circRNAs were verified by qPCR. Finally, circ_0025288 had the highest fold change in HCT116 compared to FHC which was consistent with the microarray results. Next, circ_0025288 and its candidates were further studied. Thereafter, the potential targets of circ_0025288 were predicted based on the principle of complementary pairing with miRNA seed sequences. Among these miRNAs, miR-181a-3p reportedly inhibits A549/anlotinib cell apoptosis by targeting SHQ1,[34] while miR-455-5p is a potential marker of oral cancer.[35] This study revealed the abnormal expression of circRNAs in the development and progression of CRC and provided a theoretical basis for further study of the function of circRNAs in CRC.

In summary, this study found several abnormal aberrantly expressed lncRNAs, mRNA, and circRNAs in HCT116. For further study, these differentially expressed RNAs were verified by the RT-qPCR method, their potential roles were predicted, and lncRNA-miRNA-mRNA and circRNA-miRNA networks were constructed by bioinformatics method. This lays a theoretical foundation for in-depth research on the regulatory mechanism of ncRNAs in colorectal cancer genesis. Finally, it was found that high expression of ENSG00000233429.9 and hsa_circ_0025288 were closely related to the pathogenesis of CRC.

In the future, the underlying molecular mechanisms will be further investigated through functional loss and gain experiments. Thus, these findings may provide a potential therapeutic target for CRC and a deeper understanding of the molecular mechanisms of CRC. There are some limitations in this study, such as the small sample size, and the *in vitro* study only in HCT116 and FHC cells. The next step in this study will be to verify the key differential expression of lncRNAs, mRNA, and circRNAs in CRC patients.

AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no competing interests

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AUTHOR CONTRIBUTIONS

Conceptualization: Fei Tu, Qingzhi Wang; Data curation: Mengfan Li, Lun Hai; Formal analysis: Luyao Li; Investigation: Mengfan Li; Resources: Tiesuo Zhao and Sheng Guo; Project administration: Fei Tu; Validation: Fei Tu and Mengfan Li; Visualization: Mengfan Li; Writing – original draft: Fei Tu; Writing – review and editing: Fei Tu, Zhiwei Feng, and Qingzhi Wang.

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SUPPLEMENTARY INFORMATION

Figure S1. GO Enrichment and Pathway Analysis for Dysregulated lncRNAs Gene Symbols. (A) Most Significantly Enriched GO Terms of Up-Regulated lncRNAs Gene Symbols According to Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). (B) Most Significantly Enriched GO Terms of Down-Regulated lncRNAs Gene Symbols According to Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). (C) KEGG Pathway Analysis of the Up-Regulated lncRNAs in MEC. (D) KEGG Pathway Analysis of the Down-Regulated lncRNAs in HCT116.

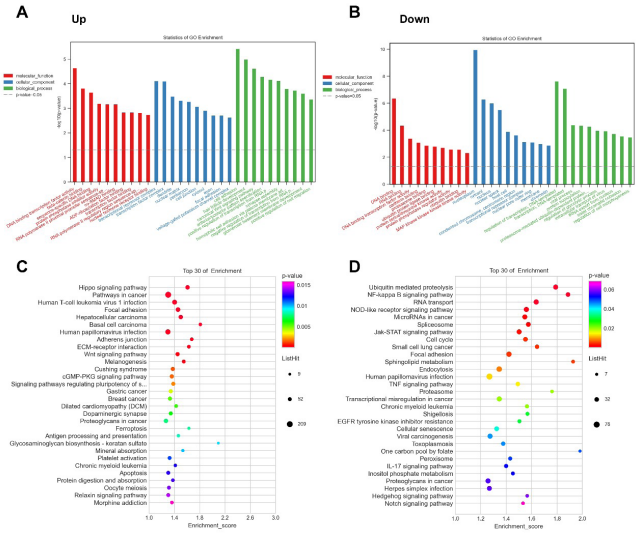

Figure S2. The lncRNA-mRNA Network of Significantly Aberrantly Expressed lncRNAs and their Target mRNAs. The Red Triangle Nodes Represent Differentially Expressed lncRNAs and the Green Oval Nodes Represent Differentially Expressed mRNAs.

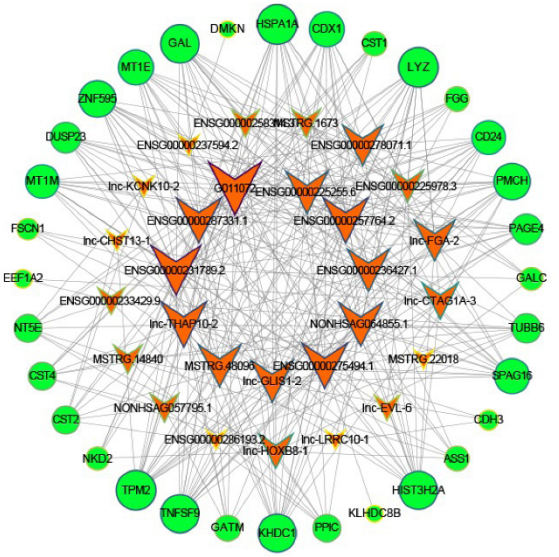

Figure S3. GO Enrichment and Pathway Analysis for Differentially Expressed circRNAs Gene Symbols. (A) Most Significantly Enriched GO Terms of the Aberrantly Expressed circRNAs Gene Symbols According to Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). (B) KEGG Pathway Analysis of the Aberrantly Expressed circRNAs in HCT116.

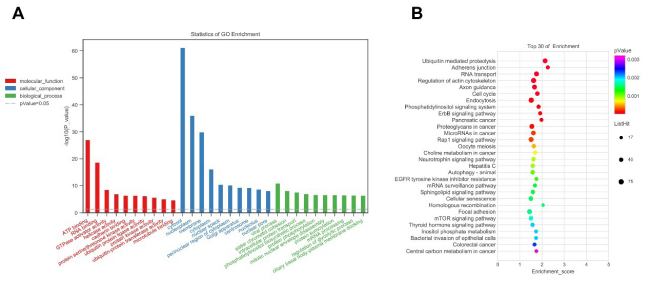

Figure S4. The Panorama Network of the Top 20 Significantly Aberrantly Expressed circRNAs (Triangle Nodes) and their Target miRNAs (Circular Nodes). The Red Triangle Nodes Represent Up-Regulated circRNAs and the Green Triangle Nodes Represent Down-Regulated circRNAs.