Identification and characterization of circRNAs as competing endogenous RNAs for miRNA-mRNA in colorectal cancer

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Background: Recent studies showed that circRNAs are involved in the biological process of some human cancers. However, little is known about their functions in colorectal cancer (CRC).

Methods: Here we first revealed the expression profiles of circRNAs in the CRC tissues and the adjacent non-tumorous tissues using high-throughput sequencing. The sequence feature, chromosome location, alternative splicing and other characteristics of the circRNAs were also explored. The miRNA and mRNA expression profiles were then obtained by analyzing relevant CRC data retrived from the TCGA database. We obtained and analyzed the competing endogenous RNA (ceRNA) network of the top 3 pairs of the largest up-regulated and down-regulated circRNAs.

Results: In this study, we obtained 50,410 circRNAs in the CRC tissue and the adjacent non-tumor tissues, of which 33.7% (16,975) were new, and revealed differential changes in circRNA expression during colorectal carcinogenesis. We have identified six potential key circRNAs (circPIEZO1-3, hsa_circ_0067163, hsa_circ_0140188, hsa_circ_0002632, hsa_circ_0001998 and hsa_circ_0023990) associated with CRC, which play important roles in carcinogenesis as ceRNA for regulation of miRNA-mRNA network. In the subsequent KEGG analysis, several CRC-related pathways were found.

Conclusions: Our findings advance the understanding of the pathogenesis of CRC from the perspective of circRNAs and provide some circRNAs as candidate diagnostic biomarkers or potential therapeutic targets.

Identification and characterization of circRNAs as competing endogenous RNAs for miRNA-mRNA in colorectal cancer

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Abstract

Background: Recent studies showed that circRNAs are involved in the biological process of some human cancers. However, little is known about their functions in colorectal cancer (CRC).

Methods: Here we first revealed the expression profiles of circRNAs in the CRC tissues and the adjacent non-tumorous tissues using high-throughput sequencing. The sequence feature, chromosome location, alternative splicing and other characteristics of the circRNAs were also explored. The miRNA and mRNA expression profiles were then obtained by analyzing relevant CRC data retrived from the TCGA database. We obtained and analyzed the competing endogenous RNA (ceRNA) network of the top 3 pairs of the largest up-regulated and down-regulated circRNAs.

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Conclusions: Our findings advance the understanding of the pathogenesis of CRC from the perspective of circRNAs and provide some circRNAs as candidate diagnostic biomarkers or potential therapeutic targets.

Introduction

Colorectal cancer (CRC) is a common malignant tumor of the digestive system in the world (1.4 million in 2012) [1], and more than 50% of the patients eventually die from this diseasee. Chemotherapy is still an indispensable treatment for CRC [2], however, with the advance of molecular biology and cell biology, targeted therapy has become a hotspot in cancer chemotherapy.

Circular RNAs (circRNAs) are a class of non coding RNAs featuring stable structure, often showing tissue/developmental-phase specific expression [3]. Compared with other non-coding RNA molecules, such as miRNAs and lncRNAs, circRNAs have more desirable biomarker features, such as the stable ring structure, that can be used for disease diagnosis, for example atherosclerosis [4] and gastric cancer [5]. In CRC research, two recent studies demonstrated that circRNA_001569 and circular BANP modulate cell proliferation in colorectal cancer [6, 7]. Recently, it was reported that hsa_circ_0020397 regulates CRC cell viability, apoptosis, and invasion [8]. Kuei-Yang Hsiao and colleagues also reported that circular RNA CCDC66 promotes colon cancer growth and metastasis [9].

In this study, we obtained the circRNA expression profiles of the CRC tissues and adjacent non-tumor tissues by high-throughput sequencing, and identified a small number of circRNAs with differential expression; then we analyzed the miRNA and mRNA data for CRC downloaded from the TCGA database; finally, we selected six circRNAs with the most significant differential expressions to analyze their circRNA-miRNA-mRNA network. In addition, Kyoto Gene and Genomic Encyclopedia (KEGG) analyses were performed.

Materials & Methods

Patients information

The CRC tissue specimens and the paired normal mucosa for circRNA detection were available from three CRC patients (two males and one female aged 58–66 years, mean age ± standard deviation (SD) 61.3 ± 4.2 years) who underwent surgery between May and October 2015 at the First Hospital of Jiaxing, China. The First Hospital of Jiaxing (Jiaxing, Zhejiang, China) granted Ethical approval to carry out the study within its facilities (Ethical Application Ref: FCFHJ-2017023). All the tissues were frozen in liquid nitrogen immediately after the surgery and then stored at −80°C until RNA extraction. All cases were newly diagnosed, histologically confirmed colorectal cancer patients, and had not received any chemotherapy or radiotherapy prior to recruitment. Written informed consent were received from all the patients.

RNA Sample quality testing

We used 1% agarose gel electrophoresis to analyze the purity and integrity of the RNA. The RNA integrity number (RIN) was measured using Agilent RNA 6000 Pico Reagents (Agilent, CA, USA) to assess the RNA quality. Sequencing was performed if the samples RIN values were greater than eight. The Qubit 2.0 instrument was used to accurately measure the RNA concentration.

Sequencing Library Preparation and circRNA Sequencing

A total amount of 1.5 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB,USA) following manufacturer's instructions. Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37℃ for 15 min followed by 5 min at 95℃ before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

After cluster generation, the prepared libraries were sequenced on an Illumina Hiseq 4000 platform and 150 bp paired-end reads were generated.

Screening and identification of colorectal cancer-associated circRNAs

To identify the circRNAs in the RNA-Seq data, the sequence reads were firstly mapped to the human reference genome (GRCh37/hg19, Feb., 2009) using TopHat2 (v2.1.0) [10]; Then, back-spliced ordering reads were extracted for circRNA prediction using CIRCexplorer [11]. These circRNAs were annotated by searching the circBase database [12] and the deepBase database [13]. Finally, differentially

expressed circRNAs were identified using edgeR [14], according to the criteria of a $|\log_2 FC| > 1.5$ and p-value < 0.05.

Prediction of the potential coding ability of circRNAs

It took two steps to predict the potential coding ability of differentially expressed circRNAs through bioinformatics method. Firstly, an online tool getorf (http://emboss.bioinformatics.nl/cgi-bin/emboss/getorf) was used to determine whether a ci***rc***RNA has a open reading frame (ORF). Then, we blasted the ci***rc***RNA sequences against all Internal Ribosome Entry Site (IRES) sequences using IRESite tool [15], and the ci***rc***RNAs with E Value < 0.05 were considered to have potential encoding capability.

Identification of differentially expressed miRNAs and mRNAs

To verify that circRNAs function as sponges or inhibitors of their interacting miRNAs, transcriptome profiling datasets were downloaded from TCGA. A data of 41 normal and 480 tumor samples for mRNA analysis were obtained. Similarly, the data of eight normal and 457 tumor samples were obtained for the miRNAs analysis by the same method. Finally, the differentially expressed miRNAs and mRNAs were identified using edgeR, according to the criteria of a fold change > 2.0 and false discovery rate (FDR) < 0.01.

miRNA spreadiction, co-expression network and function alanalysis

The putative circRNA/miRNA interactions were investigated by miRanda [16] using the miRNA list from miRBase release 20.0 [17]. The putative target genes of the miRNAs were predicted using the intersection of miRTarBase [18] and miRDB [19]. The information on the circRNAs of interest was obtained by CSCD [20].

The circRNA-miRNA-mRNA interaction network was constructed by Cytoscape. Cytoscape two plugins : ClueGO and CluePedia were used for KEGG analysis, showing only pathway with p Value < 0.05.

Results

Sequencing data

The sequencing yielded a total of 79.024 G of Raw data, and the filtered clean data totaled 72.874 G. The quality of the sequencing data was detailed in supplementary file 1.

General characteristics of circRNAs in CRC

A total of 50,410 circRNAs derived from 9,620 host genes were identified in the human CRC tissues and the adjacent non-tumorous tissues. Among them, 28,032 were found in circBase, 5,403 were

included in deepBase, and remaining 16,975 accounting for 33.7% of the total circRNAs were observed for the first time in this study.

According to their host gene location, the 50,410 circRNAs were widely distributed on all the chromosomes (Fig. 1A). Specifically, only chromosome 1 and chromosome 2 produced more than 4,000 circRNAs. Most other chromosomes generated more than one thousand circRNAs, except chromosome 21, Y and chrUn (with 542, 81 and 3 circRNAs, respectively). Our data showed that 49,801 (98.8%) circRNAs were excluded from the first or last exons of their host genes (Fig. 1B). In addition, we found that about 66.5% of the host genes produced multiple circRNA isoforms (Fig. 1C). We found that the BIRC6 host gene produces the highest numbers of circRNAs isoforms. Interestingly, it was described in other studies that BIRC6 overexpression is a predictor of poor prognosis in CRC [21] . Most exonic circRNAs consisted of multiple exons, with the most circRNAs containing two or three exons, and the maximum number of exons in a circRNA was 48 (Fig. 1D).

Insert Fig. 1 here.

Fig. 1 General characteristics of circRNAs in CRC. (A) Genomic features of circRNAs expressed in human CRC. Chromosomal distribution of the circRNAs. (B) Distribution of the back-spliced exons in circRNAs. (C) Distribution of the number of different types of circRNA transcripts from each circRNA host gene. (D) Distribution of the number of back-spliced exons in each circRNA.

Screening of the differentially expressed circRNA

The differentially expressed circRNAs between the CRC tissues and the adjacent non-tumorous tissues were identified. Finally, 98 circRNAs were identified, of which 49 were up-regulated and 49 were down-regulated (Supplementary file 2). The hierarchical clustering (Fig. 2A) and volcano plots (Fig. 2B) showed the variation of circRNA expression between the normal and the CRC samples. Additionally, the host genes of these differentially expressed circRNAs were derived from exonic regions (94), intronic regions (1, circMYO7B-3) (Fig. 2C), etc.

To predict the potential coding ability of the differentially expressed circRNAs, we found that 69 circRNAs (70%) contained at least one ORF, but only eight circRNAs had IRESs (Fig. 2D). To investigate the functional association of the host genes of the differentially expressed circRNAs in CRC, we analyzed the genes using the GeneMANIA plugin in the Cytoscape software (Fig. 2E). Most of the network interactions were co-expression, physical interactions and genetic interactions. The complex interaction between host genes suggests that this correlation may also exist between differentially expressed circRNAs.

Insert Fig. 2 here.

Fig. 2 Differential expression of circRNAs in CRC tissues. (A) Hierarchical clustering analysis of the circRNAs. CRC-D-A, CRC-D-B and CRC-D-C are adjacent normal tissue samples. The remaining three are cancer tissue samples. (B) Volcano plots are constructed using the fold-change values and q-values. The red dots in the figure represent statistically significant differentially expressed circRNAs. (C) Distribution of genomic regions that differentially expressed circRNAs derived from: exonic, intronic regions, etc. (D) Potentially encoded protein analysis of differentially expressed circRNAs. (E) GeneMANIA network of host genes of differentially expressed circRNAs.

Screening of differentially expressed miRNAs and mRNAs

According to the criteria of $|\log2FC| > 2$ and q-value < 0.01, 245 pre-miRNAs (DE_pmiRNA) and 2,083 mRNAs (DE_mRNA) were identified as aberrantly expressed in the CRC tissues compared with the adjacent non-tumorous tissues (Supplementary file 3 and Supplementary file 4). It was founded that many miRNAs and mRNAs were up-regulated or down-regulated more than 100-fold (Fig. 3A-B).

Insert Fig. 3 here.

Fig. 3 Differential expression analysis and interaction analysis of miRNAs and mRNAs. Volcano plots showing expression profile of pre-miRNAs (A) and mRNAs (B). (C) The intersection of the differentially expressed pre-miRNAs (DE_pmiRNA) and 1,666 pre-miRNAs. (D) The intersection of the differentially expressed mRNA (DE_mRNA) and 3707 miRNA target genes. (E) Comparison of data sizes before and after data processing. Purple indicates retained data and blue indicates discarded data.

Interaction between differentially expressed circRNAs, miRNAs and mRNAs

Evidence showed that circRNAs function as sponges or inhibitors of their interacting miRNAs to terminate regulation of their target genes [8, 9]. We obtained 1,666 pre-miRNAs including binding sites of the differentially expressed circRNAs, and 3,707 target genes of these pre-miRNAs by searching the three databases. Further analysis of DE_pmiRNA and these pre-miRNA, we found that 192 DE_pmiRNAs could interact with circRNAs (Fig. 3C). Similarly, we obtained 225 DE_RNAs related to the differentially expressed circRNA (Fig. 3D), and in this process, 40 DE_pmiRNAs were discarded because their target genes did not appear in this set (Fig. 3E). Interestingly, even if only 123-DE_pmiRNA s were retained, all of the differentially expressed circRNAs were still retained.

Networks Regulated by circRNAs

We selected the top 3 top down-regulated (circPIEZO1-3, hsa_circ_0067163, and hsa_circ_0140188) and up-regulated circRNAs (hsa_circ_0002632, hsa_circ_0001998 and hsa_circ_0023990) as the hub components referring to recent studies [22]. As shown in Fig. 4A, we found that all the six circRNAs belong to the exonic circRNA and are all cyclized by multiple exons.

To investigate the potential mechanisms of circRNA in the development and progression of CRC, we constructed the circRNA-miRNA-mRNA interaction network for these six circRNAs. The ceRNA interaction network consists of 6 circRNAs, 35 DE_miRNAs and 64 DE_mRNAs (Fig. 4B). By querying the clinical data in the TCGA database, we found that the expression level of six ceRNAs related mRNAs was significantly correlated with the survival time of CRC patients (Fig. 4C), suggesting that circRNAs-selected may have prognostic value. We found that high expression of hsa_circ_0023990 significantly improved survival time in patients with CRC due to high expression of SOX1, AQP6 and ITGBL1. Similarly, low expression of hsa_circ_0067163 associated with a poor survival due to a low expression of TPM2.

Insert Fig. 4 here.

Fig. 4 Information on six hub circRNAs. (A) The top three up-regulated and down-regulated circRNAs. The outer loop represents the exon that constitutes the circRNA, the innermost green ring represents the ORF, the middle red triangle represents the microRNA response element, and the blue cross point represents the RNA binding protein. (B) CircRNAs-miRNAs-mRNAs network. The red circle denotes the down-regulated circRNAs, the green circle denotes the up-regulated circRNAs, the blue inverted triangle denotes miRNAs, and the purple rectangle denotes the mRNAs. (C) Survival analysis of ceRNA - associated mRNA. (D) The KEGG pathway analysis of the top three circRNA pairs in the up-regulation and down-regulation circRNAs.

Functional enrichment analysis of circRNAs

The functional role of the most circRNAs has not been characterized, however, it would be beneficial to predict signaling pathways involving the circRNAs by bioinformatics methods. Therefore, according to the obtained ceRNA network and the target genes of the miRNA in the network, the KEGG pathway analysis of the six circRNAs was performed (Fig. 4D). There were 17 KEGG pathways significantly enriched in our study ($p < 0.01$). Among these pathways, some were directly linked to cancer pathogenesis, such as colorectal cancer, p53 signaling pathway [23], TGF-β signaling pathways [24] and microRNAs in cancer. Interestingly, although other pathways, such as cellular senescence [25] and Foxo signaling pathway [26], seemed to be not directly related to CRC, they were also found associated with multiple diseases development.

Discussion

Up to now, many circRNAs have been found in various human normal or diseased tissues. Researchers have identified 8,045 in heart, 3,982 in liver [27], 15,996 in testis [28] and 65,731 in normal human brain [29]. In our study, we predicted 50,410 circRNAs in the normal and diseased human colorectal tissues. Compared with other organs (for example heart, liver and testis), the expressions of the circRNAs in the human colorectal tissues are the most abundant. Our data showed that most circRNAs are excluded from the first or last exons of their host genes, which is consistent with previous research that back-spliced events are generally difficult to occur in the first or last exons of the host genes [30]. In the present study, we found that 66.5% of the 9,620 host genes produce multiple circRNA isoforms, suggesting that there are other factors contributing to the occurrence of back-spliced events, for example, non-repetitive sequences are largely included in these "hot-spot" genes [25].

Research showed that most circRNAs are derived from exonic regions and 5' UTR sequences [29]. Data analysis of our differentially expressed circRNAs also supports this view. Recent studies showed that circRNAs directly translate proteins and participate in various physiological processes [31, 32]. We analyzed circRNAs with differential expression and found that most of them contained ORF and IRES, indicating that these circRNAs have potential coding ability.

As is known, some oncogenes, such as RNA binding protein, ribosomal protein S5 (RPS5) and 5-hydroxytryptamine receptor 4 (HTR4), are differentially expressed in CRC compared with adjacent normal tissues [33, 34]. In our differentially expressed circRNA we found, the host genes, including hsa_circ_0128314 and hsa_circ_0005598, are HTR4 and RPS5, respectively. Therefore, we believe that some oncogenes will not affect their carcinogenic properties even if they are cyclized during transcription.

CeRNA hypothesis describes the mechanism for a class of RNAs with miRNA binding sites that competitively bind to miRNAs to inhibit their regulation of the target genes [35, 36]. The carcinogenic mechanism of circRNAs may occur through their miRNA-mediated effects on the gene expression, as circRNAs have more miRNA binding sites and are highly stable [37, 38]. In our study, based on the ceRNA hypothesis, we utilized paired circRNA, miRNA and mRNA expression profiles of the CRC patients combined with experimentally validated miRNA-target interactions to reconstruct circRNA-associated ceRNA network for the progression of CRC. However, this study has some limitations because functional analysis of circular RNA is based on bioinformatics analysis. As for our future experimental validation plan, we will focus on the in vitro validation of differential expression of hsa_circ_0023990 to verify its correlation with differentially expressed multiple mRNAs, such as SOX1, AQP6 and ITGBL1.

In the ceRNA network of the selected "hot-spot" circRNAs, we found that some miRNAs have been confirmed for their expression promoting colorectal cancer pathogenesis by other studies, such as

hsa-miR-29c-3p [39], suggesting that circRNA plays a role in the development of cancer by absorbing functional miRNAs to regulate the expression of corresponding genes.

The occurrence of colorectal cancer is not simply caused by a single signal pathway. Its occurrence and development are the result of the accumulation of multiple signal pathways, which are regulated by the network interlaced downstream of the pathway. Abnormalities in each pathway may cause disorder and/or cause colorectal cancer. The TGF-β signaling pathway regulates cell proliferation, differentiation, migration, apoptosis, and regulates stem cell repair [40]. The transcriptional co-activator with PDZ-binding motif and Yes-associated protein integrates with Wnt and TGF-β signaling in several cells and may have a significant effect on intestinal cell proliferation, differentiation and other functions [24].

Conclusions

In summary, in this study, we obtained 50,410 circRNAs in the CRC tissue and the adjacent non-tumor tissues, of which 33.7% (16,975) were new, and revealed differential changes in the circRNA expression during colorectal carcinogenesis. We have identified six potential key circRNAs associated with CRC, which play important roles in carcinogenesis as ceRNAs for regulation of miRNA-mRNA network. Our findings advance the understanding of the pathogenesis of CRC from the perspective of circRNAs and provide some circRNAs as candidate diagnostic biomarkers or potential therapeutic targets.

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Additional files

Additional file1: Summary of the quality of the raw sequencing data

Additional file2: Differentially expressed circRNAs in the CRC

Additional file3: Differentially expressed miRNAs in the CRC

Additional file4: Differentially expressed mRNAs in the CRC

Availability of data and materials

The RNA-seq data are deposited under NCBI BioProject (ID: PRJNA521856).

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Figure 1

General characteristics of circRNAs in CRC

(A) Genomic features of circRNAs expressed in human CRC. Chromosomal distribution of the circRNAs. (B) Distribution of the back-spliced exons in circRNAs. (C) Distribution of the number of different types of circRNA transcripts from each circRNA host gene. (D) Distribution of the number of back-spliced exons in each circRNA.

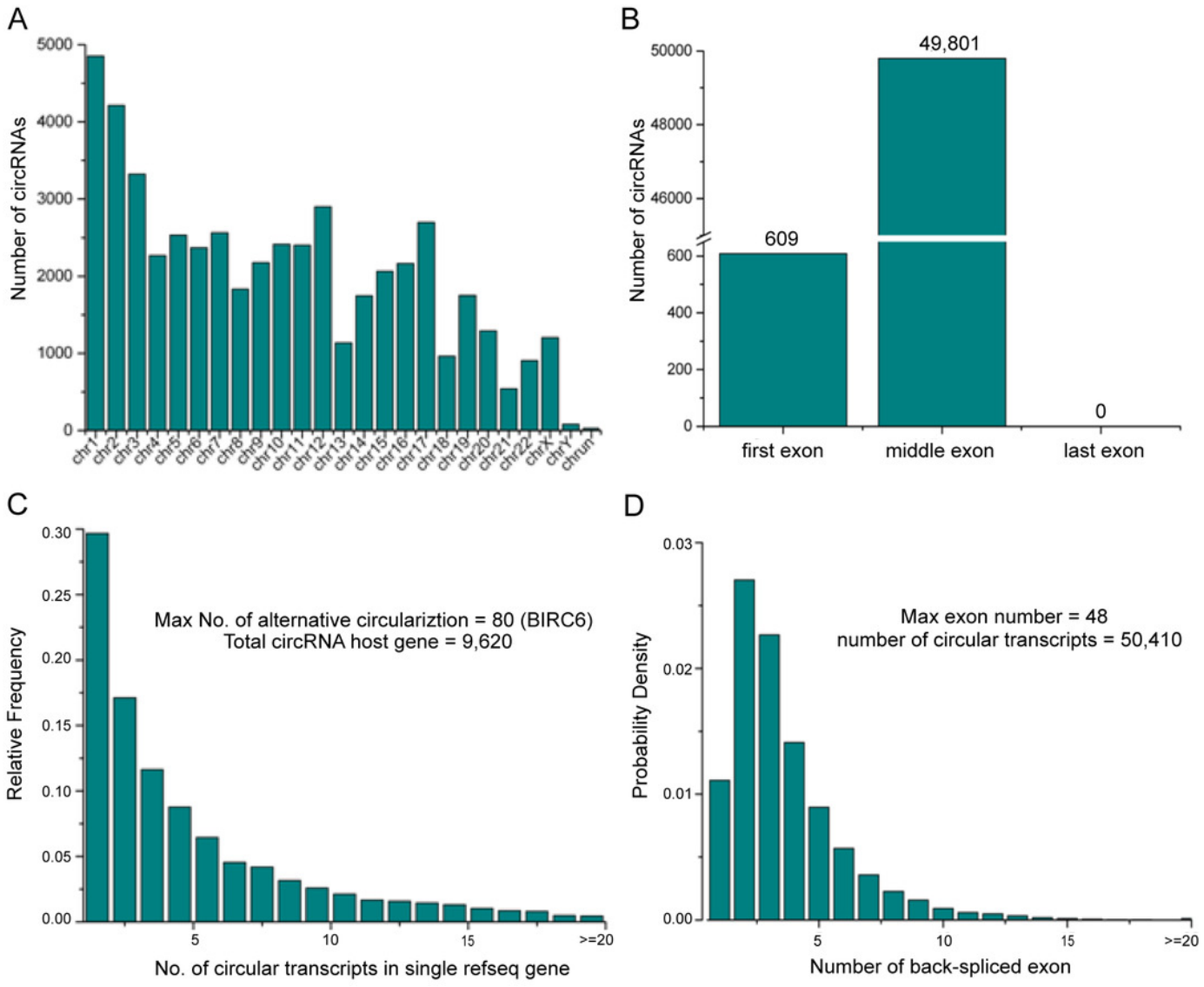


Figure 2

Differential expression of circRNAs in CRC tissues.

(A) Hierarchical clustering analysis of the circRNAs. CRC-D-A, CRC-D-B and CRC-D-C are adjacent normal tissue samples. The remaining three are cancer tissue samples. (B) Volcano plots are constructed using the fold-change values and q-values. The red dots in the figure represent statistically significant differentially expressed circRNAs. (C) Distribution of genomic regions that differentially expressed circRNAs derived from: exonic, intronic regions, etc. (D) Potentially encoded protein analysis of differentially expressed circRNAs. (E) GeneMANIA network of host genes of differentially expressed circRNAs.

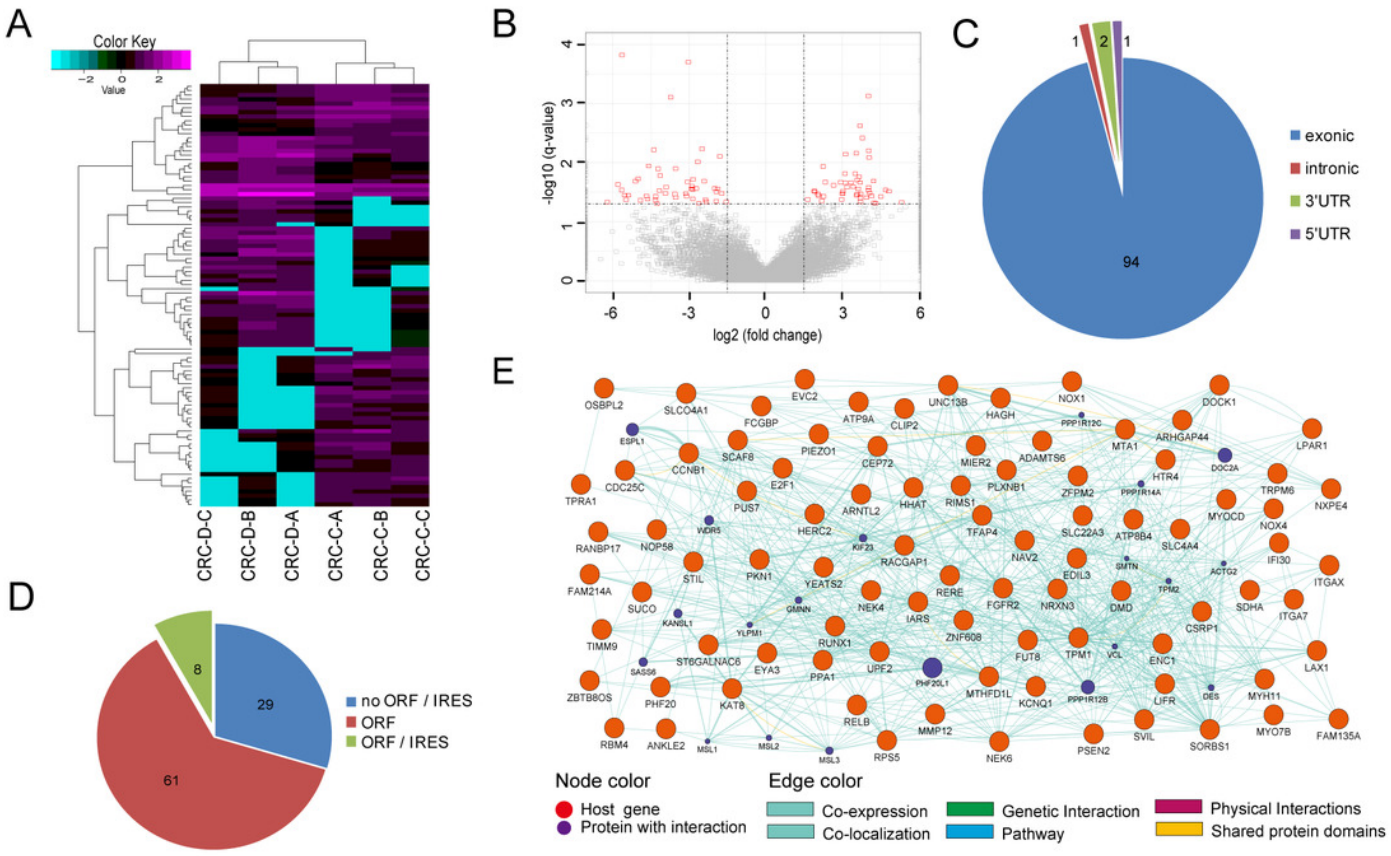


Figure 3

Differential expression analysis and interaction analysis of miRNAs and mRNAs

Volcano plots showing expression profile of pre-miRNAs (A) and mRNAs (B). (C) The intersection of the differentially expressed pre-miRNAs (DE_pmiRNA) and 1,666 pre-miRNAs. (D) The intersection of the differentially expressed mRNA (DE_mRNA) and 3707 miRNA target genes. (E) Comparison of data sizes before and after data processing. Purple indicates retained data and blue indicates discarded data.

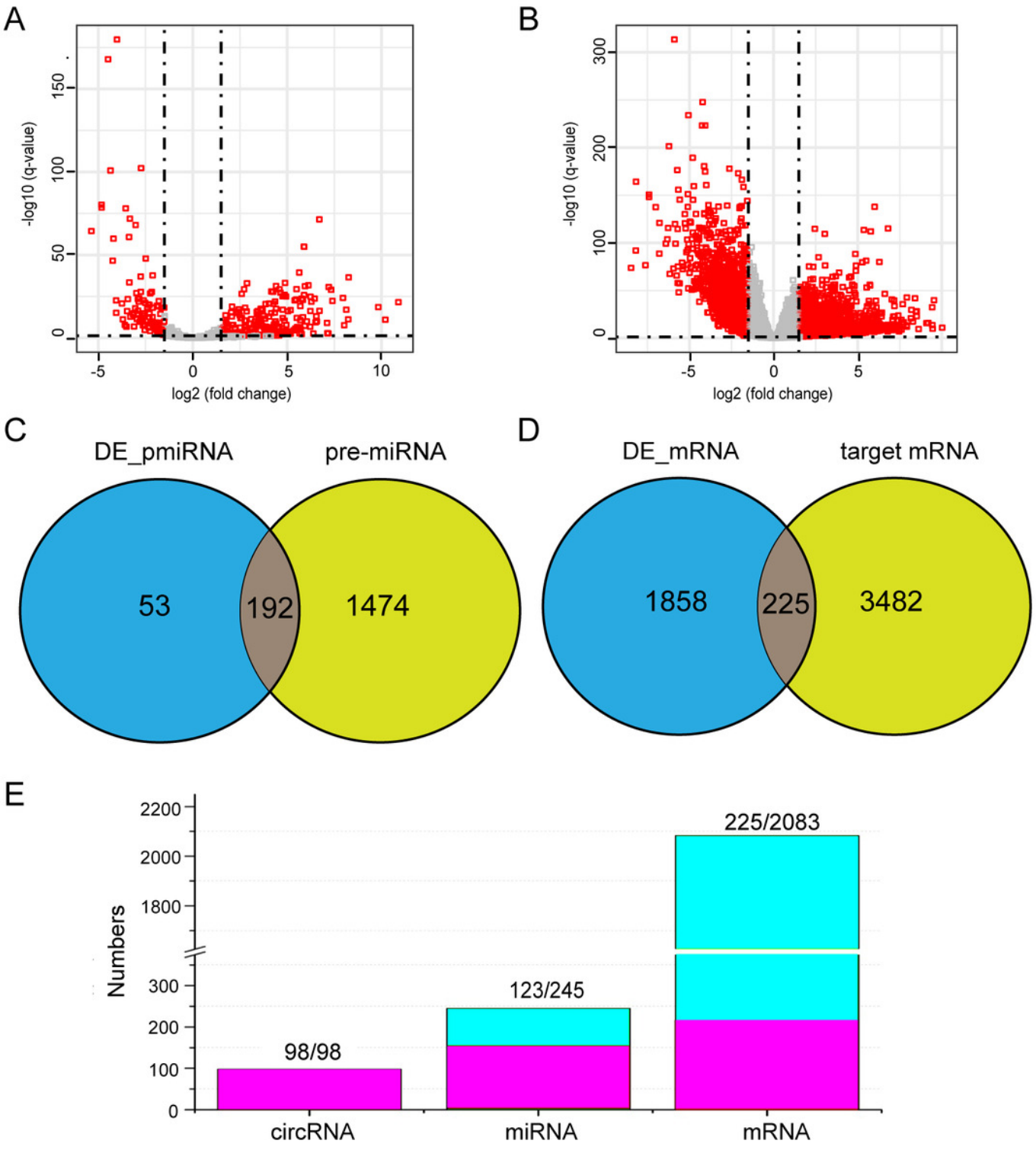
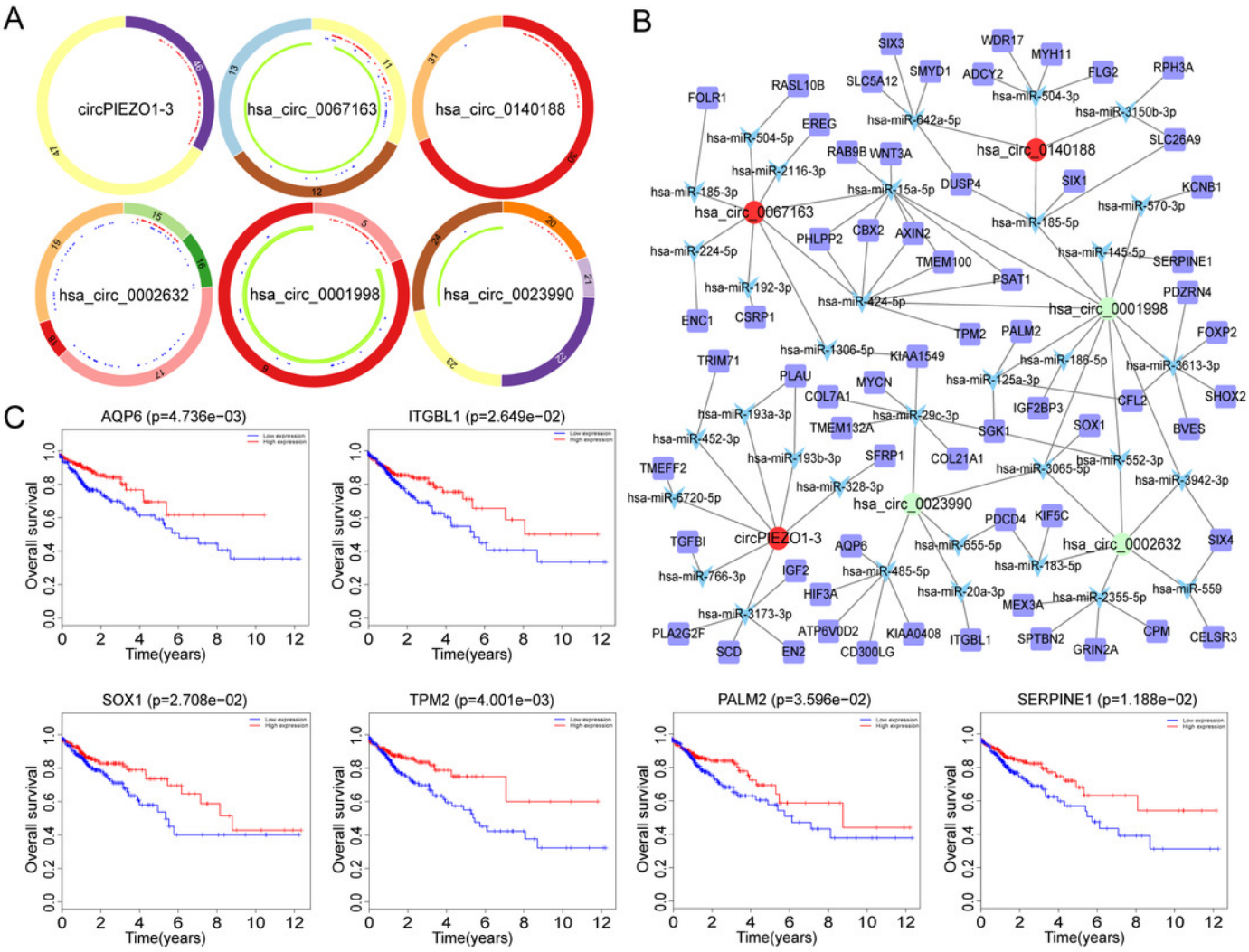

A
-log10 (q-value)
log2 (fold change)
B
-log10 (q-value)
log2 (fold change)
C
DE_pmiRNA
pre-miRNA
53
192
1474
D
DE_mRNA
target mRNA
1858
225
3482
E
Numbers
98/98
123/245
225/2083
circRNA
miRNA
mRNA

Figure 4

Information on six hub circRNAs

(A) The top three up-regulated and down-regulated circRNAs. The outer loop represents the exon that constitutes the circRNA, the innermost green ring represents the ORF, the middle red triangle represents the microRNA response element, and the blue cross point represents the RNA binding protein. (B) CircRNAs-miRNAs-mRNAs network. The red circle denotes the down-regulated circRNAs, the green circle denotes the up-regulated circRNAs, the blue inverted triangle denotes miRNAs, and the purple rectangle denotes the mRNAs. (C) Survival analysis of ceRNA - associated mRNA. (D) The KEGG pathway analysis of the top three circRNA pairs in the up-regulation and down-regulation circRNAs.


A
circPIEZO1-3
hsa_circ_0067163
hsa_circ_0140188
hsa_circ_0002632
hsa_circ_0001998
hsa_circ_0023990
B
hsa_circ_0140188
hsa_circ_0067163
hsa_circ_0001998
hsa_circ_0023990
circPIEZO1-3
hsa_circ_0002632
C
AQP6 (p=4.736e−03)
ITGBL1 (p=2.649e−02)
SOX1 (p=2.708e−02)
TPM2 (p=4.001e−03)
PALM2 (p=3.596e−02)
SERPINE1 (p=1.188e−02)
Overall survival
Time(years)
D
TGF-beta signaling pathway 1.79%**
MicroRNAs in cancer 1.79%**
Sphingolipid signaling pathway 1.79%**
Cell cycle 1.79%**
p53 signaling pathway 1.79%**
Hippo signaling pathway 1.79%**
Adherens junction 1.79%**
Tight junction 1.79%**
Transcriptional misregulation in cancer 1.79%**
Oocyte meiosis 3.57%**
Human papillomavirus infection 3.57%**
Autophagy 5.36%**
Focal adhesion 7.14%**
Colorectal cancer 7.14%**
Cellular senescence 42.86%**
FoxO signaling pathway 10.71%**