Deciphering the Roles of CircRNAs on Hepatic Metastases in Early Tumor Stage Colorectal Cancer Patients

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Research Article

Keywords: Colorectal cancer, circRNAs, ceRNA

Posted Date: June 2nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1500197/v2

Abstract

Objective

Circular RNAs (circRNAs), a specific type of non-coding RNAs, could regulate tumorigenesis and metastasis of various cancers though acting as competing endogenous RNA (ceRNAs).

Methods

Performing next generation sequencing (NGS) and bioinformatics methods, the profiles and the intricate roles of circRNAs were clarified in early stage colorectal cancer patients with hepatic metastases.

Results

CircRNAs were predicted to be involved in CRC hepatic metastasis complex processes, such as p53 binding and ErbB excision repair. Intriguingly, we detected 19 significantly differentially expressed among CRC patients with or without hepatic metastases and found circFARSA was significantly upregulated. Besides, circFARSA was found to be potential ceRNAs and a triple regulatory network of circFARSA/hsa-miR-503-5p/CCND2 was set up, which describes the possible mechanisms of CRC hepatic metastasis.

Conclusions

Our findings demonstrated that circFARSA acts as a promising biomarker in metastatic colorectal cancer (mCRC). The study provided a new perspective for targeting circFARSA in mCRC therapeutic treatment.

Introduction

Colorectal cancer (CRC) is a cancerous tumor in the digestive system that ranks as the third in regard to incidence but the second in regard to mortality [1]. 60% of CRC patients have progressed to the metastatic stage with negligible symptoms once diagnosed although benefited from local resection [2]. The 5-year survival rate is up to 90% or 82% in patients with stage I or II disease, but declines to 12% in patients with stage IV disease [3]. The important factors that affect the treatment and prognosis of CRC are tumor invasion and metastasis, especially liver metastasis [4–5]. Therefore, it is urgently needed to develop a specific biomarker for early identification and detection in CRC.

Circular RNAs (circRNAs) were considered as splicing errors in the past and rapid advances in high-throughput sequencing and bioinformatics technologies make the knowledge of circRNAs change [6]. CircRNAs, present in the cytoplasm of eukaryotic cells mostly, are a special class of noncoding RNAs (ncRNAs) molecules which do not have a 5’end cap and a 3’end poly(A) tail [7]. With the closed loop, circRNAs have better stability and certain sequence conservation than linear RNA [7]. Although there are

still largely unclear in the detailed biological functions of circRNAs, emerging researches indicate that circRNAs have important roles in the pathogenesis or progression of lung squamous cell carcinoma [8], breast cancer [9], hepatocellular cancer [10] and gastric cancer [11]. Notably, circRNAs are also aberrantly expressed in CRC and these accumulating evidences indicated that circRNAs could serve as a noninvasive biomarker for CRC [12–14].

Based on this hypothesis, we attempted to investigate the role of circRNAs in CRC hepatic metastasis. In this study, we studied the expression profiles of circRNAs in mCRC as well as predicting the targets of differentially expressed circRNAs. We found that circFARSA significantly upregulated in mCRC and a circFARSA/hsa-miR-503-5p/CCND2 regulatory axis was set up, which may provide a promising biomarker and shed new light on new therapeutic strategies treating CRC hepatic metastasis. In addition, we further excavated genomic characteristics with metastatic colorectal cancer and probed the mutation pattern and molecular network of target gene CCND2 of regulatory axis.

Materials And Methods

Sample collection and preparation.

Control and mCRC samples from four patients were used to prepare for circRNA-Seq. On 1.5% agarose gels, we monitored RNA degradation and contamination. Using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 System (Agilent Technologies, CA, USA), we measured RNA concentration as well as purity and assessed RNA integrity. We used the Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA) to removal rRNA from total 1.5 μg RNA each sample. According to the manufacturer, NEBNext^{R} Ultra^{TM} Directional RNA Library Prep Kit for Illumina^{R} (NEB, USA) was used to prepare libraries.

Under elevated temperature, we used divalent cations to carry out fragmentation in NEBNext First Strand Synthesis Reaction Buffer(5X). Random hexamer primer and Reverse Transcriptase were used to synthesize first strand cDNA. Subsequently, DNA Polymerase I and RNase H was used to synthesize second-strand cDNA. Using exonuclease/polymerase activities, we converted remaining overhangs into blunt ends. 3’ ends of DNA fragments were adenylate and then NEBNext Adaptor were ligated which have hairpin loop structure. All that were prepare for hybridization. For selecting insert fragments, we purified the library fragments with AMPure XP Beads (Beckman Coulter, Beverly, USA). The length of insert fragments were about 150~200 bp preferentially. Before PCR, 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min. Followed by which, we conducted PCR with Universal PCR primers, Phusion High-Fidelity DNA polymerase and Index(X) Primer. Finally, we purified PCR products (AMPure XP system) and assessed library quality on the qPCR and the Agilent Bioanalyzer 2100. According to the manufacturer, we used TruSeq PE Cluster Kitv3-cBot-HS(Illumia) and performed the index-coded samples clustering on acBot Cluster Generation System. Cluster was generated. Followed by which, we can sequence the library preparations on an Illumina platform and generate the reads.

Data analysis of CircRNAs .

We processed the raw data of fastq format via in-house perl scripts and removed reads containing ploy-N, reads containing adapter and low quality reads to obtain clean data. In the meantime, we calculated GC-content, Q20 and Q30 of clean data. According to clean data with high quality, we conducted the downstream analyses.

Find_circ software and CIRI (CircRNA Identifier) tools were used to identify circRNA. SAM files were scan twice by CIRI so that sufficient information was collected for circRNAs identification and characterizing. Briefly, On the first SAM alignment scanning, junction reads were detected by CIRI which were with PCC signals reflecting a circRNA candidate. We used paired-end mapping (PEM) and GT-AG splicing signals to implement preliminary filtering. Each circRNA candidate was recorded and junction reads were clustered. After that, the SAM alignment was scan by CIRI again and additional junction reads were detected. Meanwhile, further filtering was performed to eliminate false positive candidates. Finally, we can output identified circRNAs with annotation information.

The find_circ software will first be able to take 20 bp from both ends of the reads on the genomic alignment as anchor points, and then the anchor points as independent reads mapped to the reference genome and find the unique matching site. If the alignment position of the two anchor points is reversed in the linear direction, the reading of the anchor point is extended until the joint position of the circular RNA is found. When the signal is spliced for GT/AG, it is judged to be a circular RNA. The intersect of the results of the two methods will be the final prediction result.

Quantification of circRNAs expression levels

Using CIRI tools and find_circ software, we determined the expression of circRNA by the number of junction reads identify. Using the DESeq R package, we performed the differential expression analysis of two groups. Statistical routines, provided by DESeq, can determine differential expression in digital gene expression data. Statistical routines used the model on account of the negative binomial distribution. To control the false discovery rate, we used the Benjamini and Hochberg's approach to adjust the resulting P-values. Differentially expressed genes were considered as one with an adjusted P-value <0.01 and log2(Fold change) >1 or <-1. We annotated gene function based on the these databases: non-redundant protein sequence database (NR); Clusters of Protein homology database (KOG); the Swiss-Prot non-redundant protein sequence database; Gene Ontology database (GO); Kyoto Encyclopedia of Genes and Genomes database (KEGG); Clusters of Orthologous Groups of proteins database (COG);the Pfam database.

CircRNAs target prediction

Potential miRNAs that may bind to the differentially expressed circRNA were predicted by searching the CircBank database (http://www.circbank.cn/index.html) and the CircInteractome database

(https://circinteractome.nia.nih.gov/index.html). The candidate miRNAs obtained from the two databases were intersected.

MiRNAs target prediction

The target genes of miRNAs were predicted by retrieving the miRDB database (http://www.mirdb.org/) and the TargetScan database (http://www.targetscan.org). From each database, the top 15 target genes with the highest scores were selected. After selection, the candidate genes were intersected.

Whole-exome sequencing (WES)

Whole-exome sequencing for all patients were performed on Illumina Novaseq 6000 using 2 × 150 bp pair-end sequencing method, according to the manufacturer's instructions.Tumor sample was obtained via core biopsy during initial diagnostic procedure. Genomic DNA was extracted from paraffin embedded slides by using an E.Z.N.A tissue DNA Kit (Omega Biotek, Doraville, USA) according to the manufacturer's protocol.Cell-free DNA (cfDNA) was extracted as described. In brief, 10 ml of whole blood was collected and cfDNA was extracted from plasma samples using the QIAamp Circulating Nucleic Acid kit (Qiagen) according to the manufacturer's instructions.Library preparation was performed as described. Fragments of size 200–400bp were selected and followed by hybridization with capture probes baits, hybrid selection with magnetic beads and PCR amplification. Indexed samples were sequenced on Hiseq Xten VS NovaSeq 6000 (Illumina, Inc., USA) with 2 × 150 bp pair-end reads. The sequencing data in the FASTQ format were mapped to the human genome (hg19) using BWA aligner 0.7.10. Local alignment optimization, variant calling and annotation were performed using GATK 3.2, MuTect 2, and VarScan, respectively.

Quantitative reverse-transcriptase real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from different groups of cells using TRIzol Reagent Kit (Invitrogen, USA), and then were reverse-transcribed into cDNA. We fastened the products of qRT-PCR to agarose gel electrophoresis (Fermentas, USA), and normalized the expressions of circRNAs, miRNAs and mRNAs by glyceraldehyde 3-phosphate dehydrogenase (β-actin). Using the $2^{-\Delta\Delta Ct}$ method, all target RNA transcripts expressions were calculated. All experiments were repeated for three times. The mean value was used as a result of the experiment. The primers were designed for qRT-PCR analysis as follow: The sequences of primers were forward 5'-GCTCCTTCTGGAACTTTGAC-3' and reverse: 5'-TTGCTCACCCAGTAGGTCTT-3' for circFARSA; Forward 5'- tgccctagca gcgggaacag ttctgcagtg -3' and reverse 5'-agcgatcggt gctctggggt attgtttccg -3' for hsa-miR-503-5p; Forward: 5'- AGATCTATGGAGCTGCTGTGCCACGA-3' and reverse 5'-AGATCTTCACAGGTCGATATCCCGCAC-3' for CCND2.

Patient and Public Involvement

We selected 3 cases of typical metastatic colorectal cancer patients as the research object, and another case of colorectal cancer patients without liver metastasis for research. We had obtained the consent of

patients before operation and took the tissue for sequencing after operation. Patients did not participate in the process of research and paper design, only provided cancer tissue, and did not carry out any intervention on patients. In addition, we will fully inform the patients of the sequencing results through postoperative follow-up, carry out health education, and provide patients with appropriate treatment and prevention methods.

Statistical analysis

We performed all statistical analyses using SPSS version 17.0 statistical software (SPSS, Chicago, USA). The measurement data were presented as the mean ± standard error of mean (S.E.M), and statistical significance was determined by t-tests. The frequency of gene overexpression between two paired patients was compared by using the Pearson $\chi 2$ test. The relationships between gene levels and variables were analyzed by conducting the Spearman's, Pearson's, and linear regression analyses. Statistical significance was calculated using Origin 8.0 software programs (OriginLab, USA) and was considered at p-value < 0.05. GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA) was performed for all analyses.

Results

Identification of circRNAs in mCRC.

Resulting poor prognosis, five-year survival low at only 15%, metastasis is the primary cause of death in CRC [1]. Most patients with colorectal carcinoma develop metastatic disease at T2 stage. As show in Fig. 1A, three were liver mCRC, and one CRC in Four T2 stage samples. To demonstrate circRNAs possible regulatory roles in mCRC, four circRNA libraries (L01, L02, L03, L04) from mCRC (L01, L02, L03) and control sample (L04) were constructed and sequenced. Total generated raw reads were 112.79 (L01), 119.20 (L02), 118.90 (L03) and 110.95 (L04) million, respectively. There were 106.67 (L01), 112.11 (L02), 108.15 (L03) and 99.35 (L04) unique mapped reads after careful screening. A total 802 (L01), 661 (L02), 494 (L03) and 1140 (L04) circular RNAs were found through further analysis. To ensure the reliability of differentially expressed circRNAs, biological replicates were used to detect the correlation coefficient of the samples (Fig. 1B). A total of 1828 circRNAs were identified, out of which 1140 were known circRNAs and 688 were newly predicted circRNAs (Fig. 1C). CircRNAs expression were analyzed and normalized to FPKM. Box and density plots showed the integral distribution of the circRNAs (Fig. 1D-1E). The lengths of circRNAs identified in this study mostly were 400–1200 bp (Fig. 1F). As an analysis of the circRNAs expression described, the numbers of circRNAs produced from chromosomes was different from each other and the chromosome six produced the most (Fig. 1G). CircRNAs can arise from introns, exons and intergenic regions [7], which was consistent with our results. Furthermore, the predominant type was the exon derived circRNAs in every library (Fig. 1H).

The expression patterns of circRNAs in mCRC.

422 differentially expressed circRNAs were expressed in L04 vs L01, 273 (64.69%) of them were down-regulated and 149 (35.31%) were up-regulated. 410 differentially expressed circRNAs were expressed in L04 vs L02, 261 (63.66%) of them were down-regulated and 149 (36.34%) were up-regulated. Out of the 436 differentially expressed circRNAs in L04 vs L03, 324 (74.31%) of them were down-regulated and 112 (25.69%) were up-regulated. Among the 19 differentially expressed circRNAs in L04 vs L01_L02_L03, 1 (5.26%) were up-regulated and 18 (94.74%) were down-regulated (Fig. 2A). The differential circRNAs expression profile between the mCRC and control groups was revealed via hierarchical clustering (Fig. 2B). 19 differentially expressed circRNAs were expressed in all the four samples (Fig. 2C).

GO and KEGG enrichment analysis.

To understand the function of the circRNAs, we analyzed the circRNAs in the L04_vs_L01, L04_vs_L02, L04_vs_L03, L04_vs_L01_L02_L03 samples by gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis. Our data suggested that host genes related to diverse functions involved in different biological processes, molecular functions and cellular components (Fig. 3 and Supplement Fig. 1–3). The most relevant GO terms associated with biological processes were Golgi organization and neuron projection development, with cellular component were microtubule and aggresome and with molecular functions was p53 binding (Fig. 4). KEGG pathway analyses revealed several key pathways such as Lysine degradation, ErbB excision repair might play a role in mCRC (Fig. 5).

The target prediction and the roles of circRNAs acting as ceRNAs.

Acting as ceRNAs, circRNAs exhibits a vital effect in the control of gene expression mediated by miRNA. Hsa_circ_0000896 (termed circFARSA in the remainder of article), the only one upregulated circRNAs attracted our attention. CircFARSA arises from the FARSA gene, which is located at chromosome 19. To further understand the biological functions of circFARSA, potential targeting miRNAs were predicted in CircBank and CircInteractome databases by bioinformatics. The predicted intersecting miRNA were hsa-miR-503-5p, hsa-miR-330-5p and hsa-miR-1197, which might indicate the potentially functional miRNAs absorbed by circFARSA in CRC cells (Fig. 6A). Then, we continued to predict the potential target genes of hsa-miR-503-5p, hsa-miR-330-5p and hsa-miR-1197 using miRDB and TargetScan. We picked out the top 15 predicted target gene and then searched the intersection. As showed in Fig. 6B, hsa-miR-503-5p was predicted to target CCND2 and FAM122A, hsa-miR-330-5p target PALM and hsa-miR-1197 target ZNF501 and EMC7. Given that the expression of CCND2 has shown potential effects in CRC proliferation and metastasis [15, 16], hsa-miR-503-5p was selected to be the potential miRNAs targeted by circFARSA. CircFARSA induces the angiogenesis and metastasis of CRC by sponging hsa-miR-503-5p to increase CCND2 expression.

To elucidate the relationship between circFARSA, hsa-miR-503-5p and CCND2, we used qRT-PCR to measure their expression levels in our samples. CircFARSA and CCND2 were up-regulated, and hsa-miR-503-5p were down-regulated in the CRC samples (Fig. 6C). Furthermore, it was observed that a correlation between circFARSA and hsa-miR-503-5p as well as hsa-miR-503-5p and CCND2 expression in both

primary and metastases samples (Fig. 6D). These results indicated that circFARSA/hsa-miR-503-5p/CCND2 axis may regulate hepatic metastases in early tumor stage colorectal cancer patients.

Genomic landscape of target gene CCND2 in mCRC.

For the purpose of further providing more evidence to expound that circFARSA, as a regulatory axis of ceRNA, competitively binds to hsa-mir-503-5p to regulate the expression of upstream target gene CCND2. We used whole-exon sequencing (WES) to uncover the effect of target gene CCND2 mutation on circFARSA/hsa-miR-503-5p/CCND2 axis.

We assessed the genomic mutation characteristics of 39 mCRC patients with metastatic colorectal cancer and the results showed that 344 mutation genes were excavated in total, most of the gene fragments were presented with C > T or T > C transversions (Fig. 7A), but no significant difference was noted in the driving genes. Throughout 39 patients with overall mutations, most of the patients were nonsense mutations (Supplementary Fig. 4A). We accumulated each mutated differential genes in all patients (maximum score is 39) and ranked them from high to low (Supplementary Fig. 4B). The analysis showed that most of the significant mutation differential genes also had nonsense mutation (Fig. 7B). Therefore, the mutation types of 344 differential genes were consistent with 39 samples. The mutation rate of CCND1, CCND2, CCND3 in gene mutation spectrum was 94.87%, 46.15%, 87.18%, respectively, which implied that there was a 50% probability of CCND gene family mutation in mCRC patients. Thereinto, CCND2 has the highest frequency of nonsense mutations in CCND gene family (Fig. 7C). The elevated frequency of nonsense mutation may produce effects on RNA transcription or modification level. Ultimately, activating regulation of circFARSA/hsa-miR-503-5p/CCND2 axis. Next, biological function annotation of GO and KEGG pathway annotation were utilised to cluster differential genes with mutation rate close to 50%, we also applied String and Cytoscape to gather by interactive networks (confidence > 0.8) (Fig. 7D). The biological function of the mutant genes was mainly concentrated in the activation of transmembrane receptor protein tyrosine kinase (blue point) and protein kinase binding (red point). CCND gene family was mostly focused on Wnt signaling pathway. Wnt signaling pathway had been previously proved to participate the process of EMT, which is closely related to metastatic colorectal cancer [17–18]. Other pathways also indirectly regulated CCND gene family, including PI3K-Akt signaling pathway. Together, all above illustrating the significant effect of CCND2 mutation in colorectal cancer metastasis.

Discussion

Without effective and sensitive prognostic markers, it was not easy to identify in CRC primary stage by regular examinations, such as CEA and CA19-9[19]. Integrated bioinformatic analysis suggest new strategies to treatment identification and detection of CRC before invasion or metastasis. CircRNAs, the new star molecules in cancer, have exhibited important roles in the pathogenesis of the major human systems cancer[20]. We believe that it will be an important part of mCRC early identification and targeted therapy though improving our understanding of circRNA in mCRC mechanisms.

In this study, we utilized RNA-seq and found 802 (L01), 661 (L02), 494 (L03) and 1140 (L04) circRNAs in mCRC at T2 stage. Generated from diverse genomic regions chromosomes, circRNAs derived from the exon made up the largest proportion. Though circRNAs distributed in all human chromosomes, the chromosome six hold most. GO classification and KEGG pathway analyses were used for understanding the exact function of circRNAs. Intriguingly, circRNAs was associated with p53 binding, which was a key protein for cell cycle control and apoptosis in various tumors and has been reported to affects invasive behavior in CRC [21]. In addition, circRNAs play a role in ErbB excision repair. ErbB family receptors are well-known oncogenes that consist of four members including ErbB1(EGFR, HER1), ErbB2(HER2), ErbB3, and ErbB4, and could be emerging targets in mCRC [22]. In a word, circRNAs may participate in CRC hepatic metastasis complex processes.

We further revealed 1 upregulated circRNAs and 18 downregulated circRNAs in mCRC at T2 stage. The results proved that circRNAs was aberrantly expressed and have roles in mCRC at early stage. Among 17 differentially expressed circRNAs, circFARSA aroused our great interest. A study by Hang have revealed that overexpression of circFARSA significantly promoted NSCLC cells invasion and migration [23]. However, the role of circFARSA in CRC liver metastasis has not been reported.

Increasing evidence revealed that circRNAs can act as ceRNA to exhibit an important effect in gene expression [24, 25]. We try to establish a triple network of circRNA-miRNA-mRNA to identify which circRNA is specific biomarkers for mCRC. Using the bioinformatics method, circFARSA/hsa-miR-503-5p/CCND2 regulatory axis was predicted (Fig. 6E). Previous findings support the notion that hsa-miR-503-5p exerts inhibitory effects on tumor cells proliferation and survival [26, 27]. As a member of the D-type cyclin family, CCND2 has been implicated in cell cycle control, and inhibited cancer cell growth and migration ability in various malignancies [28, 29]. It is also associated with CRC liver metastasis [30, 31, 32]. Based on the genomic characteristics of the mutation spectrum with liver metastatic colorectal cancer, 344 differentially expressed genes were found. Notably, the mutation rate of CCND gene family, including CCND1, CCND2, CCND3, is more than or close to 50%. These results indicate that mutations of CCND gene family plays an indispensable role in the occurrence and metastasis of colorectal cancer. It is consistent with previous research of Jardim et al [33]. We conjecture the high frequency of nonsense mutation in target gene CCND2 may lead to changes in transcriptome levels of circFARSA/hsa-miR-503-5p. All mentioned above aid to supports the conclusion that circFARSA silencing could attenuate CRC progression through hsa-miR-503-5p-dependent CCND2 inhibition.

Conclusions

In conclusion, this work is the first to report that circFARSA is significantly up-regulated in mCRC and predict circFARSA/hsa-miR-503-5p/CCND2 regulatory that describes the possible mechanisms of CRC hepatic metastasis. The findings highlighted the promising uses of circFARSA as a novel target for mCRC treatment.

Declarations

Acknowledgments

Not applicable.

Authors' Contributions

Maosen Huang, Linyao Cheng, Haiming Ru, Chunyin Wei, Xianwei Mo and Linhai Yan critically revised the drafting work or important knowledge content, and finally approved the version to be released. Maosen Huang and Linyao Cheng collected tissue samples. Maosen Huang, Haiming Ru, Chunyin Wei, and Xianwei Mo mapped and statistically analyzed the sequencing results. Maosen Huang and Linyao Cheng did the experimental verification. Linhai Yan tracked the status of articles in time.

Funding

This work was supported by the National Natural Science Foundation of China (82160495 and 81973533). China 330 Postdoctoral Science Foundation (No. 2019M653812XB). Guangxi University High-level Innovation Team and the Project of Outstanding Scholars Program (2019AC03004) and Guangxi Science and Technology Project (AD19245197).

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Sample collection and research were in accordance with regulations issued by the National Health Commission of China and the ethical standards formulated in the Helsinki Declaration. Written informed consent was obtained from all patients. The permission for retrospective study was obtained from the institutional review board of Guangxi Medical University Cancer Hospital (KY2021279).

Consent for publication

Not applicable.

Competing interests

No competing financial interests exist.

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Figures

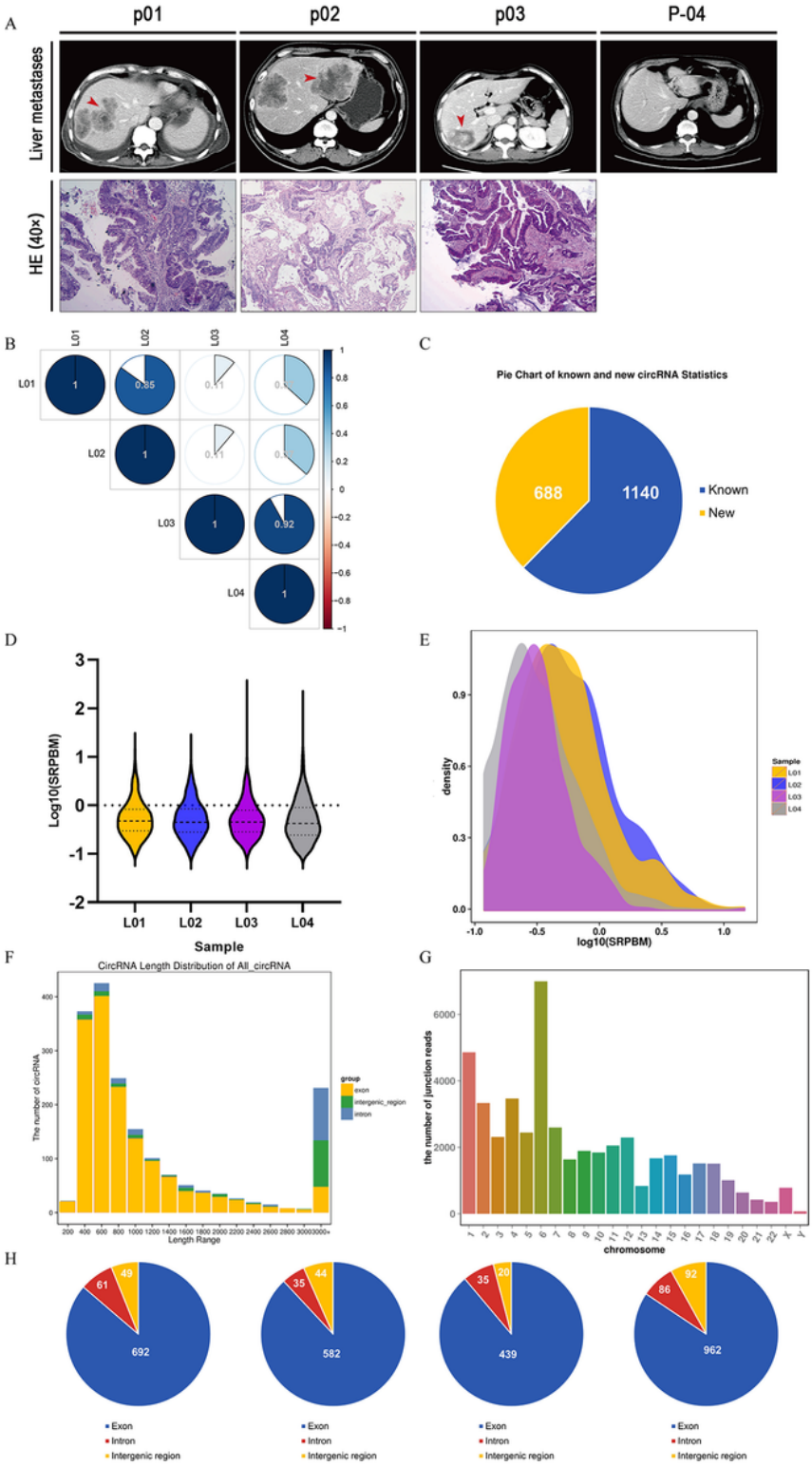

Figure 1

A The red triangles indicate multiple intrahepatic metastases. Hematoxylin confirmed that are colorectal cancer. **B** Correlation of samples. **C** Classification of the mCRC circRNAs. **D** Expression levels of circRNAs. **E** Expression density of circRNAs in the samples. **F** Length distribution of tomato circRNAs. **G** The chromosome distribution of the circRNAs. **H** The source and distribution of circRNAs.

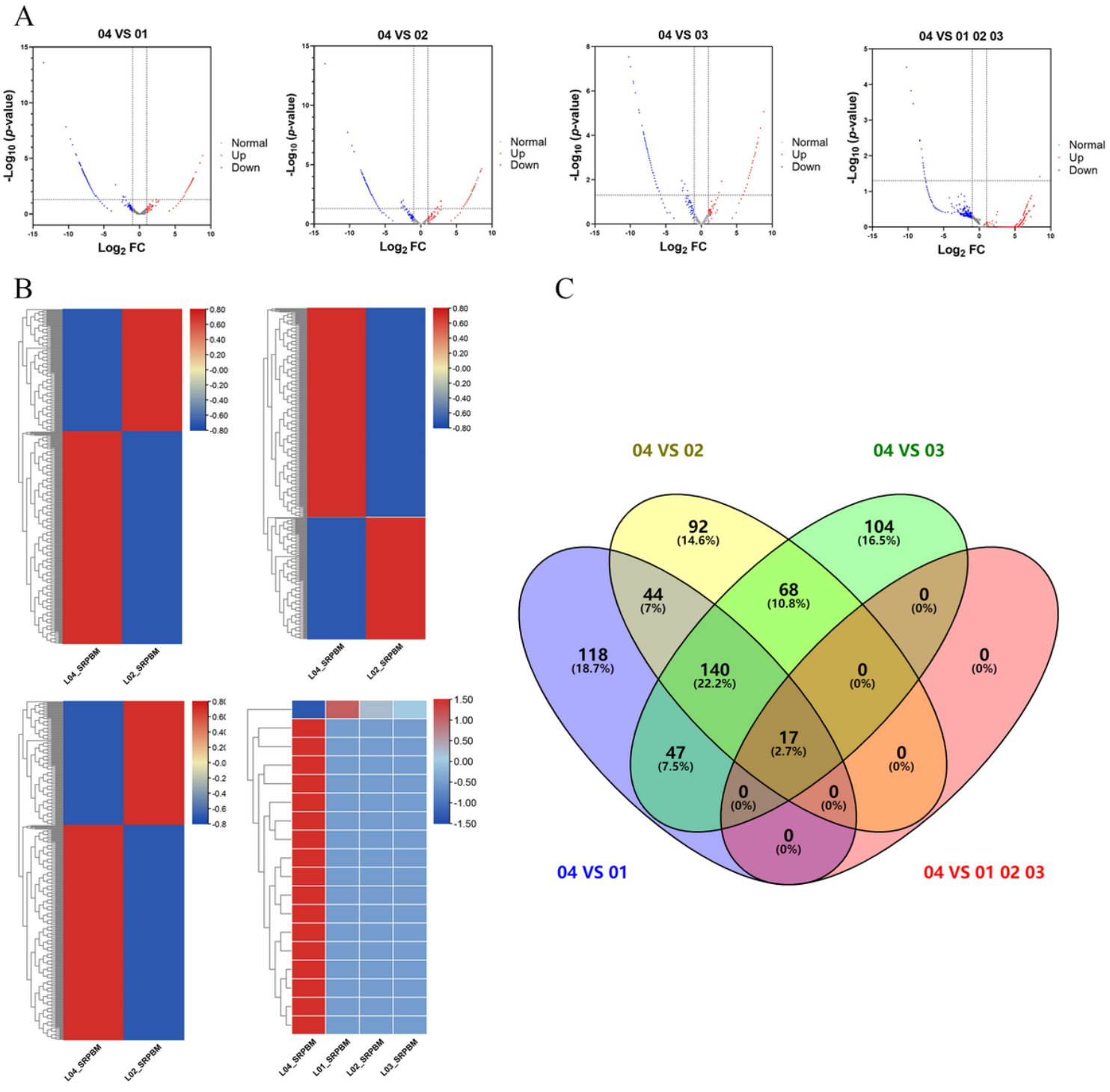

Figure 2

A Volcano plot of differentially expressed circRNAs. **B** Hierarchical clustering of differentially expressed circRNAs. **C** A Venn diagram showing circRNAs that are commonly and specifically expressed in the samples.

Figure 3

The GO classification of the targets of the circRNAs with L04 vs L01, L02 and L03.

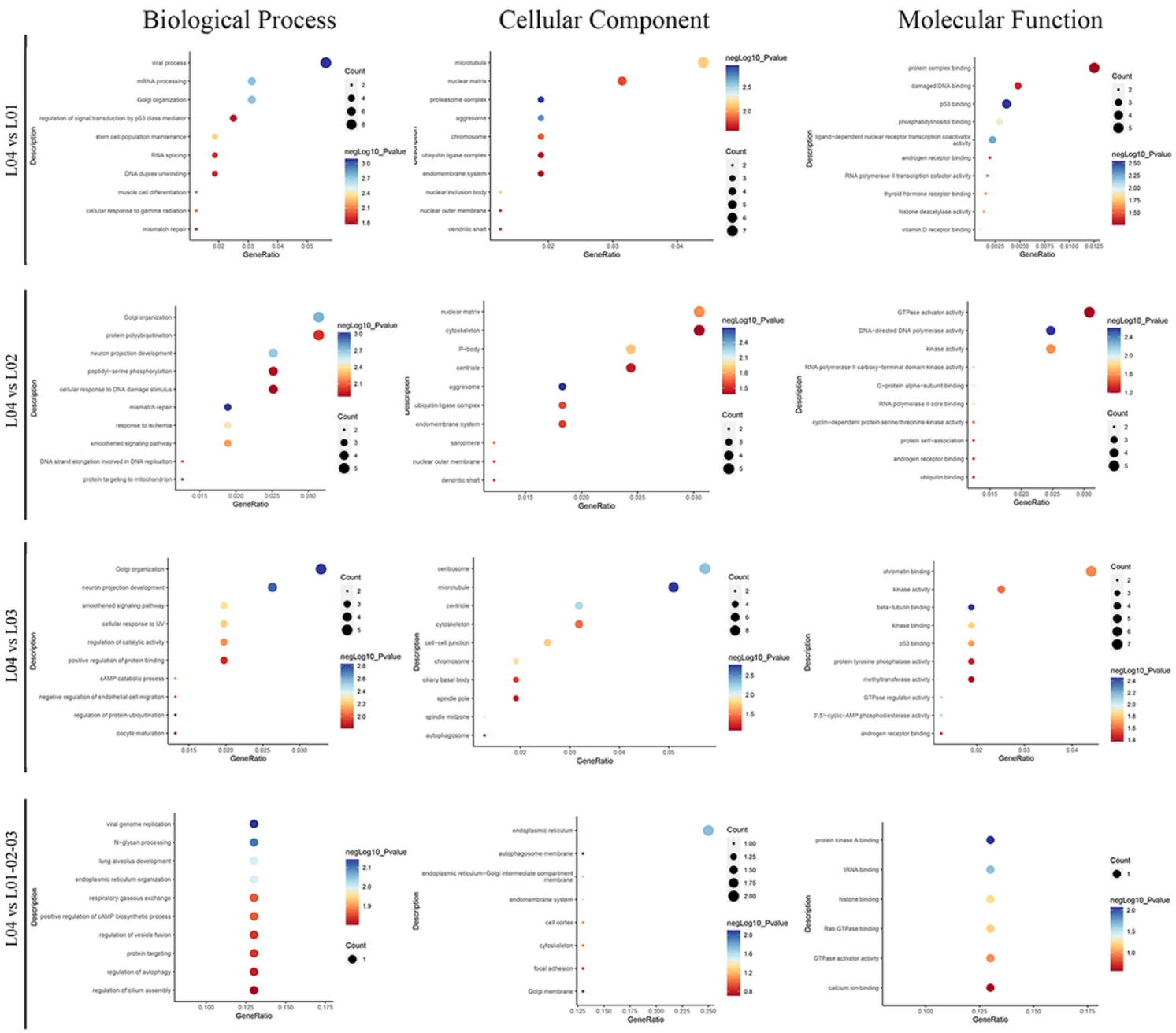

Figure 4

GO analysis of the targets of circRNAs in control and mCRC.

Figure 5

KEGG analysis of the targets of circRNAs in control and mCRC.

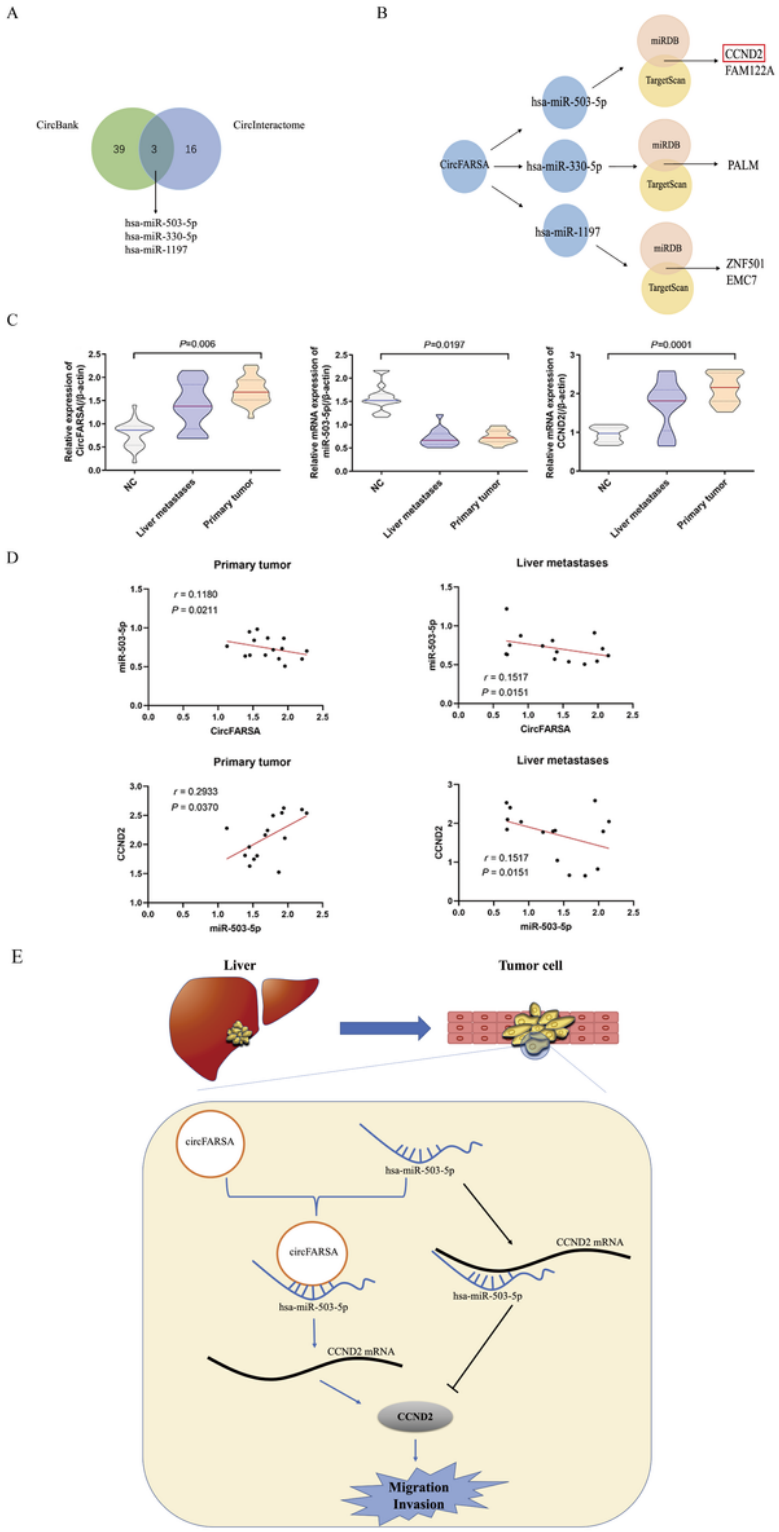

Figure 6

A The intersected miRNAs might be regulated by circFARSA predicted in CircBank and CircInteractome databases. **B** The intersection of top 15 predicted target gene might be regulated by miRNAs predicted in miRDB and TargetScan databases. **C** The expression of circFARSA, hsa-miR-503-5p and CCND2 in fresh CRC primary tumor tissues, liver metastases tissues, and their adjacent matched normal tissues. **D** The correlation between circFARSA and hsa-miR-503-5p expression or hsa-miR-503-5p and CCND2 expression

in primary tumor tissues and liver metastases tissues. **E** Schematic diagram of proposed mechanism of circFARSA in mCRC.

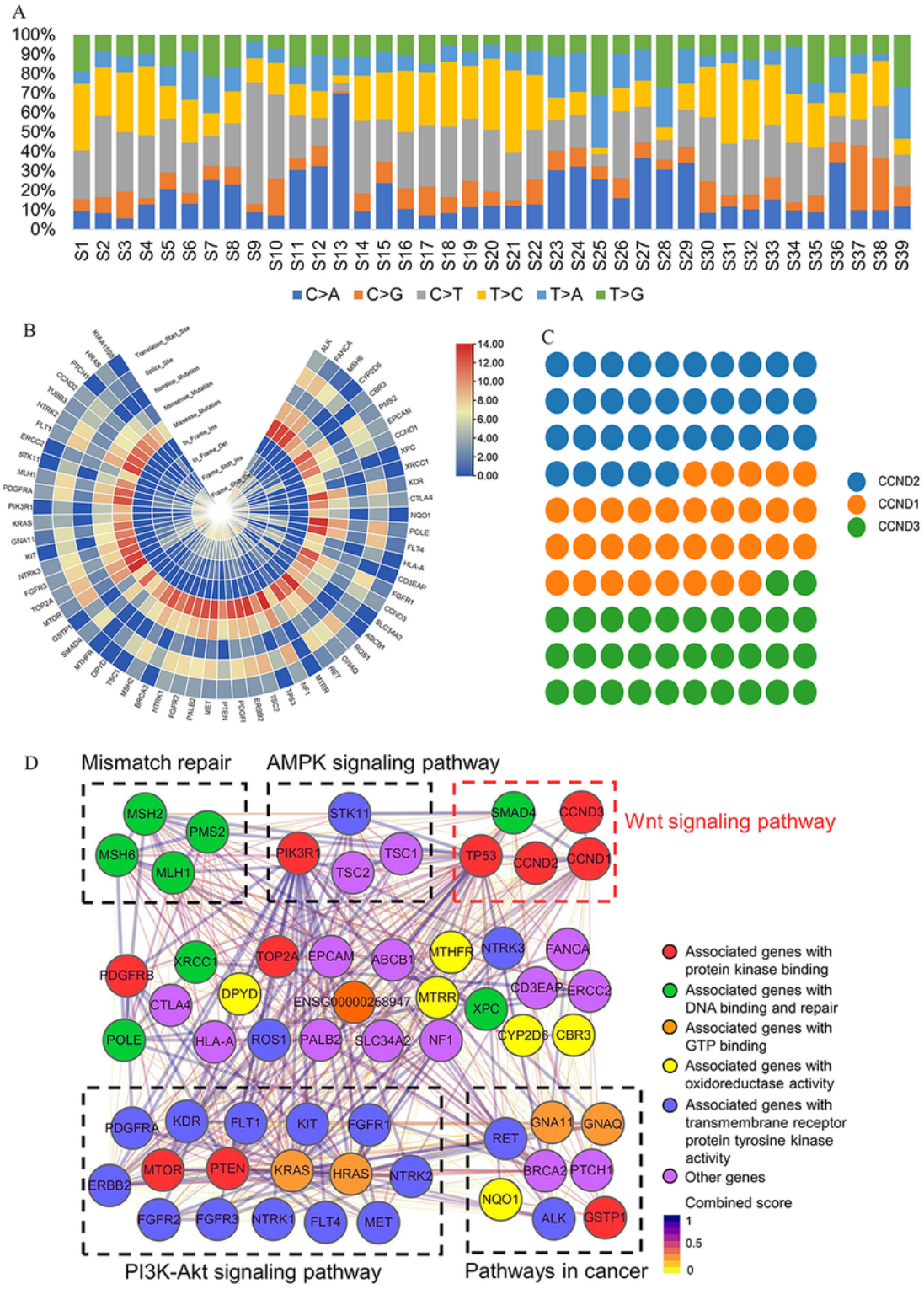

Figure 7

A Cumulative percentile analysis of gene segment transversions in 39 patients with metastatic colorectal cancer. **B** Expression of mutation type of differential genes by heatmap. **C** Percentage of mutations in

CCND gene family in metastatic colorectal cancer. **D** Molecular network map of differential expression genes by Gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementFig.1L04vsL01.GO.tif
- SupplementFig.2L04vsL02.GO.tif
- SupplementFig.3L04vsL03.GO.tif
- SupplementaryFig.4.tif