Identification of cancer-promoting circRNAs and potential contributions of these circRNAs to the pathogenesis of hepatocellular carcinoma (HCC)

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Abstract
Purpose There is growing awareness of critical roles of circular RNA (circRNA) in tumor growth and development. This study aimed to discover new cancer-promoting circRNAs and to study their potential roles in the pathogenesis of hepatocellular carcinoma (HCC).

Methods The expression profiles and clinical data of HCC-related circRNA, miRNA, and mRNA were obtained from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases. A model of the circRNA-miRNA-mRNA network was established based on analysis and interaction prediction. The main biological functions of the targeted mRNA were predicted by Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, and the protein-protein interaction (PPI) network was constructed to reveal important hub genes. The online tool OncomiR and GEPIA were used to analyze the correlation between miRNA and mRNA and clinicopathological features, and the Kaplan-Meier curve was constructed to reveal the relationship between mRNA and patient prognosis.

Results Differential analysis indicated that twenty highly expressed circRNA in GEO microarray datasets GSE78520, GSE94508, and GSE97332. Ten miRNAs were targeted by 20 circRNAs. These miRNAs are expressed at lower levels in the TCGA liver hepatocellular carcinoma (LIHC) expression profile, and most of the functions of these genes are closely related to pathological staging. These 10 miRNAs predicted a total of 7310 downstream mRNAs, of which 169 mRNAs were more abundant than normal tissues in the TCGA LIHC expression profile. GO analysis, KEGG analysis, and protein-protein interaction network analysis showed that E2F1, H2AFX, TOP2A, and RAD51 are central genes of the competitive endogenous RNA (ceRNA) network, and are mainly involved in biological mechanisms such as cell cycle, cancer-related pathways, and blood vessel morphogenesis. In addition, E2F1, H2AFX, TOP2A, and RAD51 are also closely related to the pathological stage and survival of patients.

Conclusions The analysis allowed the construction of a competitive endogenous RNA network associated with cancer-promoting circRNAs. Genes involved in this network may provide potential therapeutic targets for the diagnosis and treatment of HCC.

Introduction
Circular RNAs (circRNAs) are a class of non-coding RNAs with intact closed-loop structures[1]. In 1986, Sanger et al. first proposed circRNA, and with improvements in detection techniques, more and more circRNAs have been discovered in recent years[2]. Current research indicates that circRNA is formed by reverse shear processing of linear RNA precursors, which can be composed of an exon or an exon plus an intron[3]. Lacking a 5′ end cap structure and a 3′-end poly(A) tail, circRNA can be stably present in living organisms and can be a key biomarker for cancer[4].

Several functions of circRNA have been described, including forming a complex with RNA polymerase II to promote gene expression; binding to an RNA binding protein (RBP) to form an RNA-protein complex (RPC) to inhibit the function of a specific RBP, thereby inhibiting transcription of the parental gene; and acting as a competitive endogenous RNA (ceRNA) to sponge miRNA through miRNA response elements (MREs), thereby regulating the abundance of target genes[5]. In hepatocellular carcinoma, the circular RNA ciRS-7 can promote the expression of CCNE1, PIK3CD, and EGFR and other proteins by adsorbing miR-7, and the circular RNA circMTO1 can competitively bind and promote the expression of cyclin-dependent kinase inhibitor 1A (p21)[6, 7]. However, there has been no comprehensive analysis of circRNAs and functions for hepatocellular carcinoma (HCC).

In this study, we downloaded the HCC-related circRNA microarray dataset from the Gene Expression Omnibus (GEO) database and performed bioinformatics analysis to identify up-regulated circRNA, members of the ceRNA network, and potential functions. Finally, we constructed a circRNA-miRNA-mRNA network including six circular RNAs, four miRNAs, and four mRNAs.

Materials And Methods

Collection and selection of relevant data in the CEO and TCGA database

The gene chip and data used in this study were obtained from the GEO database (http://www.ncbi.nlm.nih.gov/gds/) and The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). Data for circRNA expression profiles of HCC and human-matched non-tumor tissues are not currently available in the TCGA database, we selected the gene chip data of relevant circRNA expression in the GEO database (dataset screening keywords: HCC and (micrornas OR miRNA) and Homo sapiens). TCGA was the source of the expression profiles of miRNAs and mRNAs.
in HCC (TCGA LIHC) and the clinical and pathological information of relevant patients. Survival analysis was performed on 364 patients with complete survival records, and correlation analysis was performed on 342 patients with complete clinicopathological features.

**Differential gene analysis in HCC**

First, differential circRNA identification was performed on each set of GEO gene chip data using the R software package (http://www.proproject.org), with log 2 values > 1 and P < 0.05. Venn diagram construction was then performed using the online tool Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/) to screen for up-regulated circular RNA in hepatocellular carcinoma. Next, TCGA expression profile data was pre-processed. First, the transcriptome expression data were converted from FPKM to TPM, and then the miRNA and mRNA data removed which with expression values equal to 0 in more than 25% of patients were removed. Finally, miRNAs with log2 < -1 and P < 0.05 and mRNAs with log2 > 1 and P < 0.05 were also screened using R software. Comparing to adjacent tissues, the differentially up-regulated circular RNA in tumor tissue was designated DUcircRNA, the differentially down-regulated miRNA in tumor tissue was designated DDmiRNA, and the differentially up-regulated mRNA was in tumor tissue designated DUmRNA.

**MRE prediction and construction of DUcircRNA-DDmiRNA-DUmRNA network**

The circular RNA Interactome (CircInteractome) (https://circinteractome.nia.nih.gov/) network tool was used to predict DUcircRNA that targeted miRNAs and their binding sites[8]. The predicted miRNA and DDmiRNAs were overlapped to obtain the final DDmiRNAs and then displayed as a heat map. The miRNA-targeted genes were predicted by microRNA Data Integration Portal (miRDIP) that uses 30 prediction algorithms (including BCmicrO, BiTargeting, CoMeTa, Cupid, DIANA, EIMMo3, GenMir++, MAMI, MBStar, MirAncesTar, MirMAP, MirSNP, MirTar, Mirza-G, MultiMiTar, PACCMIT, PITA, PicTar, RNA22, RNAhybrid, RepTar, TargetRank, TargetScan, TargetSpy, miRDB, miRTar2GO, miRcode, microrna.org, mirCoX, mirbase) (http://ophid.utoronto.ca/mirDIP/index-confirm.jsp)[9]. The following criteria were used for targeting mRNA selection: 1) mRNA were predicted by at least 10 different algorithms; 2) mRNA with miRDIP prediction score equal to 0.5 or higher; 3) before calculation,
confidence class in miRDIP was set to “Very High”. Next, the set of screened mRNAs were overlapped with the set of DUmRNA to obtain the final DUmRNA dataset for further analysis. Finally, Cytoscape (version 3.6.1) (http://cytoscape.org/) was used to map the DUCircRNA-DDmiRNA-DUmRNA network.[10].

**Functional enrichment analysis of mRNA**

We used Metascape (http://metascape.org/gp/index.html#/main/step1) to perform gene ontology (GO) and Kyoto Gene and Genomic Encyclopedia (KEGG) analysis of the final DDmRNA and screened for annotations with $P < 0.05$.[11].

**Construction of a PPI network and identification of hub genes**

STRING (http://string-db.org/) is a database of known and predicted protein-protein interactions[12]. The interactions include direct (physical) and indirect (functional) associations, and are based on computational prediction, interactions in other organisms, and interactions included in other (primary) databases. This analysis was used to construct a PPI network for final DUmRNA with an interaction score greater than 0.7 as the cutoff value.

**Statistical analysis of the correlation between miRNA and clinical features**

OncomiR (http://www.oncomir.org/oncomir/search_miR_clinical.html) is an online resource to explore miRNA dysregulation in cancer[13]. This database can be queried for miRNA association with: tumor formation, tumor stage and grade, cancer patient survival, and gene targets. Analysis of variance (ANOVA) compares miRNA expression data between the different cohorts within each clinical parameter. Multivariate Cox analysis is used to evaluate the combined effect of the miRNA data, clinical parameters, and patient survival. In the above analysis, a value of $P < 0.05$ was considered statistically significant.

**Survival analysis of hub genes and its relevance to stage**

GEPIA (http://gepia.cancer-pku.cn/) is an online analysis software based on RNA sequencing data for 9736 tumors and 8587 normal samples in the TCGA and GTex databases[14]. First, statistical analysis of the expression of hub genes in HCC and normal tissues was performed using GEPIA and the Student two-tailed t-test. Subsequently, using TCGA LIHC patient clinical information and the GEPIA
online tool, the χ2 test was used to analyze the association of hub genes and tumor stage. Finally, the survival and prognostic significance of hub genes were analyzed using the survival analysis tool and the Kaplan-Meier method. In the above analysis, a value of P < 0.05 was considered statistically significant.

Results

Eighteen HCC-related DUCircRNAs were identified

In the GEO database, three sets of HCC-related data were obtained: GSE78520 (three sets of HCC and normal liver tissue), GSE94508 (five sets of HCC and matched paracancerous tissues), and GSE97332 (seven sets of HCC and matched adjacent normal tissues). Analysis identified 175 DUCircRNAs in GSE7850, 201 DUCircRNAs in GSE94508, and 453 DUCircRNAs in GSE97332. Comparison of the identified DUCircRNAs revealed twenty DUCircRNAs that were included in all three datasets (Figure 1A, Table 1). Of these DUCircRNAs, only the mechanism of circZFR and hsa-circ-0000673 were previously reported. DUCircRNAs that were previously unreported are hsa-circRNA-103948, hsa-circRNA-104640, hsa-circRNA-101408, hsa-circRNA-102587, hsa-circRNA-102034, hsa-circRNA-101777, hsa-circRNA-001416, hsa-circRNA-102559, hsa-circRNA-102451, hsa-circRNA-104760, hsa-circRNA-101094, hsa-circRNA-101287, hsa-circRNA-101555, hsa-circRNA-400071, hsa-circRNA-102954, hsa-circRNA-100053, hsa-circRNA-104268, and hsa-circRNA-000996. In Table 1, the maternal genes, their position, and their names in circBase are listed. The differential expression of the 18 DUCircRNAs compared to normal tissues is shown by heat map (Figure 1B).

Final DDmiRNA and DUCircRNA-DDmiRNA network

The miRNAs targeted by the set of 18 final DUCircRNAs were predicted by CircInteractome, respectively, and compared with the 80 DDmiRNAs identified in TCGA. Ten miRNAs were found in both analyses (hsa-miR-136-5p, hsa-miR-145-5p, hsa-miR-187-3p, hsa-miR-370-3p, hsa-miR-377-3p, hsa-miR-383-5p, hsa-miR-758-3p, hsa-miR-874-3p, hsa-miR-326, and hsa-miR-375), and these miRNAs are considered the final DDmiRNAs (Figure 2). According to ANOVA analysis of the patient clinical data, hsa-miR-136-5p was associated with sex (P=0.0321) and pathological stage (P=0.0366); hsa-miR-187-3p was associated with sex (P=0.0253); hsa-miR-383-5p was associated with pathological
status (P=0.0340); hsa-miR-326 was related to pathological T stage (P=0.0097); and hsa-miR-375 was related to sex (P=0.00004). Under multivariate analysis, hsa-miR-326 was still closely related to pathological stage (P=0.0047) and pathological T status (P=0.004), and hsa-miR-187-3p was still related with sex (P=0.019) (Table 2).

Among the 18 DUcircRNAs, hsa-circRNA-102587, hsa-circRNA-101777, hsa-circRNA-102559, hsa-circRNA-400071, and hsa-circRNA-102954 targeted miRNAs were not included in the DDmiRNA analysis, and the remaining 13 DUcircRNAs and their targeted 10 DDmiRNAs formed 25 circRNA-miRNA network pairs, representing the DUcircRNA-DDmiRNA network (Figure 3).

**Final DUmRNA and ceRNA networks**

According to miRDIP, the 10 DDmiRNAs have the potential to target 7310 mRNAs, of which 3115 mRNAs had integrated scores > 0.5 (the highest integrated scores is 1). There were 316 DUmRNAs in the TCGA LIHC data and 169 mRNAs out of the 3115 mRNAs were identified as DU mRNA. These two sets of mRNAs were combined as the final DUmRNAs, and 169 network pairs were formed by combining these final DU mRNAs with the 10 DDmiRNAs, forming a final ceRNA network (circRNA-miRNA-mRNA network) with the DUcircRNA-DDmiRNA network (Figure 4).

**Final DUmRNA functional analysis, PPI network, and hub genes**

The list of 169 DUmRNAs were loaded to Metascape, and input as species: H. sapiens, and analysis as species: H. sapiens. The main enriched GO terms were: regulation of neuron differentiation, TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest, retrograde Trans-synaptic signaling by resonance, synaptic signaling, fat cell differentiation, blood vessel morphogenesis, G0 and Early G1, urogenital system development, connective tissue development, integration of energy metabolism, cell morphogenesis involved in differentiation, learning or memory, negative regulation of DNA binding, response to nutrients, positive regulation of cell development, resolution of D-loop structures through Synthesis-Dependent Strand Annealing (SDSA), brain development, and carbohydrate-derivative biosynthetic process. KEGG analysis showed two enriched terms: Small cell lung cancer and GABAergic synapse (Figure 5).

We next used STRING to establish a PPI network, using the Hide Disconnected Nodes in The Network
function to fully display the 47 interactions. As shown in Figure 6, in the PPI network, the line color indicates the type of interaction evidence. Default active interaction sources: Textmining, Experiments, Databases, Co-expression, Neighborhood, Gene Fusion, and Co-occurrence. Based on line color and evidence strength, E2F1, H2AFX, TOP2A, and RAD51 are hub genes associated with the HCC DUcircRNA-DDmiRNA-DU mRNA network. GO and KEGG analysis showed that the hub genes of the PPI network mainly participated in the processes of TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest and Pathways in cancer and blood vessel morphogenesis (Figure 6).

**Pathological Stage and Survival analysis of hub genes**

Difference analysis was performed using R software on E2F1, H2AFX, TOP2A, and RAD51 in the TCGA LIHC dataset including 371 HCC patient tissue samples and 50 patients with adjacent normal tissues. Boxplot was used by selecting the Match TCGA normal data option in GEPIA (Figure 7). Compared with normal tissue, E2F1 (logFC = 3.61, P = 2.95E-44), H2AFX (logFC = 1.54, P = 9.35E-16), TOP2A (logFC = 3.82, P = 4.56E-44), and RAD51 (logFC= 2.10, P = 2.69E-25) showed high expression. The log2(TPM + 1) values were used as the Y-axis for a pathological stage plot, which showed that E2F1 (F value = 6.43, Pr (>F)=0.0003), H2AFX (F value = 8.89, Pr (>F)=1.09E -05), TOP2A (F value = 8.15, Pr (>F) = 2.96E-05), and RAD51 ((F value = 7.70, Pr (>F) = 5.42E-05) are closely related to HCC stage (Figure 7). Finally, the prognostic significance of these four hub genes in 364 patients was analyzed using GEPIA. As shown in Figure 7, the four hub genes E2F1 (log-rank P = 0.0025, HR = 1.7), H2AFX (log-rank P = 0.0050, HR = 1.6), TOP2A (log-rank P = 0.0028, HR = 1.7), and RAD51 (log-rank P = 0.0054, HR = 1.6) were significantly associated with overall survival (OS) in HCC patients.

**DUcircRNA-DDmiRNA-DU mRNA network related to hub genes**

The hub gene-associated DUcircRNA-DDmiRNA-DU mRNA network was selected from the final ceRNA network. The network can be divided into two parts, the hsa-circ-101408/hsa-circ-101094/hsa-circ-000996-hsa-miR-136-5p-E2F1 axis, the hsa-circ-100053/hsa-circ101555/hsa-circ-101094/hsa-circ-101408-hsa-miR-145-5p-H2AFX axis, the hsa-circ-101408-hsa-miR-383-5P-TOP2A axis, and the hsa-circ-104760-hsa-miR-758-3p-RAD51 axis (Figure 8).

**Discussion**
The ceRNA mechanism is one of the important functions of non-coding RNA, by which mRNA translation can be regulated by competitively shared miRNAs, especially by circRNA[15, 16]. Circular RNA cSMARCA5 can promote DHX9 and TIMP3 expression by targeting miR-17-3p[17]. CircHIPK3 sponges miR-7 to increase the expression levels of FAK, IGF1R, EGFR, and YY1 and promote colorectal cancer growth and metastasis[18].

In this study, because circular RNA data was not included in TCGA, we searched the GEO database to find HCC-related circRNA microarrays, GSE7850, GSE94508, and GSE97332. We performed differential analysis of these three microarrays using R software, and then entered the DUCircRNAs data into the online tools Bioinformatics & Evolutionary Genomics to identify 20 circRNAs that were highly expressed compared to adjacent normal tissues. The ceRNA mechanism of circZFR and hsa-circ-0000673 was previously reported. In colorectal cancer, circZFR affects the expression of the FOXO4 protein by targeting miR-532-3p; in lung cancer, circZFR acts as a ceRNA on miR-4302, promoting ZNF121 expression[19, 20]. Hsa-circ-0000673 can promote HCC proliferation and metastasis by indirectly regulating the expression of SET by competitive binding to miR-767-3p[21]. So, in this study we mainly explore the ceRNA mechanism of hsa-circRNA-103948, hsa-circRNA-104640, hsa-circRNA-101408, hsa-circRNA-102587, hsa-circRNA-102034, hsa-circRNA-101777, hsa-circRNA-001416, hsa-circRNA-102559, hsa-circRNA-102451, hsa-circRNA-104760, hsa-circRNA-101094, hsa-circRNA-101287, hsa-circRNA-101555, hsa-circRNA-400071, hsa-circRNA-102954, hsa-circRNA-100053, hsa-circRNA-104268 and hsa-circRNA-000996.

To determine the ceRNA machinery and circRNA-miRNA-mRNA network of the final 18 DUCircRNAs in HCC, we used CircInteractome to predict MRE sites, and then obtained the downstream sequences of the directly bound miRNAs. At the same time, miRNA in TCGA LIHC expression profile was utilized to identify 80 DDmiRNAs in HCC patients. Subsequently, we found DDmiRNAs related to the 13 final DUCircRNAs: hsa-circRNA-103948 (hsa-miR-370-3p), hsa-circRNA-104640 (hsa-miR-370-3p, hsa-miR-785-3p, hsa-miR-375), hsa-circRNA-101408 (hsa-miR-370-3p, hsa-miR-136-5p, hsa-miR-145-5p, hsa-miR-383-5p, hsa-miR-374-3p, hsa-miR-375), hsa-circRNA-102034 (hsa-miR-326), hsa-circRNA-001416 (hsa-miR-370-3p, hsa-miR-326), hsa-circRNA-102451 (hsa-miR-326), hsa-circRNA-104760, hsa-
circRNA-101094 (hsa-miR-136-5p, hsa-miR-145-5p), hsa-circRNA-101287 (hsa-miR-377-3p), hsa-
circRNA-101555 (hsa-miR-326, hsa-miR-145-5p), hsa-circRNA-100053 (hsa-miR-187-3p, hsa-miR-145-
5p), hsa-circRNA-104268 (hsa-miR-370-3p, hsa-miR-187-3p) and hsa-circRNA-000996 (hsa-miR-370-
3p, hsa-miR-136-5p). The 10 miRNAs corresponding to the 13 DUcircRNAs are called the final
DDmiRNA. Of the final DDmiRNAs, hsa-miR-136-5p, hsa-miR-187-3p, hsa-miR-383-5p, hsa-miR-326,
and has-miR-375 are closely related to sex and pathological stage, but not significantly correlated to
clinical information of other miRNAs and patients in the TCGA database. However, many studies have
shown that inhibition of hsa-miR-145-5p expression can promote tumor proliferation and affect tumor
size; hsa-miR-370-3p can inhibit tumor size and lung metastasis in nude mice; hsa-miR-874-3p
expression was associated with tumor number, stage, size, differentiation, and vascular invasion in
120 HCC patients; hsa-miR-377-3p inhibited proliferation and apoptosis of HepG2 cells in vitro; and
the expression level of hsa-miR-758-3p is related to patient alpha fetoprotein (AFP) and stage[22–26].
Therefore, the DUcircRNA-DDmiRNA network includes 13 DUcircRNAs and 10 DDmiRNAs.
A total of 169 final DUmRNAs with sequences downstream of the final DDmiRNA were obtained from
miRDIP and TCGA LIHC data to make up a complete DUcircRNA-DDmiRNA-DUmRNA network. Analysis
of the 169 final DU mRNAs analysis showed enrichment of 18 GO terms and the two KEGG terms. The
enriched GO and KEGG terms of the PPI network of 47 final DUmRNAs were mainly TP53 Regulates
Transcription of Genes Involved in G1 Cell Cycle Arrest and Pathways in cancer and blood vessel
morphogenesis. Further analysis revealed the hub genes of the circRNA-miRNA-mRNA network as
E2F1, H2AFX, TOP2A, and RAD51.
E2F1 is a tumor suppressor in a variety of cancers, binds to gene promoter regions, and is involved in
numerous cellular pathways[27]. Its roles include regulating cell cycle, apoptosis, aging, and DNA
damage responses[28]. The different biological functions of E2F1 depend on its binding partner[29].
In HCC, E2F1 inhibits c-Myc-driven apoptosis by activating PIK3K/Akt/mTOR and COX-2 pathways[30].
H2AFX is one of the common variants in the H2A family, and is a histone that is closely related to DNA
double-strand breaks[31]. When the C-terminus of H2AX is phosphorylated, there are increased
numbers of DNA double-strand breaks, leading to gene mutation[32].
TOP2A gene is a molecular target of anthracycline antibiotics. TOP2A affects DNA topological structure, chromosome segregation, and cell cycle progression, processes important for the development and progression of tumors[33]. Previous studies have shown that TOP2A is overexpressed in HCC and is closely related to HBsAg and Ki-67[34].

The RAD51 protein is essential for homologous recombination repair processes[35]. Genetic variation in RAD51 may lead to the development of cancer, such as liver cancer, ovarian cancer, breast cancer, prostate cancer, and colorectal cancer[36].

From this analysis, we identified four important regulatory pathways in HCC that overexpress circular RNA, the hsa-circ-101408/hsa-circ-101094/hsa-circ-000996-hsa-miR-136-5p-E2F1 axis, the hsa-circ-100053/hsa-circ101555/hsa-circ-101094/hsa-circ-101408-hsa-miR-145-5p-H2AFX axis, the hsa-circ-101408-hsa-miR-383-5p-TOP2A axis, and the hsa-circ-104760-hsa-miR-758-3p -RAD51 axis.

Finally, we constructed a HCC DUcircRNA-DDmiRNA-DUmRNA network, with hub genes of E2F1, H2AFX, TOP2A, and RAD51. The included upstream circRNA and down-regulated miRNA are hsa-circ-101408, hsa-circ-000996, hsa-circ-101094, hsa-circ-101555, hsa-circ-100053, hsa-circ-104760, hsa-miR-145-5p, hsa-miR-383-5p, hsa-miR-136-5p, and hsa-miR-758-3p. Of these, hsa-miR-145-5p, hsa-miR-383-5p, hsa-miR-136-5p, and hsa-miR-758-3p are closely related to some clinical features. E2F1, H2AFX, TOP2A, and RAD51 are related to the clinical stage of the patient and also closely related to the prognosis of the patient. We indirectly inferred that hsa-circ-101408, hsa-circ-000996, hsa-circ-101094, hsa-circ-101555, hsa-circ-100053, and hsa-circ-104760 play important roles in HCC. This ceRNA network may be a potential therapeutic and diagnostic target for HCC.

Conclusions:
We allowed the construction of a competitive endogenous RNA network associated with cancer-promoting circRNAs. Genes involved in this network may provide potential therapeutic targets for the diagnosis and treatment of HCC.

Abbreviations
circRNA

circular RNA; HCC:hepatocellular carcinoma; ceRNA:competitive endogenous RNA; TCGA:The Cancer Genome Atlas; PPI:protein-protein interaction; GO:Gene Ontology; KEGG:Kyoto Encyclopedia of Genes
and Genomes; GEO:Gene Expression Omnibus; LIHC:liver hepatocellular carcinoma; RBP:RNA binding protein; miRDIP:miRNA Data Integration Portal; ANOVA:Analysis of variance; AFP:alpha fetoprotein.

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Availability of data and materials
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests

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Authors’ contributions
ZSZ and JFH designed the project. MQY and CRR analyzed and interpreted the relevant TCGA and GEO data. DLZ, XML and JZ perform part of the data processing and were major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Tables

**Table 1 Overview of the 20 DUCircRNAs**
| No. | CircRNA          | Alias          | Gene symbol | Position                      |
|-----|------------------|----------------|-------------|-------------------------------|
| 1   | hsa_circRNA_103809 | circZFR        | ZFR         | chr5:32379220-32388780        |
| 2   | hsa_circRNA_101707 | hsa_circ_0000673 | RSL1D1      | chr16:11940357-11940700       |
| 3   | hsa_circRNA_103948 | hsa_circ_0003528 | SEC24A      | chr5:134032815-134044578      |
| 4   | hsa_circRNA_104640 | hsa_circ_0001806 | CSPP1       | chr8:68018139-68028357        |
| 5   | hsa_circRNA_101408 | hsa_circ_0032698 | TTLL5       | chr14:76135758-76368567       |
| 6   | hsa_circRNA_102587 | hsa_circ_0051732 | LIG1        | chr19:48660270-48660397       |
| 7   | hsa_circRNA_102034 | hsa_circ_0043001 | RHOT1       | chr17:30500849-30502381       |
| 8   | hsa_circRNA_101777 | hsa_circ_0038718 | IL4R        | chr16:27351506-27353580       |
| 9   | hsa_circRNA_001416 | hsa_circ_0001338 | RAB43       | chr3:128824688-128825122      |
| 10  | hsa_circRNA_102559 | hsa_circ_0051220 | TMEM91      | chr19:41884185-41884424       |
| 11  | hsa_circRNA_102451 | hsa_circ_0003892 | LDLR        | chr19:11230767-11238761       |
| 12  | hsa_circRNA_104760 | hsa_circ_0003945 | UBAP2       | chr9:33953282-33956144        |
| 13  | hsa_circRNA_101094 | hsa_circ_0027477 | NUP107      | chr12:69109406-69121169       |
| 14  | hsa_circRNA_101287 | hsa_circ_0008274 | UGGT2       | chr13:96485180-96489456       |
| 15  | hsa_circRNA_101555 | hsa_circ_0001955 | CSNK1G1     | chr15:64495280-64508912       |
| 16  | hsa_circRNA_400071 | hsa_circ_0092283 | MYH9        | chr22:36681395-36681695       |
| 17  | hsa_circRNA_102954 | hsa_circ_0003923 | UBE2F       | chr2:238933982-238940895      |
| 18  | hsa_circRNA_100053 | hsa_circ_0009912 | MFN2        | chr1:12049221-12057478        |
| 19  | hsa_circRNA_104268 | hsa_circ_0078728 | WDR27       | chr6:169982931-170062520      |
| 20  | hsa_circRNA_000996 | hsa_circ_0001900 | CAMSAP1     | chr9:138773478-138774924      |
Table 2 Correlation between DEmiRs and clinicopathological features

| miRNA Name   | Clinical Parameter | ANOVA P-value | ANOVA FDR | Multivariate Log Rank P-value | Multivariate Log Rank FDR |
|--------------|--------------------|---------------|-----------|-------------------------------|----------------------------|
| hsa-miR-136-5p | Sex                | 3.21e-02      | 2.42e-01  | 5.42e-01                      | 7.82e-01                   |
| hsa-miR-187-3p | Pathologic Stage   | 3.66e-02      | 2.60e-01  | 9.00e-02                      | 2.99e-01                   |
|               | Sex                | 2.53e-02      | 2.26e-01  | 1.90e-02                      | 8.37e-02                   |
| hsa-miR-145-5p | /                  | /             | /         | /                             | /                          |
| hsa-miR-370-3p | /                  | /             | /         | /                             | /                          |
| hsa-miR-383-5p | Pathologic T Status| 3.40e-02      | 2.58e-01  | 8.28e-01                      | 9.97e-01                   |
| hsa-miR-874-3p | /                  | /             | /         | /                             | /                          |
| hsa-miR-326   | Pathologic Stage   | 5.97e-02      | 3.49e-01  | 4.70e-03                      | 4.79e-02                   |
|               | Pathologic T Status| 9.65e-03      | 1.28e-01  | 3.99e-03                      | 4.51e-02                   |
| hsa-miR-375   | Sex                | 4.03e-05      | 8.41e-03  | 4.07e-01                      | 6.81e-01                   |
| hsa-miR-377-3p| /                  | /             | /         | /                             | /                          |
| hsa-miR-758-3p| /                  | /             | /         | /                             | /                          |

DEmiRs[] differentially expressed miRNAs

Figures
Expression of DUcircRNA in the dataset. A. The Venn plot shows that 20 circRNAs are up-regulated in GSE94580, GSE97332, and GSE78502. B. The heat map shows the differences in expression of the 20 DUcircRNAs in GSE94580, GSE97332, and GSE78502.
Figure 2
Heat map illustrating the differences in the expression profiles of 10 final DDmiRNAs in TCGA LIHC.

Figure 3
CircRNA-miRNA network composed of DUcircRNA and 10 final DDmiRNAs
Figure 4

CircRNA-miRNA-mRNA network for up-regulation of circRNA in HCC.

Figure 5

GO and KEGG analysis of Final DUmRNA.
Final DDmRNA corresponding protein PPI Network and the related biological mechanisms.
Figure 7

Differences in expression of hub genes in HCC and their relationships with clinical stage and
prognosis. A. (a) In HCC patients, E2F1 is highly expressed in tumor tissues compared with normal tissues; (b) E2F1 is closely related to clinical stage of HCC patients (P=0.0003); (c) survival analysis suggests that patients with high E2F1 expression have a shorter survival time (P=0.0025). B. (a) In HCC patients, H2AFX is highly expressed in tumor tissues compared with the level in normal tissues; (b) H2AFX is closely related to clinical stage of HCC patients (P=0.00001); (c) survival analysis suggests that patients with high H2AFX expression have a shorter survival time (P=0.0054). C. (a) In HCC patients, TOP2A is highly expressed in tumor tissues compared with the level in normal tissues; (b) TOP2A is closely related to clinical stage of HCC patients (P=0.00002); (c) survival analysis suggests that patients with high TOP2A expression have a shorter survival time (P=0.003). D. (a) In HCC patients, RAD51 is highly expressed in tumor tissues compared with the level in normal tissues; (b) RAD51 is closely related to clinical stage of HCC patients (P=0.00005); (c) survival analysis suggests that patients with high RAD51 expression have a shorter survival time (P=0.0058). *p < 0.05
Figure 8

CircRNA-miRNA-mRNA network based on hub genes E2F1, H2AFX, TOP2A, and RAD51
