Circular RNA Signature in Hepatocellular Carcinoma

Li-Peng Qiu[1]*, Tao Wang[1], Qi Ge[1], Han Xu[2], Yi-Hang Wu[3], Qi Tang[1], Ke-Ping Chen[1]*

[1] Institute of Life Sciences, Jiangsu University, Zhenjiang 212013, Jiangsu, China

[2] School of Pharmacy, Jiangsu University, Zhenjiang, Jiangsu Province, 212013, China.

[3] Department of Pharmacy, College of Life Sciences, China Jiliang University, Hangzhou 310018, Zhejiang, China

*Correspondence: tulip_lipeng@163.com (L.Q.); kpchen@ujs.edu.cn (K. C.); Tel.: +86-511-8879-1702; Fax: +86-8879-1923.

Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Circular RNAs (circRNAs) are a new class of endogenous functional non-coding RNAs (ncRNAs), and have been demonstrated to play important roles in the development of HCC. This study aimed to explore the significance of circRNAs in HCC progression. HCC-associated circRNA expression profiles GSE94508 and GSE97332 were downloaded from the Gene Expression Omnibus database (GEO), and 87 differentially expressed circRNAs (DECs) between HCC tissues and paired non-cancer tissues were identified, including 76 up-regulated and 11 down-regulated circRNAs. Gene ontolog (GO) and pathway analyses of the host genes of these DECs suggested that these host genes were enriched in cell adhesion, cytosol, and protein binding, and were associated with tight junction and Wnt signaling pathways. CircRNA-miRNA interaction prediction identified 20 miRNAs that predispose to interact with DECs. Among these, four essential miRNAs, hsa-miR-7-5p, hsa-miR-145-5p, hsa-miR-203a-3p, and hsa-miR-192-5p, were reported to play pivotal roles in HCC progression by targeting multiple genes. Pathway analysis suggested that putative target genes of these essential miRNAs were involved in HCC-associated signaling pathways, such as Wnt, TGF-β, and Ras; whereas protein-protein network (PPI) analysis demonstrated that some validated target genes of these miRNAs, such as PIK3CA, AKT1, MYC, JUN, SMAD4, and SRC, were hub target genes as they have more counts of interacting protein. In the meantime, the deregulation of some DECs was validated in HCC cell lines HepG2 compared with normal liver cell line L02 by quantitative real-time polymerase chain reaction (qRT-PCR) and the Sanger sequencing. This study identified a set of DECs in HCC, and provided a comprehensive understanding of the roles of these DECs in HCC

progression.

Key words: circular RNA, miRNA sponge, hepatocellular carcinoma, bioinformatic analysis

Introduction

Hepatocellular carcinoma (HCC), the fifth common cancer in the world, ranks the third in cancer-related death [1]. Currently, the diagnosis of HCC is still poor, and therapeutic options are limited. Many HCC patients are not diagnosed until they are in advanced stages when curative therapies are too late to be applied; while for those patients who are eligible for surgical resection, liver transplantation or local ablation, although their life qualities could be improved by these therapies, their overall survival rates are still low. Therefore, novel biomarkers and therapeutic approaches are urgently needed to improve the overall survival of HCC patients.

Circular RNAs (circRNAs) are a new class of abundant endogenous functional non-coding RNAs (ncRNAs), and have been demonstrated to play important roles in different types of cancer, including gastric cancer, lung cancer, and HCC, etc.[2-4]. They form covalently closed continuous loop structures with neither polarity nor polyadenylated tails, which make them more stable and abundant than their canonical linear transcripts from the same genes in cells [5, 6]. Except for tissues, circRNAs can also be found in different body fluids, such as saliva, blood, and urine, suggesting that they may serve as non-invasive circulating biomarkers for cancer diagnosis [7]. Studies showed that circRNAs may regulate gene expression at transcriptional, post-transcriptional, and translational levels. Specifically, circular exonic circRNAs (EcircRNAs), which predominantly exist in cytoplasm, harbors miRNA response elements (MREs), and may function as miRNA sponges by competitively binding to specific miRNAs to reduce their expression, resulting in enhanced expression of target genes of these miRNAs; while circular intronic RNAs (ciRNAs) or exon-intron circRNAs (EIciRNAs) are mainly accumulate in the nucleus, and may regulate gene

transcription and post-transcription [5, 8-10]. These suggest that circRNAs may participate in various biological processes, such as cell proliferation, apoptosis, and angiogenesis, and thus contribute to cancer progression.

Most recently, some circRNAs have been reported to be deregulated in HCC tissues, and the deregulation of these circRNAs may not only associate with clinicopathological features of HCC patients, but also impact on HCC progression by targeting specific miRNAs and proteins. Several circRNAs, such as circZKSCAN1, hsa_circ_0004018, and hsa_circ_0005075, have been shown as promising biomarkers for HCC diagnosis; whereas circMTO1 and circ-ITCH may serve as potential prognostic biomarkers for poor survival of HCC patients [11-15]. Furthermore, a few circRNAs have been reported to interfere with HCC progression by modulating the proliferation, apoptosis, migration and invasion via targeting different miRNAs and proteins. For example, circMTO1 inhibits the proliferation of HCC cells by sponging miR-9 and increasing the expression of miR-9 target gene p21, a cell cycle inhibitory protein; hsa_circ_100338 promotes the migration and invasion of HCC cells by targeting miR-141; while ciRS-7 facilitates the proliferation and invasion of HCC cells by competitively binding to hsa-miR-7-5p (miR-7) and promoting the expression of multiple miR-7 target genes, including CCNE1, PIK3CD, and EGFR [14, 16-19]. These results indicate that circRNAs may play critical roles in regulating HCC progression.

In current study, we downloaded the HCC-associated circRNA expression profiles GSE94508 and GSE97332 from the Gene Expression Omnibus database (GEO), analyzed the differentially expressed circRNAs (DECs) in both profiles between HCC liver tissues and paired non-tumor tissues, and investigated their possible target genes and pathways involved in HCC using bioinformatic approaches, to provided possible

functions and underlying mechanisms of these DECs in HCC tumorigenesis and progression.

Materials and methods

Microarray analysis of gene expression

Two circRNA expression profiles, GSE94508 and GSE97332, were downloaded from the GEO (www.ncbi.nlm.nih.gov/geo/), both of which were completed on the Agilent-069978 Arraystar Human CircRNA microarray V1 GPL19978 platform [20]. The GSE94508 dataset contained 5 pairs of HCC and paracancerous liver tissues, and the GSE97332 dataset contained 7 pairs of HCC and matched non-tumor liver tissues.

Identification of DECs

GEO2R [21] was used to analysis the raw data TXT files downloaded from GEO. The absolute value of log fold change (FC) > 1.0 and *p* value <0.05 were used as cut-off criteria. CircRNAs with statistical significance between HCC and non-tumor tissues in GSE94508 and GSE97332 were screened, separately. Then, circRNAs up-regulated or down-regulated in both profiles were selected and identified.

Functional enrichment analysis

Gene ontolog (GO) annotation, including biological process (BP), cellular component (CC), and molecular function (MF), was analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; https://david. ncifcrf.gov) [22-24]. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Panther (http.//www.pantherdb.org/) pathway analysis were used to classify genomic and gene functional information [25-26]. The significant enrichment results were obtained with *p* values <0.05.

Prediction of circRNA-miRNA and miRNA-mRNA interactions

Online tools miRDB (http.//www.mirdb.org/) and circinteractome (https.//circinteractome.nia.nih.gov/) were used to predict the possible interactions between circRNAs and miRNAs [27-29]; miRDB and TargetScan (http.//www.targetscan.org/mamm_31/) were applied for predicting target genes of the four essential miRNAs; while mirTarBase (http.//mirtarbase.mbc.nctu.edu.tw/php/index.php) was used to obtain experimentally strongly supported targets genes (by Reporter assay, Western blot, or qPCR) of these miRNAs [30-32].

Construction of protein-protein interaction (PPI) network

Experimentally supported target genes of the four essential miRNAs were put into the Search Tool for the Retrieval of Interacting Genes database (STRING; https.//string-db.org/), and an interaction network chart with a combined score > 0.4 was saved and exported [33]. Subsequently, top 50 genes with more counts of interacting protein were selected, and the PPI network was visualized using Cytoscape software (version 3.6.1; http.//cytoscape.org/) [34]. The values of gene interactions predicted by STRING were also imported into Cytoscape to identify hub genes among potential targets.

Cell culture

HCC cell line HepG2 was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA), and human normal liver cell line L02 was cultured in 1640 in a humidified 37℃ incubator with 5% CO_2. Both media were supplemented with 10% FBS (Gibco.USA), 2 mmol/L L-glutamine and 100U/mL penicillin/100 μg/mL streptomycin (Life Technologies, CA, USA).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs from HepG2 and L02 cells were extracted using TRIzol reagent (Life Technologies, CA, USA) according to the manufacture's protocol. RNA quality and quantity was meatured using Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments Inc, Winooski, VT, USA), Total RNAs were reversely transcribe using HiScript Q RT SuperMix for qPCR with gDNA wiper (Vazyme Biotech, Nanjing, China), and qPCR assays were performed in triplicate using AceQ qPCR SYBR Green Master Mix kit (Vazyme Biotech, Nanjing, China) on the ABI QuantStudio 3 real-time PCR system (ThermoFisher Scientific, USA). The convergent primers used for detecting circRNAs were synthesized from Shanghai Generay Biotech (Shanghai, China), and β-actin was used as an internal control. The sequences of primers for qPCR were listed in Table 1, and the specificity of PCR products was evaluated by dissociation curve analysis and the Sanger sequencing (Sango Biotech, Shanghai, China).

Table 1. CircRNA primers used for qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
has_circ_0001806	CCATCCCATCAGTTCATCCT	TTCACCTCCAAAGAGCATCC
has_circ_0003528	GTAACCAGCAGCCTGGACTC	GCAACTTGCTGACCAGAACA
has_circ_0008583	TTACGGGAGCAGATGATGAA	CCAAGAAGGAAGATGGGCTA
has_circ_0009910	CAGGTTCTGGACGTCAAAGG	TCACCTCAGCCATGTGTCTC
has_circ_0032704	TTGTTCCTCATCGCAGCAGT	ATAGAGGCGCACGTCAAACT
has_circ_0065214	TCATGTCTGTGGGACTCTGC	GGGCGAGTAATCCTTCACAG
has_circ_0007762	CATTCAGATGGCACCTTGAC	GTGCCCACATAGAGCCACTT
β-actin	AGAAAATCTGGCACCACACC	CAGAGGCGTACAGGGATAGC

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, CA, USA). All Data were shown as mean ± SEM. Student's *t*-test was used to compare the differences of circRNA expression between L02 and HepG2 cells, and a p value <0.05 was considered statistically significant.

Results

Selection of DECs

Two circRNA expression profiles, including GSE94508 and GSE97332, were downloaded from GEO, and GEO2R method was applied to analysis DECs between HCC liver tissues and paired non-tumor tissues. Results showed that many circRNAs were differentially expressed in HCC tissues. In GSE94508, 560 circRNAs were identified to be differentially expressed, including 214 up-regulated and 346 down-regulated circRNAs; while in GSE97332, 892 differentially expressed circRNAs were identified, including 453 up-regulated and 439 down-regulated circRNAs. Among them, 87 circRNAs, including 76 up-regulated and 11 down-regulated circRNAs, were observed in both circRNA expression profiles (Figure 1A and 1B, and Table 2).

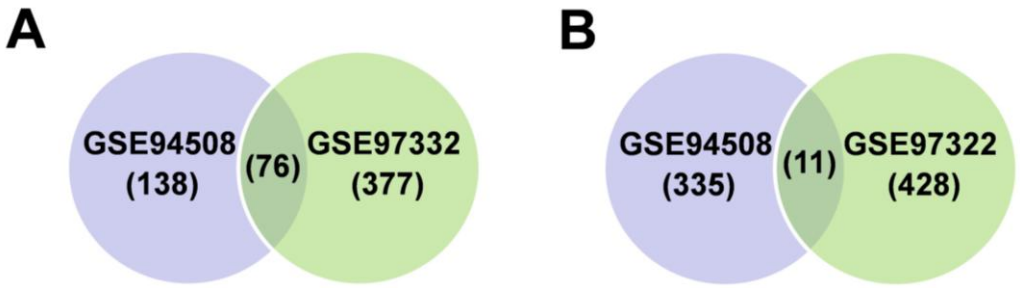


Figure 1. Differentially expressed circular RNAs (DECs) between hepatocellular carcinoma (HCC) and paired non-tumor liver tissues. (A-B) Venn diagram representing the overlap of the identified DECs in HCC tissues compared with paired non-tumor liver tissues from the results of microarray GSE94508 (5 pairs) and GSE97332 (7 pairs). (A) Up-regulated circRNAs in HCC tissues. (B) Down-regulated circRNAs in HCC tissues.

Table 2. Differentially expressed circRNAs (DECs) between hepatocellular carcinoma (HCC) and paired non-tumor liver tissues

No.	CircRNA_ID	Chromosome location	Gene symbol	Accession Number
Up-regulated circRNAs				
1	hsa_circ_0000291	chr11:35163017-35163328+	CD44	NM_001202557
2	hsa_circ_0000511	chr14:20811282-20811431-	RPPH1	NR_002312

3	hsa_circ_0000554	chr14:74551677-74551959+	LIN52	NM_001024674
4	hsa_circ_0000644	chr15:81195758-81195954-	KIAA1199	NM_018689
5	hsa_circ_0000673	chr16:11940357-11940700-	RSL1D1	NM_015659
6	hsa_circ_0000747	chr17:27209637-27210251+	FLOT2	NM_004475
7	hsa_circ_0000981	chr2:20240809-20240905-	LAPTM4A	NM_014713
8	hsa_circ_0001228	chr22:37868480-37870715-	MFNG	NM_002405
9	hsa_circ_0001279	chr3:33109721-33110462-	GLB1	NM_001079811
10	hsa_circ_0001338	chr3:128824688-128825122-	RAB43	NM_001204883
11	hsa_circ_0001489	chr5:59770958-59771235+	PDE4D	NM_001165899
12	hsa_circ_0001806	chr8:68018139-68028357+	CSPP1	NM_024790
13	hsa_circ_0001828	chr8:142139086-142139265+	DENND3	NM_014957
14	hsa_circ_0001834	chr9:2017333-2017502+	SMARCA2	NM_003070
15	hsa_circ_0001901	chr9:138773785-138774005-	CAMSAP1	NM_015447
16	hsa_circ_0001917	chrX:41519691-41530783-	CASK	NM_001126055
17	hsa_circ_0001955	chr15:64495280-64508912-	CSNK1G1	NM_022048
18	hsa_circ_0002191	chr9:97535283-97563284+	C9orf3	NM_001193331
19	hsa_circ_0002702	chr9:35546426-35548532+	RUSC2	NM_001135999
20	hsa_circ_0003528	chr5:134032815-134044578+	SEC24A	NM_021982
21	hsa_circ_0003645	chr16:19656207-19663412+	C16orf62	NM_020314
22	hsa_circ_0003892	chr19:11230767-11238761+	LDLR	NM_000527
23	hsa_circ_0003923	chr2:238933982-238940895+	UBE2F	NM_080678
24	hsa_circ_0003945	chr9:33953282-33956144-	UBAP2	NM_018449
25	hsa_circ_0003958	chr7:27668989-27672064-	HIBADH	NM_152740
26	hsa_circ_0004004	chr5:172359438-172362313+	ERGIC1	NM_001031711
27	hsa_circ_0004976	chr2:25990451-25994409-	ASXL2	NM_018263
28	hsa_circ_0005397	chr17:30500849-30503232+	RHOT1	NM_001033568
29	hsa_circ_0005785	chr12:110819556-110834257-	ANAPC7	NM_016238
30	hsa_circ_0006517	chr3:188202379-188242575+	LPP	NM_005578
31	hsa_circ_0006789	chrX:118544152-118544325+	SLC25A43	NM_145305
32	hsa_circ_0007196	chr3:50144199-50147121+	RBM5	NM_005778
33	hsa_circ_0008274	chr13:96485180-96489456-	UGGT2	NM_020121
34	hsa_circ_0008310	chr17:7849045-7849304+	CNTROB	NM_001037144
35	hsa_circ_0008563	chr1:21599191-21599404-	ECE1	NM_001113347
36	hsa_circ_0008583	chr3:196817782-196846401-	DLG1	NM_004087
37	hsa_circ_0009910	chr1:12049221-12052747+	MFN2	NM_014874
38	hsa_circ_0012107	chr1:44290402-44303983+	ST3GAL3	NM_174963
39	hsa_circ_0013048	chr1:82302569-82372915+	LPHN2	NM_012302
40	hsa_circ_0014879	chr1:160206924-160231148-	DCAF8	NR_028103
41	hsa_circ_0016404	chr1:212977661-212977993+	TATDN3	NM_001146171
42	hsa_circ_0018004	chr10:27024168-27024508+	PDSS1	NM_014317
43	hsa_circ_0026143	chr12:49722709-49723237+	TROAP	NM_005480
44	hsa_circ_0027478	chr12:69109406-69125499+	NUP107	NM_020401
45	hsa_circ_0028196	chr12:110826316-110834257-	ANAPC7	NM_016238
46	hsa_circ_0029325	chr12:125270902-125284788-	SCARB1	NM_005505
47	hsa_circ_0031132	chr14:21464367-21464870+	METTL17	NM_022734
48	hsa_circ_0032704	chr14:76173360-76187046+	TTLL5	NM_015072
49	hsa_circ_0033408	chr14:102842986-102931626+	TECPR2	NM_014844
50	hsa_circ_0036005	chr15:67004005-67008836+	SMAD6	NR_027654
51	hsa_circ_0038718	chr16:27351506-27353580+	IL4R	NM_000418
52	hsa_circ_0039053	chr16:30510394-30510810+	ITGAL	NM_002209
53	hsa_circ_0043438	chr17:37583953-37584043-	MED1	NM_004774
54	hsa_circ_0045006	chr17:59152280-59161925+	BCAS3	NM_001099432
55	hsa_circ_0045862	chr17:76082583-76083174+	TNRC6C	NM_001142640
56	hsa_circ_0048937	chr19:6934997-6937659+	EMR1	NM_001974
57	hsa_circ_0049997	chr19:17626981-17628198+	PGLS	NM_012088

58	hsa_circ_0051220	chr19:41884185-41884424+	TMEM91	NM_001098821
59	hsa_circ_0051732	chr19:48660270-48660397-	LIG1	NM_000234
60	hsa_circ_0060055	chr20:33866724-33872064-	EIF6	NM_181468
61	hsa_circ_0062682	chr22:26936754-26937684-	TPST2	NM_001008566
62	hsa_circ_0064288	chr3:11348416-11350535+	ATG7	NM_006395
63	hsa_circ_0065214	chr3:47466974-47476627-	SCAP	NM_012235
64	hsa_circ_0067934	chr3:170013698-170015181+	PRKCI	NM_002740
65	hsa_circ_0072088	chr5:32379220-32388780-	ZFR	NM_016107
66	hsa_circ_0073271	chr5:88044886-88047860-	MEF2C	NM_002397
67	hsa_circ_0074903	chr5:168110970-168112932-	SLIT3	NM_003062
68	hsa_circ_0078738	chr6:170033042-170058454-	WDR27	NM_182552
69	hsa_circ_0082182	chr7:128317617-128323309+	FAM71F2	NM_001128926
70	hsa_circ_0082564	chr7:137569740-137570248-	CREB3L2	NM_194071
71	hsa_circ_0083766	chr8:27382878-27394372+	EPHX2	NM_001979
72	hsa_circ_0091331	chrX:109310574-109352374+	TMEM164	NM_032227
73	hsa_circ_0092283	chr22:36681395-36681695-	MYH9	NM_002473
74	hsa_circ_0092298	chr5:178287348-178287568+	ZNF354B	NM_058230
75	hsa_circ_0092310	chr6:43467278-43467478+	TJAP1	NM_001146018
76	hsa_circ_0092327	chr19:17972481-17972901+	RPL18A	NM_000980
Down-regulated circRNAs				
1	hsa_circ_0003859	chr19:41122794-41125398+	LTBP4	NM_001042544
2	hsa_circ_0004913	chr17:62248459-62265775-	TEX2	NM_018469
3	hsa_circ_0005428	chr1:47761436-47770668-	STIL	NM_001048166
4	hsa_circ_0007762	chr6:147581750-147599340+	STXBP5	NM_001127715
5	hsa_circ_0008160	chr21:38439561-38441924-	PIGP	NR_028352
6	hsa_circ_0036044	chr15:68434283-68457142+	PIAS1	NM_016166
7	hsa_circ_0038929	chr16:29810948-29811369+	KIF22	NM_007317
8	hsa_circ_0043302	chr17:36353600-36353765-	LOC440434	NR_036750
9	hsa_circ_0051637	chr19:47285639-47285806-	SLC1A5	NM_005628
10	hsa_circ_0056548	chr2:135878388-135881816+	RAB3GAP1	NM_001172435
11	hsa_circ_0078279	chr6:151226785-151293194+	MTHFD1L	NM_001242767

GO and pathway analysis of the host genes of DECs

Since some circRNAs function by regulating their parental genes, GO analysis was carried out to annotate the host genes of the 87 DECs. Biological process analysis showed that these host genes were enriched in cell adhesion, positive/ negative regulation of gene expression, and Golgi organization. Cellular component analysis indicated that these host genes were remarkably enriched in cytosol, membrane and Golgi apparatus. For molecular function, the most significantly enriched GO term was protein binding (Figure 2A). KEGG and Panther pathway analysis demonstrated that tight junction, aldosterone synthesis and secretion, inflammation mediated by chemokine and cytokine signaling pathway, and Wnt signaling pathway may involved

in circRNA-mediated regulatory network in the pathogenesis of HCC (Figure 2B).

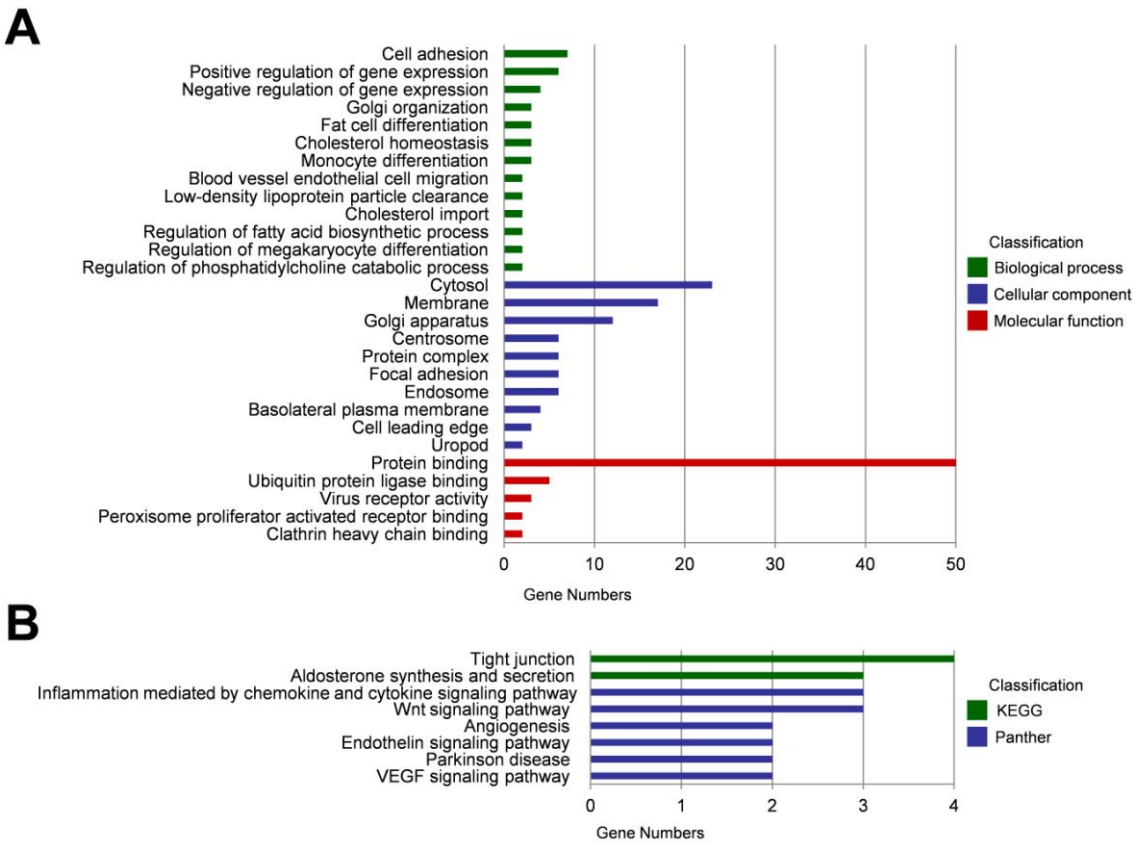


Figure 2. Functional annotation of the host genes of DECs. (A) Gene ontolog (GO) enrichment analyses of the host genes of DECs, including biological process, cellular component, and molecular function. (B) KEGG and Panther pathway enrichment analyses of the host genes of DECs.

Prediction of circRNA-miRNA interactions

Increasing evidences have demonstrated that circRNAs may competitively bind to miRNAs and increase the expression of the target genes of these miRNAs. For the 87 DECs, online tools miRDB and circinteractome were used to predict the possible interactions between circRNAs and miRNAs, and 343 and 1085 circRNA-miRNA interactions were obtained from miRDB and circinteractome, respectively. Furthermore, 20 consensus miRNAs from both prediction tools were identified, and DECs potentially bind to these miRNAs were shown in Table 3. Results showed that one specific circRNA may bind to more miRNAs, while different circRNAs could interact with one

specific miRNAs, indicating that circRNAs may impact on HCC progression by modulating various miRNAs.

Table 3. Consensus miRNAs and DECs potentially interact with them.

MiRNAs	DECs predicted to interact with
hsa-miR-1248	hsa_circ_0032704, hsa_circ_0000673, hsa_circ_0039053, hsa_circ_0016404, hsa_circ_0028196, hsa_circ_0003958, hsa_circ_0004976, hsa_circ_0008583, hsa_circ_0003528, hsa_circ_0001806, hsa_circ_0078279, hsa_circ_0005428
hsa-miR-1236-3p	hsa_circ_0032704, hsa_circ_0016404, hsa_circ_0027478, hsa_circ_0065214, hsa_circ_0001806, hsa_circ_0065214, hsa_circ_0002191, hsa_circ_0014879, hsa_circ_0007762, hsa_circ_0003859
hsa-miR-330-5p	hsa_circ_0001338, hsa_circ_0000291, hsa_circ_0005397, hsa_circ_0003892, hsa_circ_0007196, hsa_circ_0082182, hsa_circ_0002702, hsa_circ_0043302, hsa_circ_0026143, hsa_circ_0001955
hsa-miR-615-5p	hsa_circ_0008563, hsa_circ_0045006, hsa_circ_0092283, hsa_circ_0009910, hsa_circ_0002702, hsa_circ_0001228, hsa_circ_0006789, hsa_circ_0000511, hsa_circ_0003859
hsa-miR-1299	hsa_circ_0039053, hsa_circ_0002702, hsa_circ_0003645, hsa_circ_0002191, hsa_circ_0056548, hsa_circ_0007762, hsa_circ_0001806, hsa_circ_0001955, hsa_circ_0083766
hsa-miR-486-3p	hsa_circ_0002702, hsa_circ_0003859, hsa_circ_0001806, hsa_circ_0000554, hsa_circ_0000291, hsa_circ_0001806, hsa_circ_0033408, hsa_circ_0092310
hsa-miR-370-3p	hsa_circ_0004976, hsa_circ_0003528, hsa_circ_0074903, hsa_circ_0003945, hsa_circ_0001338, hsa_circ_0026143, hsa_circ_0062682
hsa-miR-589-5p	hsa_circ_0005428, hsa_circ_0000291, hsa_circ_0003892, hsa_circ_0008583, hsa_circ_0002191, hsa_circ_0078279
hsa-miR-145-5p	hsa_circ_0001955, hsa_circ_0028196, hsa_circ_0083766, hsa_circ_0001489, hsa_circ_0027478, hsa_circ_0005428
hsa-miR-1286	hsa_circ_0000747, hsa_circ_0026143, hsa_circ_0038718, hsa_circ_0051732, hsa_circ_0003958, hsa_circ_0002702
hsa-miR-7-5p	hsa_circ_0028196, hsa_circ_0065214, hsa_circ_0005785, hsa_circ_0003892, hsa_circ_0001489, hsa_circ_0092327
hsa-miR-637	hsa_circ_0016404, hsa_circ_0039053, hsa_circ_0026143, hsa_circ_0004913, hsa_circ_0003892, hsa_circ_0005397
hsa-miR-377-3p	hsa_circ_0036044, hsa_circ_0003859, hsa_circ_0072088, hsa_circ_0008274, hsa_circ_0003645
hsa-miR-203a-3p	hsa_circ_0039053, hsa_circ_0013048, hsa_circ_0032704, hsa_circ_0078279
hsa-miR-1296-5p	hsa_circ_0001955, hsa_circ_0001489, hsa_circ_0001279, hsa_circ_0082564
hsa-miR-192-5p	hsa_circ_0003528, hsa_circ_0078738, hsa_circ_0007196
hsa-miR-1256	hsa_circ_0033408, hsa_circ_0002191, hsa_circ_0001955
hsa-miR-1289	hsa_circ_0049997, hsa_circ_0004976, hsa_circ_0008160
hsa-miR-215-5p	hsa_circ_0003528, hsa_circ_0078738
hsa-miR-1261	hsa_circ_0072088, hsa_circ_0009910

Target genes of some essential miRNAs and their function analysis

Among the 20 consensus miRNAs, four essential miRNAs, including miR-7, hsa-miR-145-5p (miR-145), hsa-miR-203a-3p (miR-203a), and hsa-miR-192-5p (miR-192), were reported to be down-regulated in HCC, and play pivotal roles in HCC

progression by targeting multiple protein-coding genes. Using online tools miRDB and TargetScan, 1480 target genes of these four miRNAs were predicted, and then presented in Venn diagram (Figure 3). Two common target genes (SLC4A4 and ZDHHC9) were found for miR-7, miR-145 and miR-203a; two target genes (CPEB4, DYRK1A) were found for miR-145, miR-203a, and miR-192; and one target gene (MECP2) for miR-7, miR-203a and miR-192; while none of these genes was predicted to be targeted by all the four miRNAs. KEGG and Panther pathway analyses revealed that these target genes were presented in many HCC-associated signaling pathways, including Wnt, TGF-β, Ras, etc., suggesting their possible roles in HCC pathogenesis (Figure 4).

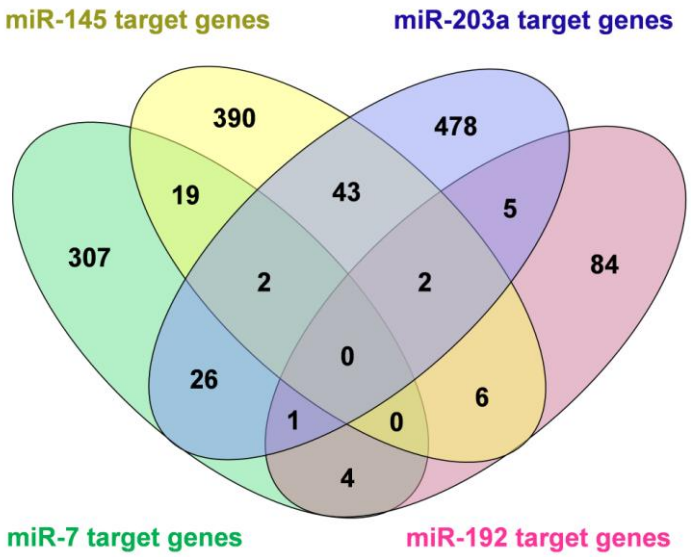


Figure 3. Venn diagram of target genes of the four essential microRNAs (miRNAs), including hsa-miR-7-5p (miR-7), hsa-miR -145-5p (miR-145), hsa-miR-203a-3p (miR-203), and hsa-miR-192-5p (miR-192). 1480 target genes of the four miRNAs were predicted using online tools miRDB and TargetScan.

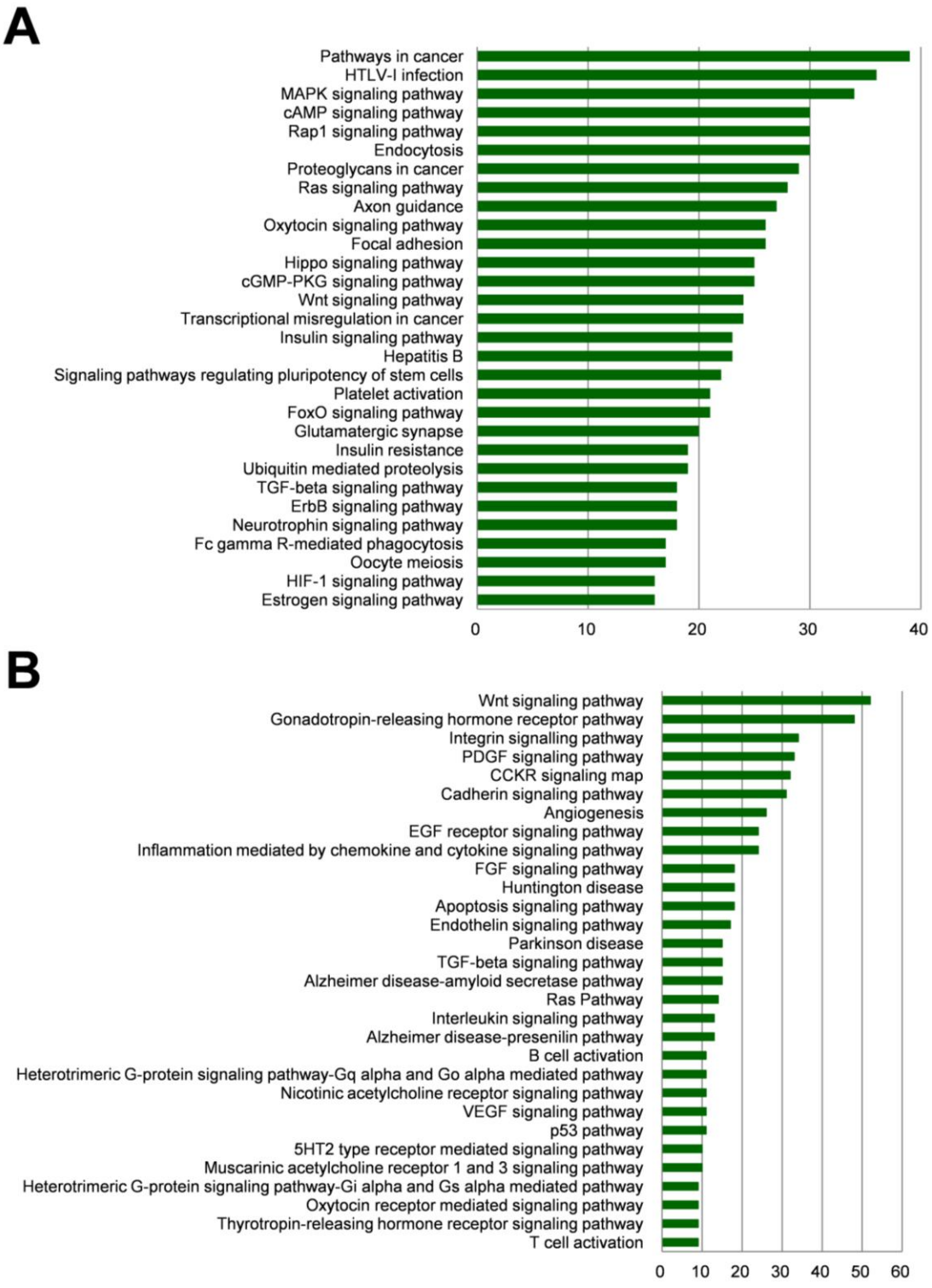


Figure 4. Pathway analysis of target genes of the four essential miRNAs. (A-B) KEGG pathway (A) and Panther pathway (B) functional analyses of these target genes.

Construction of essential miRNAs-centered regulatory network

To analysis the significance of target genes of the four essential miRNAs, their

experimentally strongly supported targets genes were obtained from mirTarBase. Among these, some genes, such as ABCC1 and CUL5, have been demonstrated to be targeted by more than one essential miRNAs, suggesting a complex regulatory network involving the four essential miRNAs and their target genes. Furthermore, protein-protein interaction (PPI) was analyzed using online tool STRING, and the PPI network of the top 50 genes which have more counts of interacting protein was visualized using Cytoscape software (Figure 5). Subsequently, four essential miRNAs and their potential interacting circRNAs were combined into the regulatory network. In this figure, proteins with bigger circles showed more interactions with other proteins than those with smaller circles.

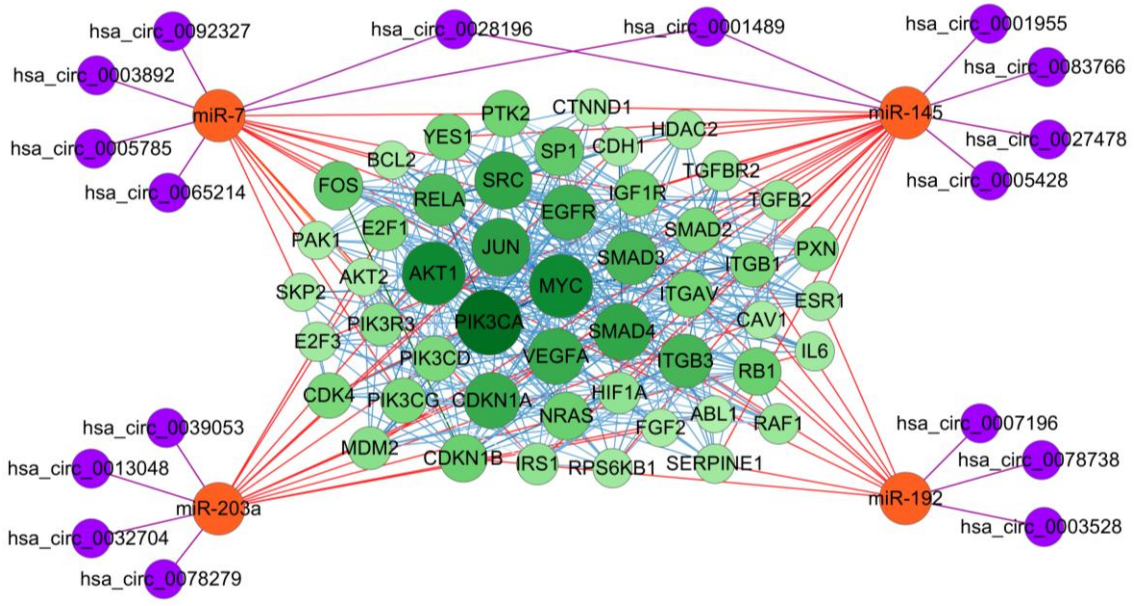
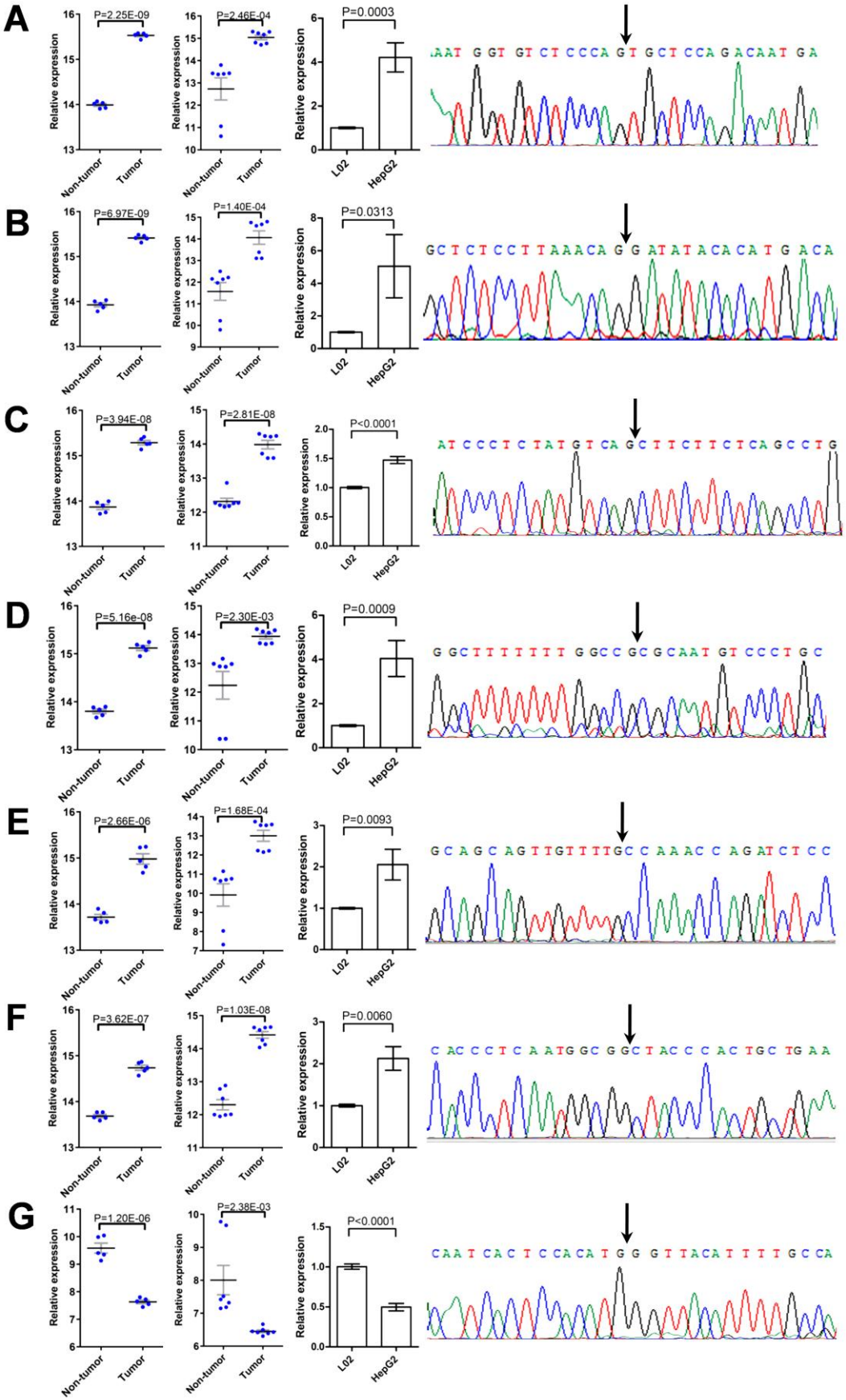


Figure 5. Essential miRNAs-centered circRNA-miRNA-target gene network. The experimentally supported target genes of the four essential miRNAs were obtained using online tool mirTarBase. MiRNA-mRNA interaction was represented as orange edges, protein-protein interaction was represented as blue edges, and circRNA-miRNA interaction was represented as purple edges. PIK3CA, AKT1, MYC, JUN, SMAD4, and SRC were shown as hub target genes as they have more counts of interacting protein.

Validation of DECs

To verify the deregulation of DECs, nine of them were randomly chosen for further validation. The expression of these DECs in L02 and HepG2 cells were detected using qRT-PCR, and the specificity of qPCR products was evaluated by dissociation curve analysis and sequencing. Among them, the expression of two circRNAs, including hsa_circ_0001489 and hsa_circ_0039053, was low in both cell lines, and was failed to be detected. Six circRNAs were validated to be greatly up-regulated in HepG2 cells compared with L02 cells, including hsa_circ_0001806, hsa_circ_0003528, hsa_circ_0008583, hsa_circ_0009910, hsa_circ_0032704, and hsa_circ_0065214 (Figure 6A-6F); while hsa_circ_0007762 was found to be greatly down-regulated in HepG2 cells compared with L02 cells (Figure 6G). The expression pattern of these circRNAs in HCC cell lines was similar to that in HCC specimens from the two GEO HCC datasets.


A
P=2.25E-09
P=2.46E-04
P=0.0003
Relative expression
Non-tumor
Tumor
L02
HepG2
B
P=6.97E-09
P=1.40E-04
P=0.0313
C
P=3.94E-08
P=2.81E-08
P<0.0001
D
P=5.16e-08
P=2.30E-03
P=0.0009
E
P=2.66E-06
P=1.68E-04
P=0.0093
F
P=3.62E-07
P=1.03E-08
P=0.0060
G
P=1.20E-06
P=2.38E-03
P<0.0001

263

Figure 6. Validation of DECs. (A-G) The deregulation of seven DECs, including hsa_circ_0001806 (A), hsa_circ_0003528 (B), hsa_circ_0008583 (C), hsa_circ_0009910 (D), hsa_circ_0032704 (E), hsa_circ_0065214 (F), and hsa_circ_0007762 (G), was validated by qRT-PCR and sequencing. For each DEC, from left to right: comparison of relative expression of the DEC between HCC tissues and paired non-tumor tissues, as extracted from GSE94508 (five pairs) and GSE97332 (seven pairs), respectively; comparison of relative expression of the DEC between HCC cell line HepG2 and normal liver cell line L02, as determined by qRT-PCR; and the specificity of the convergent PCR products, as verified by the Sanger sequencing of the DEC in back-splice junction. Data were shown as mean ± SEM, and student's *t*-test was used to compare the differences of circRNA expression between two groups.

Discussion

Emerging evidences indicate that circRNAs are frequently deregulated in HCC tissues. Some deregulated circRNAs are promising biomarkers for HCC diagnosis and prognosis, while others may regulate HCC progression through diverse mechanisms by functioning as miRNA sponges, RNA-binding protein sponges, or transcriptional regulators [4]. In the current study, we identified 87 DECs from the HCC circRNA expression profiles of GSE94508 and GSE97332 downloaded from GEO database, and analyzed the functions and possible underlying mechanisms of these DECs in HCC tumorigenesis and progression using bioinformatic methods. In the meantime, the deregulation of some DECs was validated in HCC cells by qRT-PCR and the Sanger sequencing.

Among the 87 DECs identified in our study, four up-regulated circRNAs, including hsa_circ_0000673, hsa_circRNA_0072088, hsa_circ_0009910, and hsa_circ_0067934, were reported to play important roles in the progression of different cancers, such as HCC, gastric cancer (GC), colorectal cancer (CRC), and esophageal squamous cell

carcinoma (ESCC), etc. [35-40]. Two circRNAs, hsa_circ_0000673 and hsa_circRNA_0072088, were up-regulated in HCC and lung cancer, separately, and play tumor-promoting roles by modulating miR-767-3p/SET axis and miR-4302/znf121/MYC axis, respectively [35, 37]. However, they were down-regulated, and may act as tumor suppressors in other cancers. Hsa_circ_0000673 was reported to inhibit tumor suppressor gene RUNX3 by targeting miR-532-5p, leading to inhibition of GC progression; while hsa_circRNA_0072088 promote FOXO4 by targeting miR-532-3p, resulting in suppression of CRC [36, 38]. Other two circRNAs, hsa_circ_0009910 and hsa_circ_0067934, were demonstrated to be up-regulated in osteosarcoma and ESCC, separately, and play oncogenic roles in these cancers. Hsa_circ_0009910 was capable of sponging miR-499a and enhancing the expression of its target gene IL6R, and thus accelerating carcinogenesis of osteosarcoma; while hsa_circ_0067934 could promote the proliferation and migration of ESCC cells, and its expression level in ESCC was associated with tumor differentiation and TNM stages [39, 40]. Interestingly, our study demonstrated the up-regulation of hsa_circ_009910 in HCC, indicating its possible role in HCC progression. The precise function and possible mechanisms of this circRNA in HCC deserves further investigation.

Recent research works suggest that some circRNAs may regulate the transcription of their parental genes [41]. In the current study, the possible GO functional terms and signaling pathways of the host genes of these DECs were characterized. Results showed that these host genes were enriched in cell adhesion, cytosol, and protein binding, all of which were associated with HCC progression. Pathway analysis from KEGG and Panther demonstrated that tight junction and Wnt signaling pathway are among the most relevant pathways for HCC. Tight junction signaling contributes to pathogenesis of GC

and CRC, plays functional roles in epithelial-to-mesenchymal transition and viral entry, and could be used as potential targets for gastrointestinal and liver disease; while Wnt signaling pathway is well known to impact on hepatocarcinogenesis and HCC progression [42-46]. These results suggested that these DECs may participate in HCC progression by regulating their parental genes.

Many miRNAs have been demonstrated to regulate HCC progression by modulating the expression of one or more HCC-associated genes [47, 48]. However, the functions of miRNAs can be interfered by their possible interaction with circRNAs and lncRNAs. One of the most interesting examples is the interaction between ciRS-7 and miR-7. CiRS-7 is a special and unique natural circRNA which harbors more than 70 binding sites for miR-7, which could bind to miR-7 and facilitate the expression of miR-7 target genes, such as CCNE1, PIK3CD, and EGFR, and thus block the inhibitory effect of miR-7 on HCC [17-19]. Similarly, circRNAs circMTO1 and circHIPK3 were found to modulate HCC progression by competitively bind to miR-9 and miR-124-3p, and increase the expression of multiple oncogenic genes, such as p21, Norch1, AQP3, etc. [14, 49]. In the current study, the interactions between DECs and miRNAs were predicted. Among all the DECs, hsa_circ_0005428 contains the most number of interactions and could potentially bind to 16 different miRNAs, whereas hsa_circ_0001834 and hsa_circ_000511 have only one potential miRNA binding site. Among the top 20 miRNAs which predispose to interact with DECs, each one contains at least two potential DECs binding sites. Specially, hsa-miR-1248 has as many as 12 DECs binding sites. Given the importance of miRNAs in HCC progression, it is possible that DECs could impact on HCC progression by targeting specific miRNAs to increasing the expression of their target genes.

Among the top 20 miRNAs, four essential miRNAs, including miR-7, miR-145, miR-203, and miR-192, were reported to be down-regulated in HCC, and could inhibit HCC progression by modulating the expression of multiple target genes; while their modulation on target genes may be blocked by their interaction with non-coding RNAs. MiR-7 could be sponged by ciRS-7 and lncRNA KCNQ1OT1, and reduced expression of miR-7 leads to increased expression of its target genes CCNE1, PIK3CD, EGFR, and ABCC1, resulting in promotion of HCC progression [18, 19, 50]; whereas miR-145 could be blocked by lincRNA-ROR and pseudogene OCT4-pg4, and the reduction of this miRNA led to enhanced expression of its target genes RAD18, ZEB2, and OCT4, which in turn aggravates the development of HCC [51-53]. MiR-203 could interact with three lncRNAs, including CRNDE, UCA1 and HULC, and the interaction of which greatly elevated the expression of miR-203 target genes BCAT1, SNAI2, and ADAM9, leading to acceleration of HCC progression [54-56]; while miR-192, however, could be sponged by lncRNA HOTTIP, and the decrease of this miRNA led to exacerbated expression of its target gene GLS1, and resulting in enhanced progression of HCC [57]. Regarding to their pivotal roles in HCC progression, these four essential miRNAs were selected for further analysis. Their predicted and validated target genes were obtained, and pathway and protein-protein interaction network analyses were carried out to investigate the possible functions and related pathways of these target genes in HCC progression. KEGG and Panther pathway analyses suggested that the predicted target genes of these miRNAs are involved in various pathways, including Wnt, TGF-β, oxytoxin, Ras, etc., all of which are closely related to hepatogenesis and HCC progression. A four essential miRNAs-centered circRNA-miRNA-mRNA network was established by combination of the potential interacting DECs of the four miRNAs with

the PPI network based on the top 50 experimentally supported target genes of these miRNAs. Results suggested that some target genes of these miRNAs, such as PIK3CA, AKT1, MYC, JUN, SMAD4, and SRC, may impact on cell function by interacting with different molecules in cells and interfering with various signaling pathways, while specific circRNAs may control the expression of these genes by indirectly targeting essential miRNAs. As expected, we found that the expression of two circRNAs, including hsa_circ_0032704 and hsa_circ_0065214, were greatly increased in HCC tissues and cell lines, suggesting that these two circRNAs may function in HCC progression by targeting HCC suppressor genes miR-203a and miR-7, respectively, leading to enhanced expression of oncogenic proteins and promotion of HCC progression. In following investigations, the cellular location of these two circRNAs needs to be determined, and their function and molecular mechanisms, including possible interactions with miRNAs, need to be explored.

In conclusion, this study reveals aberrant circRNA profile of HCC, and discussed the possible roles of these deregulated circRNAs in HCC progression. Further investigations are needed to fully elucidate the function and underlying mechanisms of these deregulated circRNAs and their regulatory networks in tumorigenesis and HCC progression.

Abbreviations

HCC, Hepatocellular carcinoma; circRNAs, circular RNAs; ncRNAs, non-coding RNAs; GEO, the Gene Expression Omnibus database; DECs, differentially expressed circRNAs; GO, Gene ontolog; PPI, protein-protein network; qRT-PCR, quantitative real-time polymerase chain reaction; EcircRNAs, circular exonic circRNAs; MREs, miRNA response elements; ciRNAs, circular intronic RNAs; EIciRNAs, exon-intron

circRNAs; miR-7, hsa-miR-7-5p; FC, fold change; BP, biological process; CC, cellular component; MF, molecular function; DAVID, the Database for Annotation, Visualization, and Integrated Discovery; KEGG, Kyoto Encyclopedia of Genes and Genomes; STRING, the Search Tool for the Retrieval of Interacting Genes database; miR-145, hsa-miR-145-5p; miR-203a, hsa-miR-203a-3p; miR-192, hsa-miR-192-5p; GC, gastric cancer; CRC, colorectal cancer; ESCC, esophageal squamous cell carcinoma.

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Competing Interests:

The authors have declared that no competing interest exists.

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