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Original Paper

Expression Profiling Identifies Circular RNA Signature in Hepatoblastoma

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Key Words
Circular RNA • Hepatoblastoma • MicroRNA sponge

Abstract
Background/Aims: Hepatoblastoma is the most common malignant pediatric liver cancer. circular RNAs (circRNAs) play important roles in fine-tuning gene expression and are often deregulated in cancers. However, the expression profile and clinical significance of circRNAs in hepatoblastoma is still unknown. ***Methods:*** Circular RNA microarray was conducted to identify hepatoblastoma-related circRNAs. GO analysis, pathway analysis, and miRNA response elements analysis was conducted to predict the potential roles of differentially expressed circRNAs in hepatoblastoma. MTT assays, Ki67 staining, and Transwell assays were conducted to clarify the role of circRNA in hepatoblastoma *in vitro*. Bioinformatics analysis and *in vitro* experiments were conducted to clarify the mechanism of circRNA-mediated gene regulation in hepatoblastoma cell. ***Results:*** 869 differentially expressed circRNAs were identified between hepatoblastoma and adjacent normal liver samples, including 421 up-regulated circRNAs and 448 down-regulated circRNAs. The significant enriched GO term of hepatoblastoma-related circRNAs in biological process, cellular component, and molecular function were "chromosome organization", "cytoplasm", and "organic cyclic compound binding". Tight junction signaling pathway was ranked the Top 1 potentially affected by circRNA-mediated regulatory network. circ_0015756 was significantly up-regulated in human hepatoblastoma specimens and metastatic hepatoblastoma cell lines. circ_0015756 silencing decreased hepatoblastoma cell viability, proliferation, and invasion *in vitro*. circ_0015756 acted as miR-1250-3p sponge to regulate hepatoblastoma cell function. ***Conclusions:*** circRNAs are involved in the pathogenesis of hepatoblastoma. circ_0015756 is a promising target for the prognosis, diagnosis, and treatment of hepatoblastoma.

Introduction

Hepatoblastoma is one of the major liver cancers in infants and toddlers, accounting for about 1% of pediatric cancers. It develops after a short latency period. Less than 50% of

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cases are resectable at the time of the diagnosis [1, 2]. The occurrence of hepatoblastoma is associated with the familial adenomatous polyposis, low or high birth weights, and constitutional trisomy [3, 4]. Although the pathogenesis of hepatoblastoma has been studied for several years, the precise etiology is still unknown.

So far, the available methods for hepatoblastoma treatment mainly include surgical resection, adjuvant chemotherapy, and liver transplantation. However, a high percentage of patients have a high risk of relapse or metastasis. The mortality rate is still over 35% in high-risk patients [2, 5]. Thus, further clarifying the pathogenesis of hepatoblastoma is still required for improving diagnosis, prevention, and treatment of hepatoblastoma.

Circular RNAs (circRNAs) is a novel class of RNA transcripts that are widely expressed in mammal genome. The 5' and 3' ends are covalently linked to form a closed circular structure. circRNAs are usually more stable than their host genes, linear mRNAs. Several circRNAs are highly conserved expressed across different species [6]. Previous studies have shown that circRNAs are involved in many developmental and physiological processes, including cell proliferation, apoptosis, differentiation, and angiogenesis [7]. Moreover, dysregulated expression of circRNAs has been implicated in many cancers, such as glioma, ovarian cancer, and gastric cancer [8-10].

Considering the critical roles of circRNAs in cancers, we speculated that circRNAs might be involved in the pathogenesis of hepatoblastoma. We compared circRNA expression profiles between hepatoblastoma and paired adjacent normal tissue samples. We identified 869 differentially expressed circRNAs in hepatoblastoma tissues. And then, we focused on hsa_circ_0015756, one of the most up-regulated circRNAs. We found that circ_0015756 was significantly up-regulated in clinical samples and metastatic hepatoblastoma cell lines. circ_0015756 acted as miR-1250-3p sponge to regulate hepatoblastoma cell function.

Materials and Methods

Human tissue specimens and cell culture

Human hepatoblastoma tissues were collected from the patients undergoing resection of hepatoblastoma in Children hospital, Fudan University (China). A written informed consent was obtained from every patient or their guardians. This study was approved by the Institute Research Ethics Committee in this hospital.

Tumor cell lines, HepG2, HuH-6, and SMMC-7221, and nonmalignant QSG-7701 and L02 hepatocytes were cultured in the Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO_2 at 37 °C

RNA extraction

Total RNAs from the hepatoblastoma and adjacent normal liver tissues were extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol.. Total RNA from the whole blood was extracted using a rapid blood total RNA extraction kit (Biotech, China). RNA quality for microarray analysis was estimated by the Agilent Bioanalyzer 2100 (Agilent technologies). The amount of extracted RNA was measured using a Nanodrop 2000 (Thermo Scientific, USA). RNA integrity was estimated by using the 1% formaldehyde denaturing gel electrophoresis.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNAs were reversely transcribed using the PrimeScript RT reagent Kit (TaKaRa). qPCR assays were conducted using the SYBR Green PCR kit (Takara) in the ABI Prism 7300 sequence detection system (Applied Biosystems). Relative gene expression was normalized to GAPDH expression level. The dissociation curve was used to estimate the specificity of PCR products.

Microarray analysis

Three hepatoblastoma and adjacent normal liver samples were used for circRNA microarray. The patients were 23 (female), 23 (female), and 24(male) months old. All histologic classification is epithelial type. The total RNAs were treated with RNase R to remove linear RNAs to enrich circRNA transcripts. The enriched circRNAs were amplified and transcribed into the fluorescent cRNA. The labeled cRNAs

were purified by RNeasy Mini Kit (Qiagen), and then hybridized with the circRNA microarray slide. The hybridized arrays were washed, fixed, and scanned. Scanned images were imported into the Agilent Feature Extraction software for raw data extraction. Quantile normalization of raw data and data processing was conducted using the R software package. After quantile normalization of the raw data, low intensity filtering was performed. The log2-ratio was used for quantile normalization. Differentially expressed circRNAs with statistical significance were identified through fold change filtering or volcano plot filtering. Hierarchical clustering was performed to show the distinguishable circRNA expression pattern among different samples [10, 11]. Circular RNAs exhibiting fold change>2 and *P*-value<0.05 were selected as significantly differentially expressed circRNAs.

Bioinformatics analysis

The host genes of differentially expressed circRNAs were put into to the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov) for the annotation and functional analysis, including Gene Ontology (GO) and KEGG pathway analysis. The potential circRNA-binding miRNAs were predicted based on the miRanda and TargetScan algorithm.

Cell viability assay

MTT assays were conducted to detect cell viability [12, 13]. Briefly, HepG2, HuH-6, or SMMC-7221 cells were seeded at 1.5×10^4 cells/well in a 96-well plate. After the required treatments, they were incubated with MTT (0.5 mg/ml) for 3 h at 37°C After the medium removal, DMSO was used to dissolve the formazan crystal. Absorbance at 570 nm was determined by a microplate reader (Molecular Devices).

Cell proliferation assay

Cell proliferation was detected by using Ki67 staining [14]. HepG2, HuH-6, or SMMC-7221 cells were fixed in 4% formaldehyde for 10 min, and then blocked with 5% BSA for 1 h. They were incubated with Ki67 antibody (Abcam, dilution: 1:200) for overnight at 4°C, and then stained with the Cy3-conjugated secondary antibody. Slides were stained with 4', 6-diamidino-2-phenylindole (DAPI) to label nuclei.

Cell invasion assay

Twenty-four-well culture plates with 8-μm micropore inserts were used for cell invasion assays [15]. The top side of the insert was coated with Matrigel (BD Biosciences). After the required treatment, HepG2, HuH-6, or SMMC-7221 cells were placed into the upper well, cultured for 24 h, and allowed to invade into the Matrigel layer. The cells on the inserts were fixed with 3% paraformaldehyde, stained with crystal violet, and counted.

Statistical analysis

All data were shown as mean±SEM. Comparison between different groups were performed using the Student's *t* test or one-way ANOVA followed by Tukey's post-hoc analysis. *P*<0.05 was considered to be statistically significant.

Results

Screening for hepatoblastoma-related circRNAs by circRNA microarray

We employed the SBC Human (4×180K) circular RNA microarray (Shanghai Biotechnology Co., Ltd.) to identify hepatoblastoma-related circRNAs. The box plot showed the distribution of circRNA expression profiling. After the normalization, the distributions of log2 ratios across different samples were displayed in Fig. 1A. We then used the volcano plot to identify differentially expressed circRNAs between hepatoblastoma (HB) and the matched normal tissues (non-HB) (Fig. 1B).

We set the threshold as fold change >2.0, and identified 869 differentially expressed circRNAs, including 421 up-regulated circRNAs and 448 down-regulated circRNAs (Table 1, HB versus non-HB). We then conducted hierarchical clustering analysis to obtain the systematic comparison of circRNAs expression across different samples. HB samples were clustered

Table 1. Differentially expressed circRNAs between hepatoblastoma (HB)

circRNA	Fold change	circRNA	Fold change	circRNA	Fold change
Up-regulated					
hsa_circ_0080961	62.15	hsa_circ_0054583	3.83	hsa_circ_0091670	2.78
hsa_circ_0067745	38.64	hsa_circ_0039825	3.82	hsa_circ_0002171	2.78
hsa_circ_0062163	38.63	hsa_circ_0071410	3.76	hsa_circ_0036290	2.77
hsa_circ_0015756	29.24	hsa_circ_0013784	3.75	hsa_circ_0029729	2.77
hsa_circ_0079549	28.16	hsa_circ_0021485	3.72	hsa_circ_0048196	2.77
hsa_circ_0017515	26.72	hsa_circ_0029734	3.71	hsa_circ_0091868	2.77
hsa_circ_0045510	24.84	hsa_circ_0060646	3.70	hsa_circ_0074484	2.77
hsa_circ_0030791	23.97	hsa_circ_0057397	3.70	hsa_circ_0087461	2.76
hsa_circ_0005931	23.46	hsa_circ_0054558	3.69	hsa_circ_0042282	2.76
hsa_circ_0078710	21.70	hsa_circ_0032537	3.67	hsa_circ_0036896	2.75
hsa_circ_0078340	19.64	hsa_circ_0066998	3.64	hsa_circ_0002407	2.75
hsa_circ_0057371	17.22	hsa_circ_0060472	3.64	hsa_circ_0031895	2.74
hsa_circ_0028107	16.62	hsa_circ_0005875	3.62	hsa_circ_0015492	2.74
hsa_circ_0020984	14.39	hsa_circ_0071115	3.62	hsa_circ_0022449	2.74
hsa_circ_0079944	13.79	hsa_circ_0054560	3.61	hsa_circ_0039519	2.74
hsa_circ_0086370	13.63	hsa_circ_0054569	3.60	hsa_circ_0011485	2.74
hsa_circ_0063053	12.65	hsa_circ_0090566	3.60	hsa_circ_0089543	2.73
hsa_circ_0055922	12.50	hsa_circ_0089443	3.60	hsa_circ_0089695	2.73
hsa_circ_0003845	11.69	hsa_circ_0002360	3.59	hsa_circ_0074651	2.73
hsa_circ_0078715	11.49	hsa_circ_0042487	3.59	hsa_circ_0079331	2.73
hsa_circ_0005505	11.19	hsa_circ_0038160	3.58	hsa_circ_0000370	2.73
hsa_circ_0045276	11.13	hsa_circ_0066991	3.56	hsa_circ_0049241	2.72
hsa_circ_0017254	11.13	hsa_circ_0021486	3.56	hsa_circ_0035169	2.72
hsa_circ_0003271	11.05	hsa_circ_0037850	3.55	hsa_circ_0005610	2.72
hsa_circ_0039993	10.71	hsa_circ_0051509	3.54	hsa_circ_0089992	2.71
hsa_circ_0075851	10.58	hsa_circ_0085931	3.53	hsa_circ_0048945	2.71
hsa_circ_0045271	10.43	hsa_circ_0002624	3.52	hsa_circ_0036614	2.71
hsa_circ_0006057	10.31	hsa_circ_0022797	3.51	hsa_circ_0011905	2.71
hsa_circ_0064842	10.12	hsa_circ_0066015	3.51	hsa_circ_0010768	2.71
hsa_circ_0076156	10.00	hsa_circ_0081225	3.51	hsa_circ_0036616	2.71
hsa_circ_0084957	9.93	hsa_circ_0027006	3.47	hsa_circ_0081978	2.71
hsa_circ_0064841	9.86	hsa_circ_0027005	3.45	hsa_circ_0072493	2.70
hsa_circ_0031283	9.81	hsa_circ_0033867	3.44	hsa_circ_0046379	2.70
hsa_circ_0080013	9.80	hsa_circ_0024270	3.43	hsa_circ_0048967	2.70
hsa_circ_0060540	9.70	hsa_circ_0002782	3.43	hsa_circ_0091886	2.70
hsa_circ_0057389	9.58	hsa_circ_0057344	3.41	hsa_circ_0025002	2.69
hsa_circ_0064481	9.31	hsa_circ_0091694	3.41	hsa_circ_0049418	2.69
hsa_circ_0086376	9.12	hsa_circ_0059090	3.39	hsa_circ_0041407	2.69
hsa_circ_0008634	8.98	hsa_circ_0088205	3.37	hsa_circ_0029529	2.69
hsa_circ_0001599	8.95	hsa_circ_0002710	3.37	hsa_circ_0051027	2.68
hsa_circ_0088345	8.53	hsa_circ_0024150	3.36	hsa_circ_0001326	2.68
hsa_circ_0080011	8.25	hsa_circ_0029044	3.30	hsa_circ_0048188	2.68
hsa_circ_0017253	8.18	hsa_circ_0081968	3.29	hsa_circ_0008194	2.67
hsa_circ_0078705	7.99	hsa_circ_0004940	3.29	hsa_circ_0040045	2.67
hsa_circ_0064817	7.65	hsa_circ_0043297	3.28	hsa_circ_0011497	2.67
hsa_circ_0085895	7.33	hsa_circ_0066065	3.27	hsa_circ_0067306	2.67
hsa_circ_0089453	7.07	hsa_circ_0064827	3.27	hsa_circ_0001416	2.67
hsa_circ_0008085	7.06	hsa_circ_0040116	3.27	hsa_circ_0068496	2.67
hsa_circ_0043108	6.96	hsa_circ_0029329	3.26	hsa_circ_0030221	2.67
hsa_circ_0032973	6.89	hsa_circ_0025721	3.26	hsa_circ_0005852	2.66
hsa_circ_0066588	6.75	hsa_circ_0031932	3.26	hsa_circ_0041351	2.66
hsa_circ_0080224	6.66	hsa_circ_0005613	3.24	hsa_circ_0029154	2.65
hsa_circ_0023866	6.54	hsa_circ_0089424	3.24	hsa_circ_0045487	2.65
hsa_circ_0060395	6.53	hsa_circ_0017078	3.18	hsa_circ_0022208	2.65
hsa_circ_0046749	6.41	hsa_circ_0048810	3.17	hsa_circ_0036658	2.65
hsa_circ_0008412	6.41	hsa_circ_0074036	3.17	hsa_circ_0027035	2.65
hsa_circ_0006192	6.40	hsa_circ_0035142	3.17	hsa_circ_0021305	2.65
hsa_circ_0069254	6.40	hsa_circ_0079665	3.16	hsa_circ_0026479	2.64
hsa_circ_0025989	6.28	hsa_circ_0083696	3.15	hsa_circ_0046281	2.64
hsa_circ_0000295	6.22	hsa_circ_0056819	3.15	hsa_circ_0074137	2.64
hsa_circ_0082074	6.07	hsa_circ_0041151	3.14	hsa_circ_0043840	2.64
hsa_circ_0025497	6.06	hsa_circ_0041148	3.13	hsa_circ_0020120	2.64
hsa_circ_0023451	6.05	hsa_circ_0006392	3.11	hsa_circ_0009861	2.63
hsa_circ_0080221	5.97	hsa_circ_0060493	3.10	hsa_circ_0082217	2.62
hsa_circ_0000895	5.94	hsa_circ_0024683	3.10	hsa_circ_0067799	2.61
hsa_circ_0036879	5.92	hsa_circ_0030394	3.10	hsa_circ_0014857	2.61
hsa_circ_0057413	5.89	hsa_circ_0036644	3.10	hsa_circ_0026457	2.61
hsa_circ_0075846	5.86	hsa_circ_0058873	3.09	hsa_circ_0029503	2.61
hsa_circ_0045509	5.85	hsa_circ_0057880	3.09	hsa_circ_0044544	2.61
hsa_circ_0045269	5.83	hsa_circ_0048738	3.08	hsa_circ_0061481	2.60
hsa_circ_0072155	5.78	hsa_circ_0035528	3.08	hsa_circ_0026011	2.60
hsa_circ_0069256	5.62	hsa_circ_0067684	3.08	hsa_circ_0080113	2.60
hsa_circ_0006731	5.56	hsa_circ_0011693	3.07	hsa_circ_0075320	2.59
hsa_circ_0075265	5.55	hsa_circ_0001217	3.07	hsa_circ_0041476	2.59
hsa_circ_0040631	5.55	hsa_circ_0056646	3.07	hsa_circ_0080009	2.59
hsa_circ_0059572	5.47	hsa_circ_0016130	3.07	hsa_circ_0038008	2.59
hsa_circ_0038938	5.45	hsa_circ_0041492	3.06	hsa_circ_0037847	2.58
hsa_circ_0067047	5.44	hsa_circ_0078190	3.06	hsa_circ_0066847	2.58
hsa_circ_0025997	5.38	hsa_circ_0008421	3.06	hsa_circ_0028286	2.58
hsa_circ_0082069	5.36	hsa_circ_0074645	3.06	hsa_circ_0048942	2.58
hsa_circ_0057401	5.36	hsa_circ_0039786	3.04	hsa_circ_0089521	2.58
hsa_circ_0090559	5.30	hsa_circ_0001218	3.04	hsa_circ_0090560	2.57
hsa_circ_0038723	5.28	hsa_circ_0038153	3.03	hsa_circ_0076017	2.57
hsa_circ_0017627	5.25	hsa_circ_0034158	3.02	hsa_circ_0062758	2.57
hsa_circ_0017638	5.23	hsa_circ_0075071	3.02	hsa_circ_0009760	2.56
hsa_circ_0076085	5.14	hsa_circ_0061871	3.01	hsa_circ_0015010	2.56
hsa_circ_0082068	5.09	hsa_circ_0013171	3.00	hsa_circ_0017090	2.56
hsa_circ_0044335	5.02	hsa_circ_0040874	3.00	hsa_circ_0036625	2.56
hsa_circ_0017250	5.00	hsa_circ_0081105	2.99	hsa_circ_0069560	2.55
hsa_circ_0006616	4.99	hsa_circ_0031238	2.97	hsa_circ_0075854	2.55
hsa_circ_0027719	4.90	hsa_circ_0019978	2.96	hsa_circ_0062684	2.55
hsa_circ_0076632	4.88	hsa_circ_0071119	2.96	hsa_circ_0035158	2.55
hsa_circ_0029542	4.87	hsa_circ_0028087	2.96	hsa_circ_0034060	2.55

continued

circRNA	Fold change	circRNA	Fold change	circRNA	Fold change
hsa_circ_0017473	4.74	hsa_circ_0080213	2.95	hsa_circ_0087371	2.54
hsa_circ_0021496	4.73	hsa_circ_0028453	2.94	hsa_circ_0087812	2.54
hsa_circ_0016101	4.73	hsa_circ_0039787	2.94	hsa_circ_0081133	2.54
hsa_circ_0065159	4.72	hsa_circ_0058886	2.94	hsa_circ_0050073	2.54
hsa_circ_0004077	4.70	hsa_circ_0041457	2.94	hsa_circ_0081465	2.54
hsa_circ_0085116	4.65	hsa_circ_0012918	2.93	hsa_circ_0076494	2.53
hsa_circ_0050065	4.63	hsa_circ_0003774	2.93	hsa_circ_0077032	2.53
hsa_circ_0082078	4.60	hsa_circ_0024589	2.93	hsa_circ_0089544	2.53
hsa_circ_0021072	4.53	hsa_circ_0048514	2.93	hsa_circ_0029829	2.53
hsa_circ_0066839	4.46	hsa_circ_0032119	2.92	hsa_circ_0050070	2.53
hsa_circ_0029608	4.44	hsa_circ_0028210	2.91	hsa_circ_0075952	2.53
hsa_circ_0031930	4.42	hsa_circ_0051188	2.90	hsa_circ_0011483	2.52
hsa_circ_0058094	4.40	hsa_circ_0003541	2.90	hsa_circ_0009794	2.52
hsa_circ_0028112	4.40	hsa_circ_0046247	2.90	hsa_circ_0086247	2.52
hsa_circ_0001223	4.36	hsa_circ_0009640	2.90	hsa_circ_0026065	2.51
hsa_circ_0082685	4.35	hsa_circ_0083630	2.89	hsa_circ_0048963	2.51
hsa_circ_0061074	4.34	hsa_circ_0026425	2.89	hsa_circ_0038122	2.50
hsa_circ_0067416	4.32	hsa_circ_0045285	2.89	hsa_circ_0037671	2.49
hsa_circ_0019274	4.31	hsa_circ_0003887	2.89	hsa_circ_0080074	2.49
hsa_circ_0006877	4.27	hsa_circ_0074468	2.88	hsa_circ_0055528	2.49
hsa_circ_0038941	4.22	hsa_circ_0091915	2.87	hsa_circ_0067163	2.49
hsa_circ_0011308	4.21	hsa_circ_0007881	2.87	hsa_circ_0007116	2.48
hsa_circ_0089414	4.19	hsa_circ_0024545	2.87	hsa_circ_0019517	2.47
hsa_circ_0060803	4.14	hsa_circ_0090183	2.87	hsa_circ_0065487	2.47
hsa_circ_0025500	4.11	hsa_circ_0069176	2.86	hsa_circ_0011689	2.47
hsa_circ_0063115	4.09	hsa_circ_0011196	2.85	hsa_circ_0021446	2.47
hsa_circ_0058121	4.09	hsa_circ_0080382	2.84	hsa_circ_0000069	2.47
hsa_circ_0018321	4.07	hsa_circ_0016544	2.83	hsa_circ_0043777	2.46
hsa_circ_0057438	4.06	hsa_circ_0026460	2.83	hsa_circ_0007569	2.46
hsa_circ_0087451	4.03	hsa_circ_0010029	2.83	hsa_circ_0089986	2.46
hsa_circ_0058130	4.00	hsa_circ_0051390	2.83	hsa_circ_0042003	2.46
hsa_circ_0060802	3.98	hsa_circ_0007856	2.82	hsa_circ_0091990	2.45
hsa_circ_0085782	3.95	hsa_circ_0004853	2.82	hsa_circ_0088447	2.45
hsa_circ_0032128	3.95	hsa_circ_0057439	2.81	hsa_circ_0081579	2.44
hsa_circ_0018063	3.93	hsa_circ_0041648	2.80	hsa_circ_0051730	2.44
hsa_circ_0000446	3.92	hsa_circ_0066451	2.80	hsa_circ_0040971	2.43
hsa_circ_0003854	3.91	hsa_circ_0071396	2.79	hsa_circ_0036306	2.43
hsa_circ_0039304	3.91	hsa_circ_0071748	2.79	hsa_circ_0068942	2.42
hsa_circ_0070420	3.89	hsa_circ_0090808	2.79	hsa_circ_0050223	2.78
hsa_circ_0089967	3.87	hsa_circ_0036615	2.79		
hsa_circ_0047955	3.84	hsa_circ_0063877	2.78		
Down-regulated					
hsa_circ_0040081	90.87	hsa_circ_0031247	4.28	hsa_circ_0084356	3.05
hsa_circ_0041731	81.83	hsa_circ_0063376	4.26	hsa_circ_0015341	3.04
hsa_circ_0043534	43.84	hsa_circ_0014719	4.26	hsa_circ_0055433	3.04
hsa_circ_0004983	37.86	hsa_circ_0054905	4.24	hsa_circ_0051415	3.04
hsa_circ_0023110	24.31	hsa_circ_0022445	4.24	hsa_circ_0049908	3.04
hsa_circ_0023114	24.22	hsa_circ_0069330	4.20	hsa_circ_0012604	3.04
hsa_circ_0066077	20.69	hsa_circ_0072777	4.20	hsa_circ_0027801	3.03
hsa_circ_0023117	20.67	hsa_circ_0031735	4.18	hsa_circ_0030290	3.02
hsa_circ_0057336	19.44	hsa_circ_0078556	4.17	hsa_circ_0065252	3.02
hsa_circ_0072359	18.57	hsa_circ_0084464	4.15	hsa_circ_0015418	3.01
hsa_circ_0070972	18.27	hsa_circ_0027798	4.13	hsa_circ_0070881	3.00
hsa_circ_0043549	16.38	hsa_circ_0043710	4.13	hsa_circ_0020022	2.99
hsa_circ_0034606	15.77	hsa_circ_0012603	4.12	hsa_circ_0005231	2.99
hsa_circ_0015301	15.37	hsa_circ_0067769	4.11	hsa_circ_0019346	2.99
hsa_circ_0000467	14.99	hsa_circ_0036501	4.11	hsa_circ_0024097	2.99
hsa_circ_0015287	13.62	hsa_circ_0092041	4.11	hsa_circ_0072264	2.98
hsa_circ_0010171	13.26	hsa_circ_0014883	4.10	hsa_circ_0003534	2.98
hsa_circ_0072361	12.78	hsa_circ_0014142	4.10	hsa_circ_0025215	2.98
hsa_circ_0072219	12.77	hsa_circ_0027604	4.09	hsa_circ_0056414	2.97
hsa_circ_0043079	12.67	hsa_circ_0053305	4.07	hsa_circ_0009613	2.97
hsa_circ_0083809	12.35	hsa_circ_0063629	4.07	hsa_circ_0058170	2.97
hsa_circ_0011046	10.87	hsa_circ_0062303	4.06	hsa_circ_0008318	2.96
hsa_circ_0075826	10.51	hsa_circ_0057551	4.06	hsa_circ_0082379	2.96
hsa_circ_0005676	10.20	hsa_circ_0052554	4.05	hsa_circ_0056419	2.95
hsa_circ_0022560	10.02	hsa_circ_0032015	4.03	hsa_circ_0067792	2.93
hsa_circ_0021417	9.57	hsa_circ_0083398	4.03	hsa_circ_0028192	2.92
hsa_circ_0019160	9.52	hsa_circ_0023521	4.02	hsa_circ_0083262	2.92
hsa_circ_0020957	9.12	hsa_circ_0059548	3.99	hsa_circ_0077286	2.91
hsa_circ_0079633	8.93	hsa_circ_0077510	3.98	hsa_circ_0052759	2.91
hsa_circ_0018122	8.44	hsa_circ_0031221	3.97	hsa_circ_0060265	2.91
hsa_circ_0008627	8.37	hsa_circ_0005131	3.97	hsa_circ_0005428	2.90
hsa_circ_0004233	8.37	hsa_circ_0012602	3.97	hsa_circ_0031919	2.90
hsa_circ_0063424	8.09	hsa_circ_0047578	3.96	hsa_circ_0054360	2.89
hsa_circ_0056878	7.91	hsa_circ_0002329	3.95	hsa_circ_0025196	2.88
hsa_circ_0018446	7.88	hsa_circ_0024719	3.95	hsa_circ_0045707	2.88
hsa_circ_0019158	7.72	hsa_circ_0061380	3.94	hsa_circ_0067796	2.88
hsa_circ_0076139	7.64	hsa_circ_0060087	3.94	hsa_circ_0010394	2.88
hsa_circ_0063268	7.59	hsa_circ_0055315	3.93	hsa_circ_0034724	2.86
hsa_circ_0012847	7.57	hsa_circ_0082348	3.92	hsa_circ_0008707	2.85
hsa_circ_0014249	7.55	hsa_circ_0012855	3.92	hsa_circ_0014164	2.85
hsa_circ_0077880	7.35	hsa_circ_0046140	3.91	hsa_circ_0067712	2.85
hsa_circ_0013183	7.26	hsa_circ_0011106	3.91	hsa_circ_0042447	2.84
hsa_circ_0029775	7.22	hsa_circ_0070679	3.91	hsa_circ_0064156	2.84
hsa_circ_0019244	7.09	hsa_circ_0069032	3.90	hsa_circ_0015108	2.84
hsa_circ_0013176	7.07	hsa_circ_0014665	3.89	hsa_circ_0042697	2.84
hsa_circ_0044796	7.05	hsa_circ_0031085	3.88	hsa_circ_0084265	2.84
hsa_circ_0005932	7.02	hsa_circ_0075387	3.87	hsa_circ_0040839	2.84
hsa_circ_0063754	6.88	hsa_circ_0079430	3.86	hsa_circ_0057644	2.84
hsa_circ_0037373	6.88	hsa_circ_0002045	3.85	hsa_circ_0017432	2.83
hsa_circ_0078301	6.79	hsa_circ_0029699	3.83	hsa_circ_0085367	2.82
hsa_circ_0052694	6.71	hsa_circ_0087614	3.83	hsa_circ_0083307	2.81
hsa_circ_0022557	6.69	hsa_circ_0085365	3.83	hsa_circ_0035964	2.81
hsa_circ_0044797	6.67	hsa_circ_0007128	3.82	hsa_circ_0013999	2.81
hsa_circ_0057770	6.64	hsa_circ_0046923	3.81	hsa_circ_0004722	2.81

continued

circRNA	Fold change	circRNA	Fold change	circRNA	Fold change
hsa_circ_0037380	6.38	hsa_circ_0022441	3.80	hsa_circ_0023715	2.80
hsa_circ_0090859	6.37	hsa_circ_0012174	3.80	hsa_circ_0031427	2.80
hsa_circ_0073036	6.32	hsa_circ_0069845	3.79	hsa_circ_0064722	2.79
hsa_circ_0014355	6.32	hsa_circ_0079500	3.79	hsa_circ_0042030	2.79
hsa_circ_0029703	6.26	hsa_circ_0084888	3.78	hsa_circ_0069827	2.79
hsa_circ_0029716	6.18	hsa_circ_0060762	3.76	hsa_circ_0004693	2.79
hsa_circ_0075402	6.15	hsa_circ_0073054	3.76	hsa_circ_0075644	2.79
hsa_circ_0083395	6.07	hsa_circ_0027827	3.75	hsa_circ_0092138	2.79
hsa_circ_0075378	6.05	hsa_circ_0072102	3.75	hsa_circ_0047001	2.77
hsa_circ_0058043	6.03	hsa_circ_0020357	3.74	hsa_circ_0040071	2.77
hsa_circ_0076143	5.99	hsa_circ_0087128	3.71	hsa_circ_0046915	2.76
hsa_circ_0019349	5.98	hsa_circ_0091723	3.69	hsa_circ_0001347	2.76
hsa_circ_0029720	5.97	hsa_circ_0064483	3.68	hsa_circ_0031167	2.76
hsa_circ_0033829	5.93	hsa_circ_0084241	3.68	hsa_circ_0083543	2.76
hsa_circ_0047580	5.92	hsa_circ_0077400	3.67	hsa_circ_0057146	2.76
hsa_circ_0084773	5.91	hsa_circ_0014709	3.66	hsa_circ_0058162	2.75
hsa_circ_0047658	5.79	hsa_circ_0068472	3.64	hsa_circ_0031032	2.75
hsa_circ_0058045	5.77	hsa_circ_0091731	3.62	hsa_circ_0028186	2.75
hsa_circ_0083397	5.76	hsa_circ_0014521	3.61	hsa_circ_0014061	2.75
hsa_circ_0058055	5.68	hsa_circ_0014264	3.61	hsa_circ_0070019	2.74
hsa_circ_0085316	5.66	hsa_circ_0022447	3.60	hsa_circ_0036598	2.74
hsa_circ_0091219	5.65	hsa_circ_0009737	3.59	hsa_circ_0070577	2.74
hsa_circ_0052701	5.63	hsa_circ_0070497	3.57	hsa_circ_0056756	2.73
hsa_circ_0035981	5.60	hsa_circ_0023800	3.57	hsa_circ_0075342	2.73
hsa_circ_0056930	5.59	hsa_circ_0064230	3.57	hsa_circ_0060283	2.73
hsa_circ_0052681	5.59	hsa_circ_0027103	3.54	hsa_circ_0014202	2.73
hsa_circ_0054479	5.57	hsa_circ_0011712	3.52	hsa_circ_0078083	2.73
hsa_circ_0069473	5.52	hsa_circ_0057726	3.51	hsa_circ_0011849	2.73
hsa_circ_0070624	5.46	hsa_circ_0052804	3.50	hsa_circ_0009735	2.72
hsa_circ_0044894	5.43	hsa_circ_0055508	3.49	hsa_circ_0053267	2.72
hsa_circ_0025873	5.40	hsa_circ_0060097	3.49	hsa_circ_0015202	2.72
hsa_circ_0072774	5.40	hsa_circ_0015097	3.47	hsa_circ_0059588	2.71
hsa_circ_0041171	5.37	hsa_circ_0020021	3.47	hsa_circ_0052575	2.70
hsa_circ_0029815	5.37	hsa_circ_0033606	3.47	hsa_circ_0006955	2.69
hsa_circ_0008640	5.35	hsa_circ_0000766	3.45	hsa_circ_0073644	2.69
hsa_circ_0077385	5.34	hsa_circ_0090840	3.43	hsa_circ_0033040	2.69
hsa_circ_0009474	5.32	hsa_circ_0022554	3.42	hsa_circ_0014253	2.69
hsa_circ_0016574	5.27	hsa_circ_0014162	3.41	hsa_circ_0041928	2.68
hsa_circ_0006178	5.27	hsa_circ_0043694	3.39	hsa_circ_0066612	2.68
hsa_circ_0013597	5.25	hsa_circ_0010395	3.39	hsa_circ_0014551	2.68
hsa_circ_0069772	5.24	hsa_circ_0007889	3.39	hsa_circ_0019364	2.68
hsa_circ_0072610	5.20	hsa_circ_0059982	3.38	hsa_circ_0013994	2.68
hsa_circ_0033128	5.19	hsa_circ_0058642	3.38	hsa_circ_0078068	2.67
hsa_circ_0029380	5.19	hsa_circ_0072363	3.37	hsa_circ_0054137	2.67
hsa_circ_0010392	5.14	hsa_circ_0060092	3.34	hsa_circ_0089264	2.66
hsa_circ_0064574	5.14	hsa_circ_0030220	3.34	hsa_circ_0009766	2.65
hsa_circ_0044676	5.11	hsa_circ_0021919	3.33	hsa_circ_0013155	2.65
hsa_circ_0031654	5.10	hsa_circ_0056752	3.32	hsa_circ_0084484	2.64
hsa_circ_0084771	5.08	hsa_circ_0055009	3.31	hsa_circ_0007657	2.64
hsa_circ_0047464	5.06	hsa_circ_0004605	3.31	hsa_circ_0013513	2.64
hsa_circ_0035483	5.03	hsa_circ_0018126	3.31	hsa_circ_0014612	2.63
hsa_circ_0014263	5.03	hsa_circ_0030680	3.31	hsa_circ_0004337	2.63
hsa_circ_0058054	5.01	hsa_circ_0008384	3.30	hsa_circ_0011340	2.62
hsa_circ_0083807	5.00	hsa_circ_0052769	3.30	hsa_circ_0078065	2.62
hsa_circ_0082377	4.99	hsa_circ_0026522	3.27	hsa_circ_0072280	2.61
hsa_circ_0061371	4.99	hsa_circ_0088890	3.27	hsa_circ_0083305	2.61
hsa_circ_0019354	4.88	hsa_circ_0088712	3.27	hsa_circ_0070028	2.60
hsa_circ_0072385	4.84	hsa_circ_0035966	3.26	hsa_circ_0028044	2.60
hsa_circ_0014529	4.84	hsa_circ_0019085	3.26	hsa_circ_0011270	2.60
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hsa_circ_0063261	4.73	hsa_circ_0059491	3.23	hsa_circ_0012501	2.59
hsa_circ_0013386	4.70	hsa_circ_0021914	3.20	hsa_circ_0020852	2.58
hsa_circ_0014074	4.68	hsa_circ_0059641	3.20	hsa_circ_0055291	2.58
hsa_circ_0006780	4.68	hsa_circ_0022665	3.20	hsa_circ_0025022	2.58
hsa_circ_0072226	4.67	hsa_circ_0015579	3.20	hsa_circ_0026505	2.58
hsa_circ_0016478	4.66	hsa_circ_0074886	3.20	hsa_circ_0000286	2.58
hsa_circ_0014143	4.61	hsa_circ_0039754	3.19	hsa_circ_0046597	2.58
hsa_circ_0068096	4.59	hsa_circ_0031863	3.18	hsa_circ_0046867	2.56
hsa_circ_0035990	4.58	hsa_circ_0084948	3.18	hsa_circ_0036599	2.56
hsa_circ_0082592	4.58	hsa_circ_0077412	3.18	hsa_circ_0072484	2.55
hsa_circ_0086475	4.56	hsa_circ_0086406	3.18	hsa_circ_0008133	2.55
hsa_circ_0084421	4.54	hsa_circ_0015197	3.17	hsa_circ_0027623	2.55
hsa_circ_0084301	4.53	hsa_circ_0052705	3.16	hsa_circ_0078070	2.55
hsa_circ_0091733	4.53	hsa_circ_0038389	3.15	hsa_circ_0000697	2.55
hsa_circ_0088884	4.51	hsa_circ_0024076	3.15	hsa_circ_0089259	2.55
hsa_circ_0014519	4.48	hsa_circ_0008123	3.14	hsa_circ_0042046	2.55
hsa_circ_0017802	4.48	hsa_circ_0004507	3.13	hsa_circ_0001877	2.54
hsa_circ_0012612	4.45	hsa_circ_0005340	3.12	hsa_circ_0055236	2.54
hsa_circ_0008897	4.44	hsa_circ_0021295	3.11	hsa_circ_0078071	2.53
hsa_circ_0052556	4.43	hsa_circ_0022063	3.11	hsa_circ_0001886	2.53
hsa_circ_0012607	4.41	hsa_circ_0080088	3.10	hsa_circ_0044275	2.53
hsa_circ_0024080	4.41	hsa_circ_0059499	3.10	hsa_circ_0027523	2.52
hsa_circ_0004704	4.40	hsa_circ_0028184	3.09	hsa_circ_0054271	2.50
hsa_circ_0006529	4.40	hsa_circ_0052762	3.09	hsa_circ_0026123	2.50
hsa_circ_0072388	4.39	hsa_circ_0072083	3.08	hsa_circ_0069781	2.47
hsa_circ_0084775	4.39	hsa_circ_0021923	3.08	hsa_circ_0072386	2.46
hsa_circ_0082587	4.36	hsa_circ_0092159	3.06	hsa_circ_0063172	2.45
hsa_circ_0014259	4.31	hsa_circ_0058165	3.06	hsa_circ_0044312	2.45
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hsa_circ_0035985	4.29				

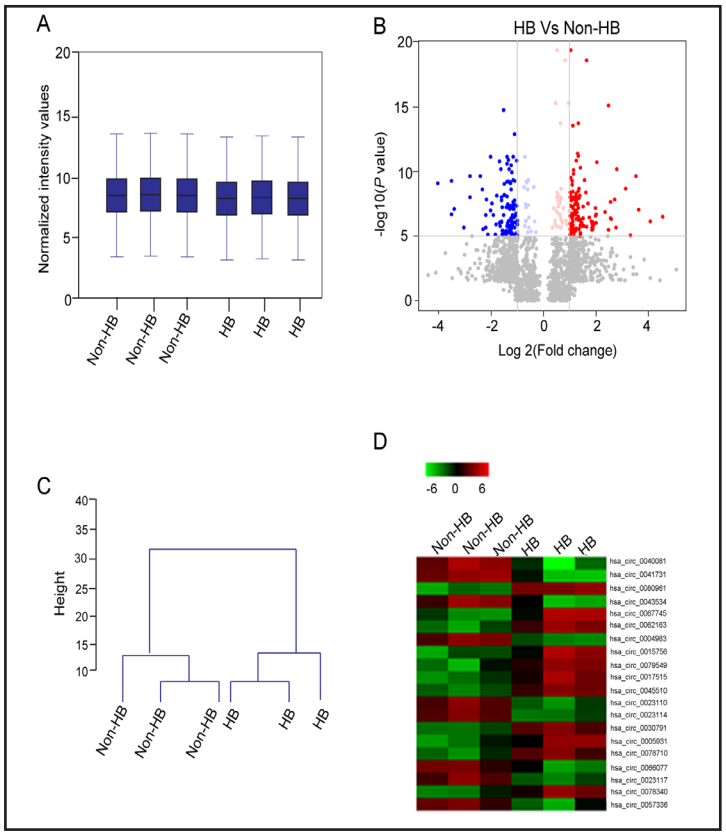

Fig. 1. Screening for hepatoblastoma-related circRNAs by circRNA microarray (A) The box plot shows circRNA expression distribution. After the normalization, the distributions of log2 ratios among different samples are shown. The box plot consists of boxes with a central line and two tails. The central line represents the median of the data. The tails represent the upper and lower quartiles. (B) Volcano plot displays the differentially expressed circRNAs between non-HB and HB samples. (C) Sample cluster analysis was performed to detect circular RNA expression among different samples. (D) Heatmap displays the top 20 dysregulated circRNAs between non-HB and HB samples. The color scale shows the relative expression level of circRNAs across different samples.

into the same branch. Non-HB samples were clustered into the other branch (Fig. 1C). We also used the top 20 dysregulated circRNAs to conduct hierarchical clustering analysis. The up-regulated or down-regulated circRNAs were clustered into the same sample (Fig. 1D).

To verify the microarray result, we randomly chose 20 dysregulated circRNAs for expression verification by qRT-PCRs, including 10 up-regulated circRNAs and 10 down-regulated circRNAs. Sixteen of 20 circRNAs were verified by qRT-PCRs, showing the similar expression pattern as previously detected by microarray (Table 2).

Table 2. Verification of circRNA microarray profiling by qRT-PCRs

circRNA name	HB versus Non-HB			
	microarray			qRT-PCR
hsa_circ_0080961	62.15	up	Not	
hsa_circ_0067745	38.64	up	4.34	up
hsa_circ_0062163	38.63	up	5.59	up
hsa_circ_0015756	29.24	up	32.85	up
hsa_circ_0079549	28.16	up	6.43	up
hsa_circ_0017515	26.72	up	22.72	up
hsa_circ_0045510	24.84	up	6.98	up
hsa_circ_0030791	23.97	up	Not	
hsa_circ_0005931	23.46	up	8.50	up
hsa_circ_0078710	21.70	up	6.26	up
hsa_circ_0040081	90.87	down	16.83	down
hsa_circ_0041731	81.83	down	24.27	down
hsa_circ_0043534	43.84	down	3.87	down
hsa_circ_0004983	37.86	down	Not	
hsa_circ_0023110	24.31	down	13.26	down
hsa_circ_0023114	24.22	down	11.58	down
hsa_circ_0066077	20.69	down	Not	down
hsa_circ_0023117	20.67	down	7.42	down
hsa_circ_0057336	19.44	down	15.92	down
hsa_circ_0072359	18.57	down	9.58	down

Functional annotation of the host genes of differentially expressed circRNAs

A majority of circRNAs are originated from the exonic or intronic sequences of their host genes [16]. Some circRNAs play important role in regulating the expression of their parental genes. We thus conducted GO analysis and pathway analysis of the host genes of differentially expressed circRNAs to predict circRNA function. The significantly enriched GO term of host genes of differentially expressed circRNAs in biological process was chromosome organization (Fig. 2A). The most significantly enriched GO term in cellular component was cytoplasm (Fig. 2B). The

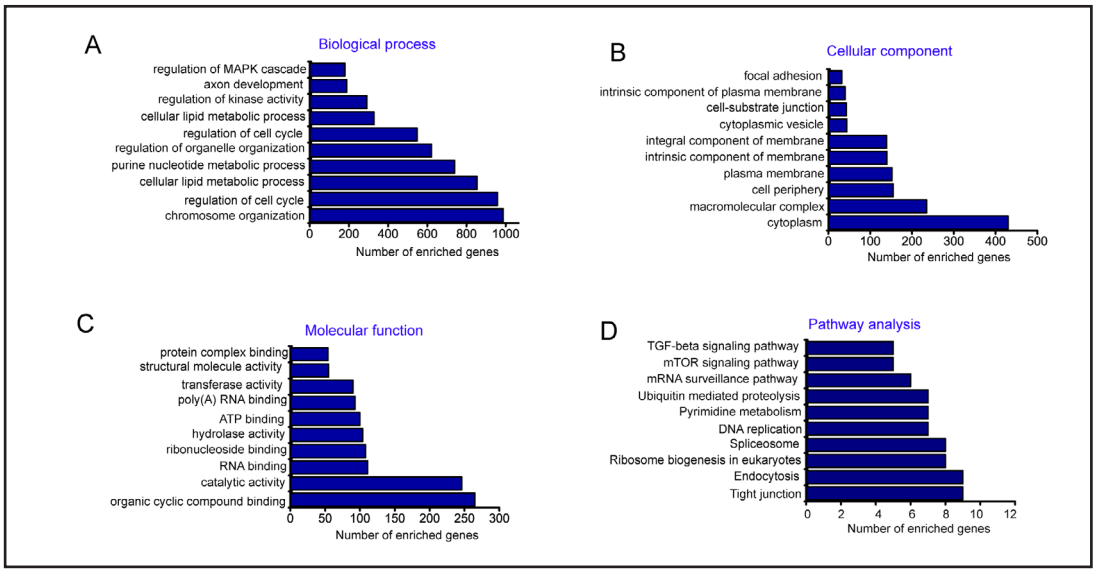

Fig. 2. Functional annotation of the host genes of differentially expressed circRNAs. (A-C) Functional annotation of the host genes of differentially expressed circRNAs was conducted based on gene ontology (GO) categorization and pathway analysis. GO analysis was conducted to obtain three main categories, including cellular component, molecular function and biological process. (D) The bar plot shows the result of pathway analysis, which demonstrates the top 10 signaling pathways potentially involved in circRNA-mediated regulatory network in hepatoblastoma.

most significantly enriched GO term in molecular function was organic cyclic compound binding (Fig. 2C). Pathway analysis showed that "tight junction signaling pathway" was ranked the top 1, which was potentially affected by circRNA-mediated regulatory network (Fig. 2D).

Prediction of circRNA-binding miRNAs

Increasing studies have revealed that most circRNAs have conserved seed sequence for miRNAs. They could play their roles by acting as microRNA (miRNA) sponges [17]. We thus predicted whether the top 10 up-regulated circRNAs and the top 10 down-regulated circRNAs could bind to some miRNAs based on TargetScan and miRana. The results showed that 18 circRNAs potentially could bind to at least five different miRNAs (Table 3), suggesting that most of differentially expressed circRNAs may act as miRNA sponges to regulate the expression of target genes.

Table 3. Identification of differentially expressed circRNA-binding miRNAs

circRNA	MRE1	MRE2	MRE3	MRE4	MRE5
hsa_circ_0080961	miR-6829-5p	miR-593-5p	miR-4758-3p	miR-4417	miR-1245b-5p
hsa_circ_0067745	miR-4778-3p	miR-583	miR-4311	miR-4289	miR-651-3p
hsa_circ_0062163	miR-1231	miR-7111-3p	miR-4486	miR-4732-5p	miR-6851-3p
hsa_circ_0015756	miR-6134	miR-7854-3p	miR-4778-3p	miR-1250-3p	miR-4659a-3p
hsa_circ_0079549	miR-4793-3p	miR-6499-3p	miR-6516-5p	miR-27b-3p	miR-27a-3p
hsa_circ_0017515	-	-	-	-	-
hsa_circ_0045510	miR-548m	miR-3605-3p	miR-432-3p	miR-6893-3p	miR-4663
hsa_circ_0030791	miR-216b-3p	miR-6875-3p	miR-3064-3p	miR-125b-2-3p	miR-4776-3p
hsa_circ_0005931	miR-3972	miR-1202	miR-203a-5p	miR-548au-3p	miR-5047
hsa_circ_0078710	-	-	-	-	-
hsa_circ_0040081	miR-4435	miR-505-5p	miR-3678-3p	miR-4802-3p	miR-4715-3p
hsa_circ_0041731	miR-4448	miR-4268	miR-6772-3p	miR-6084	miR-7108-3p
hsa_circ_0043534	miR-7974	miR-3653-5p	miR-5193	miR-5739	miR-221-5p
hsa_circ_0004983	miR-205-5p	miR-6128	miR-4691-3p	miR-219b-5p	miR-4437
hsa_circ_0023110	miR-1908-3p	miR-6778-3p	miR-6825-5p	miR-6836-3p	miR-4254
hsa_circ_0023114	miR-5193	miR-660-3p	miR-6775-3p	miR-1343-5p	miR-1291
hsa_circ_0066077	miR-4632-3p	miR-4731-5p	miR-4459	miR-5589-5p	miR-4783-5p
hsa_circ_0023117	miR-6778-3p	miR-6836-3p	miR-6791-3p	miR-6829-3p	miR-214-3p
hsa_circ_0057336	miR-4666b	miR-298	miR-374b-3p	miR-4537	miR-7112-3p
hsa_circ_0072359	miR-4469	miR-204-5p	miR-211-5p	miR-5096	miR-502-5p

circRNAs are potentially involved in the pathogenesis of hepatoblastoma

qRT-PCR verification assays in Table 1 revealed that circ_0015756 was the most significantly up-regulated circRNA in hepatoblastoma samples. We further collected human specimens, and detected circ_0015756 expression pattern in hepatoblastoma samples.

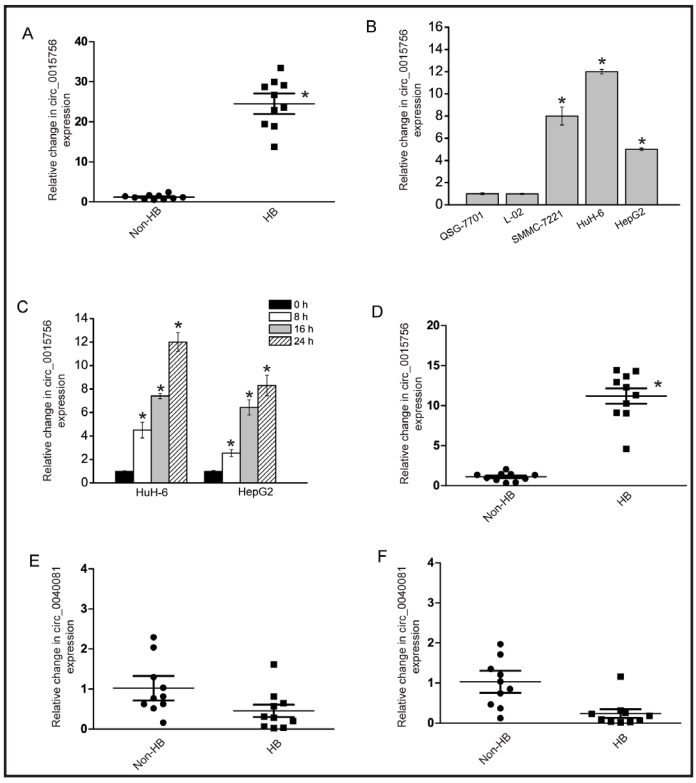

Fig. 3. circRNAs are potentially involved in the pathogenesis of hepatoblastoma. (A) Total RNAs were extracted from 10 hepatoblastoma specimens (HB) and 10 matched adjacent non-cancerous liver tissues. qRT-PCRs were conducted to detect circ_0015756 expression (n=3; Mann-Whitney U-test). (B) qRT-PCRs were conducted to detect circ_0015756 expression in nonmalignant QSG-7701 and L02 hepatocytes, malignant SMMC-7221, HuH-6, and HepG2 (n=4). Statistical differences were analyzed by one-way ANOVA followed by Tukey's post hoc analyses. (C) Hepatoblastoma cell lines, HuH-6 and HepG2 cell, were exposed to hypoxic condition for the indicated time periods, respectively. qRT-PCRs were conducted to detect circ_0015756 expression (n=4). Statistical differences were analyzed by one-way ANOVA followed by Tukey's post hoc analyses. (D and E) Total RNAs were extracted from the peripheral blood of 10 hepatoblastoma patients and 10 healthy controls. qRT-PCRs were conducted to detect circ_0015756 (D) or circ_0040081 expression (E; n=3; Mann-Whitney U-test). (F) Total RNAs were extracted from 10 hepatoblastoma specimens and 10 matched adjacent non-cancerous liver tissues. qRT-PCRs were conducted to detect circ_0040081 expression (n=3; Mann-Whitney U-test).

circ_0015756 was significantly up-regulated in hepatoblastoma tissues compared with the adjacent normal liver samples (Fig. 3A and Table 4). We also detected increased circ_0015756 expression in all examined hepatocellular carcinoma and hepatoblastoma cells compared with the nonmalignant QSG-7701 and L02 hepatocytes (Fig. 3B). Hypoxia is a pathological factor for tumorigenesis. Hepatoblastoma cells were exposed to hypoxic condition to mimic tumor microenvironment. circ_0015756 expression was found to be progressively up-regulated (Fig. 3C). Previous study has shown that peripheral blood circular RNA is potentially used as a diagnostic biomarker for human disease [18]. We also determined whether circulating circ_0015756 expression was altered in the peripheral blood of hepatoblastoma patients. circ_0015756 expression was significantly up-regulated in the peripheral blood of hepatoblastoma patients (Fig. 3D). We also determined the expression pattern of a down-regulated circRNA, circ_0040081. The result showed that circulating circ_0040081

Table 4. Clinical and pathological features of hepatoblastoma subjects. * On the date of serum sample collection; M, male; F, female

Subject	Age (Month)*	Gender	Diagnosis type	Alpha-fetoprotein (ng/ml)
1	21	M	Epithelial	1623
2	24	F	Epithelial	>121000
3	14	M	Epithelial	>121000
4	24	M	Epithelial	>121000
5	28	F	Epithelial	>121000
6	23	F	Epithelial	>121000
7	35	F	Mixed epithelial and mesenchymal	>121000
8	16	M	Mixed epithelial and mesenchymal	>121000
9	5	M	Mixed epithelial and mesenchymal	>121000
10	15	F	Embryonal and Fetal	>121000

expression was significantly down-regulated in the peripheral blood of hepatoblastoma patients (Fig. 3E). circ_0040081 was significantly down-regulated in the hepatoblastoma tissues compared with the adjacent normal liver samples (Fig. 3F). Taken together, these results suggest that circRNAs are potentially involved in the pathogenesis of hepatoblastoma.

circ_0015756 silencing decreases cell viability, proliferation, and invasion in vitro

To identify the role of circ_0015756 in the pathogenesis of hepatoblastoma *in vitro*, we first designed a siRNA based on the backsplice sequence of circ_0015756. circ_0015756 siRNA transfection significantly decreased circ_0015756 expression in HuH-6 cells (Fig. 4A). MTT assay showed that circ_0015756 silencing significantly decreased the viability of HuH-6 cells (Fig. 4B). Ki67 staining showed that circ_0015756 silencing significantly decreased the proliferation of HuH-6 cells (Fig. 4C). Transwell assays showed that circ_0015756 silencing significantly decreased the invasion ability of HuH-6 cells (Fig. 4D). In addition, we investigated the effect of circ_0015756 silencing on SMMC-7221 and HepG2 cell function. The result showed that circ_0015756 silencing significantly decreased the viability, proliferation, and invasion ability of SMMC-7221 and HepG2 cells (Fig. 5 and Fig. 6).

circ_0015756 acts as miR-1250-3p sponge in hepatoblastoma cell

circRNAs could act as miRNA sponges, and regulate the availability of miRNA for binding mRNAs [19]. We thus determined whether circ_0015756 acted as a miRNA sponge. miR-6134, miR-7854-3p, miR-4778-3p, miR-1250-3p, and miR-4659a-3p was predicated as the potential miRNA target on circ_0015756. Subsequently, the full sequence of circ_0015756 was cloned into the downstream of luciferase gene (RLuc-circ_0015756-WT), and then transfected into HuH-6 cells with different miRNA mimics. The activity of RLuc-circ_0015756-WT was significantly reduced by miR-1250-3p mimic transfeciton, but not by other miRNA mimics transfeciton (Fig. 7A). To avoid the unspecific binding, we also mutated miR-1250-

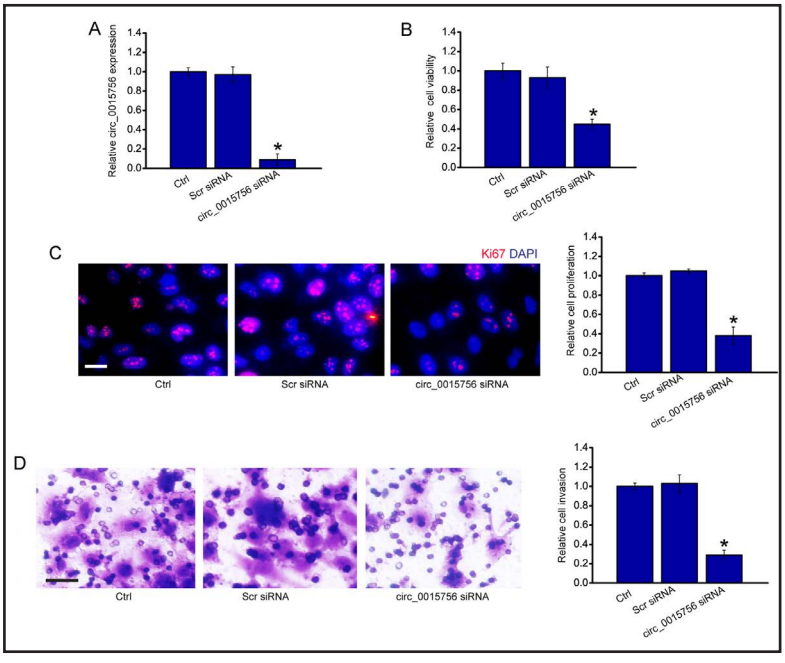

Fig. 4. circ_0015756 silencing decreases cell viability, proliferation, and invasion in vitro. (A) HuH-6 cells were transfected with scrambled siRNA (Scr), circ_0015756 siRNA, or left untreated (Ctrl) for 24 h. qRT-PCRs were conducted to detect circ_0015756 levels. The data was shown as the fold change compared with Ctrl group. "*" indicated significant difference compared with Ctrl group (n=4; One-way ANOVA followed by Tukey's post hoc analyses). (B-D) HuH-6 cells were transfected with scrambled (Scr) siRNA, circ_0015756 siRNA, or left untreated (Ctrl) for 24 h. Cell viability was detected by MTT method (B; n=4; One-way ANOVA followed by Tukey's post hoc analyses). Ki67 immunofluorescence staining and quantitative analysis showed that circ_0015756 knockdown decreased HuH-6 cell proliferation. Scale bar, 10 μm (C; n=4; One-way ANOVA followed by Tukey's post hoc analyses). Transwell assays showed that circ_0015756 silencing inhibited HuH-6 cell invasion. Scale bar, 20 μm (D; n=4; One-way ANOVA followed by Tukey's post hoc analyses).

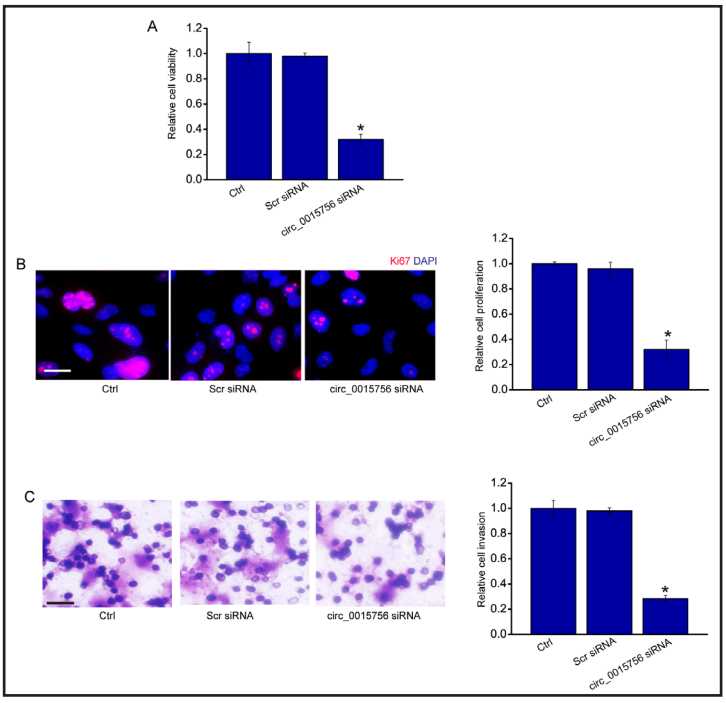

Fig. 5. circ_0015756 silencing affects the viability, proliferation, and invasion of SMMC-7221cells. (A) SMMC-7221 cells were transfected with scrambled siRNA (Scr), circ_0015756 siRNA, or left untreated (Ctrl) for 24 h. Cell viability was detected by MTT method (n=4; One-way ANOVA followed by Tukey's post hoc analyses). (B) Ki67 immunofluorescence staining and quantitative analysis showed that circ_0015756 knockdown decreased SMMC-7221 cell proliferation. Scale bar, 10 μm (n=4; One-way ANOVA followed by Tukey's post hoc analyses). (C) Transwell assays showed that circ_0015756 silencing inhibited SMMC-7221 cell invasion. Scale bar, 20 μm (n=4; One-way ANOVA followed by Tukey's post hoc analyses).

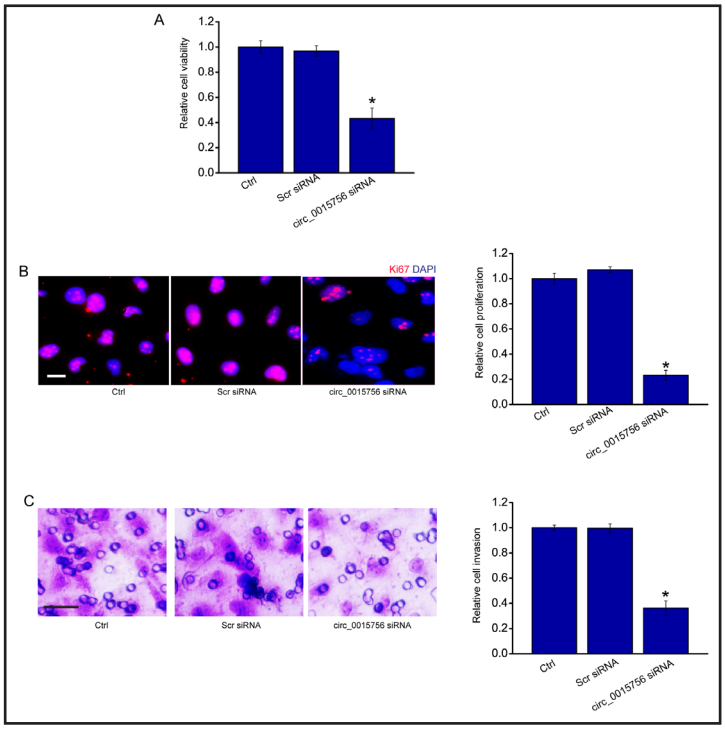

Fig. 6. circ_0015756 silencing affects the viability, proliferation, and invasion of HepG2 cells. (A) HepG2 cells were transfected with scrambled siRNA (Scr), circ_0015756 siRNA, or left untreated (Ctrl) for 24 h. Cell viability was detected by MTT method (n=4; One-way ANOVA followed by Tukey's post hoc analyses). (B) Ki67 immunofluorescence staining and quantitative analysis showed that circ_0015756 knockdown decreased HepG2 cell proliferation. Scale bar, 10 μm (n=4; One-way ANOVA followed by Tukey's post hoc analyses). (C) Transwell assays showed that circ_0015756 silencing inhibited HepG2 cell invasion. Scale bar, 20 μm (n=4; One-way ANOVA followed by Tukey's post hoc analyses).

3p binding site of circ_0015756 to generate RLuc-circ_0015756-Mut. miR-1250-3p mimic transfection significantly decreased RLuc-circ_0015756-WT activity, but had no effect on RLuc-circ_0015756-Mut activity (Fig. 7B). These data suggest that miR-1250-3p directly regulates circ_0015756 expression.

We then investigated the role of miR-1250-3p in HuH-6 cells. miR-1250-3p mimic transfection significantly decreased cell viability, proliferation, and invasion of HuH-6

cells, which could mimic the effects of circ_0015756 silencing on HuH-6 cells (Fig. 7C-E). Moreover, circ_0015756 overexpression could reverse the effects of miR-1250-3p mimic on HuH-6 cells (Fig. 7C-E).

Discussion

More recently, circRNAs have aroused great attention as novel diagnostic markers for diseases, including cancers. However, their expression profile and clinical significance in hepatoblastoma is still unknown [20]. We reveal 869 differentially expressed circRNAs in hepatoblastoma samples. Of them, circ_0015756 is significantly up-regulated in hepatoblastoma specimens and metastatic hepatoblastoma cell lines. circ_0015756 regulates hepatoblastoma cell function by acting as miR-1250-3p sponge.

circRNAs were originally considered as the byproducts of RNA splicing without functional significance. However, increasing studies have shown that circRNAs play important roles in several biological processes [21]. circRNAs are usually generated from

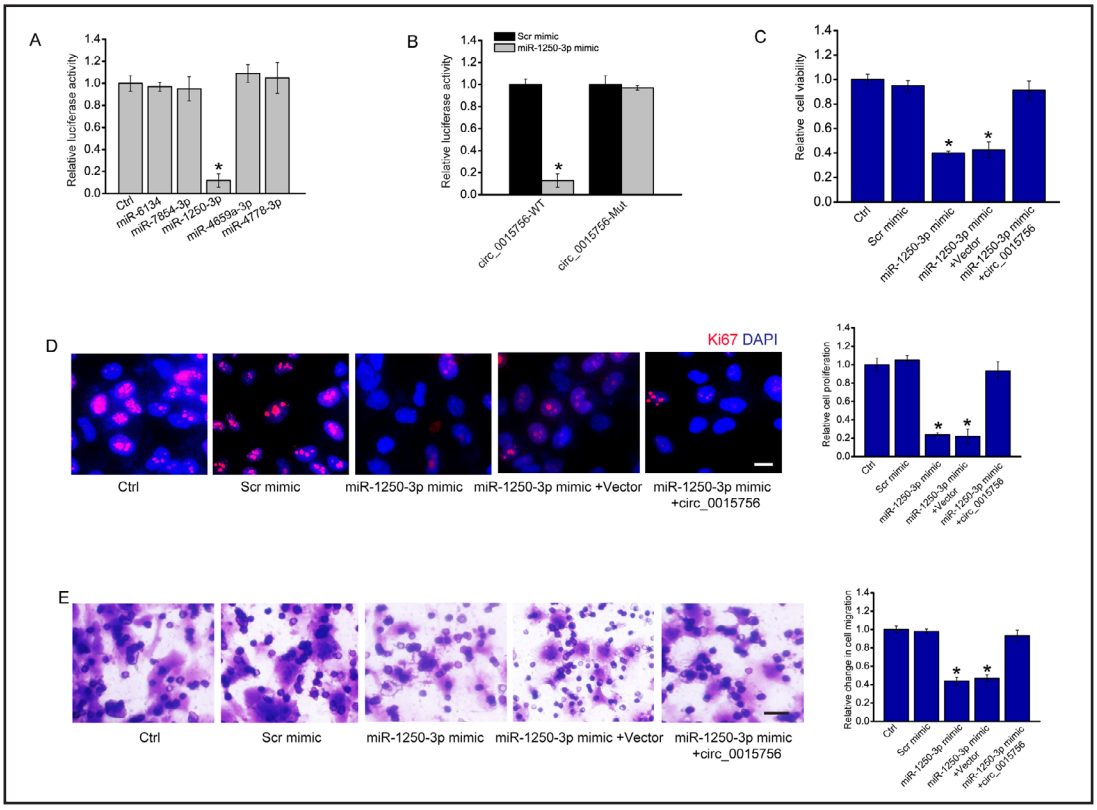

Fig. 7. circ_0015756 siRNA acts as miR-1250-3p sponge in hepatoblastoma cell. (A) HuH-6 cells were co-transfected RLuc-circ_0015756-WT with different miRNA mimics. Luciferase activity was detected by the dual luciferase assay (Promega). The group transfected with RLuc-circ_0015756-WT vector was used as the control group. Luciferase activity was detected 24 h after transfection (n=4; One-way ANOVA followed by Tukey's post hoc analyses). (B) RLuc-circ_0015756-WT or RLuc- circ_0015756-Mut was co-transfected with miR-1250-3p mimic into HuH-6 cells in parallel with the vector. Luciferase activity was detected 24 h after transfection. The data was shown as the relative change compared with the control group (n=4; One-way ANOVA followed by Tukey's post hoc analyses). (C-E) HuH-6 cells were treated as shown for 24 h. Cell viability was detected by MTT assays (C; n=4; One-way ANOVA followed by Tukey's post hoc analyses). Ki67 immunofluorescence staining and quantitative analysis showed that miR-1250-3p mimic decreased HuH-6 cell proliferation. Scale bar, 10 μm (D; n=4; One-way ANOVA followed by Tukey's post hoc analyses). Transwell assays showed that miR-1250-3p mimic inhibited HuH-6 cell invasion. Scale bar, 20 μm (E; n=4; One-way ANOVA followed by Tukey's post hoc analyses).

the exonic or intronic sequences of host genes. Thus, it is possible to predict circRNA function via the functional analysis of their parental genes. GO enrichment analysis shows that genes producing these differentially expressed circRNAs are enriched in chromosome organization, cytoplasm, and organic cyclic compound binding. circRNAs mainly play their role in cytoplasm [20]. Chromosome organization dysfunction is tightly associated with gene expression dysfunction [22]. Cyclic compound binding is associated with signaling pathway activation and inactivation. Tight junction signaling pathway is ranked the top 1 signaling pathway affected by circRNA-mediated regulatory network. Tight junction signaling has been implicated in the pathogenesis of many cancers [23, 24]. Since the pathogenesis of cancer is also associated with abnormal gene expression and signaling pathway dysfunction. Thus, it is not surprised that circRNA-mediated regulatory network is involved in the pathogenesis of hepatoblastoma.

circRNAs play a critical role in fine-tuning miRNA-mediated gene expression by sequestering miRNAs [6]. For example, ciRS-7 contains multiple miRNA-7 binding sites, thereby acting as an endogenous miRNA sponge [25]. circ-ITCH acts as miRNA (miR-7, miR-17, and miR-214) sponge and plays an inhibitory role in esophageal squamous cell carcinoma [26]. circHIPK3 can act as the sponge of 9 miRNAs, which has about 18 potential binding sites [27]. We annotate circRNA/miRNA interaction for some differentially expressed circRNAs and performe a detailed annotation for the conserved MREs. The majority of differentially expressed circRNAs in hepatoblastoma contain more than five different miRNA binding sites. miRNAs play crucial role in several biological processes, including cell viability, proliferation, differentiation, and apoptosis [28]. The critical role of miRNAs in hepatoblastoma has been gradually recognized [29]. circRNAs could act as miRNA sponges to regulate the expression of target genes of miRNAs. Thus, it is not surprised that circRNA-miRNA-mRNA network is involved in the progression and development of hepatoblastoma.

Hepatoblastoma is usually characterized by abnormal hepatoblastoma cell proliferation and invasion [2, 5]. circ_0015756 silencing significantly decreases hepatoblastoma cell viability, proliferation, and invasion. Thus, we speculated that compared to normal liver cells, circ_0015756 up-regulation hepatoblastoma cell would take longer to become senescence. circRNAs are believed to negatively regulate miRNAs, and contribute substantially to the competing endogenous RNA (ceRNA) network [7]. miR-1250-3p mimic transfection significantly decreases cell viability, proliferation ability, and invasion ability of hepatoblastoma cells. circ_0015756 overexpresson could reverse the effects of miR-1250-3p mimic. Thus, circ_0015756 could act as miR-1250-3p sponges, and regulate the availability of miRNA for binding mRNA targets.

Conclusion

This study reveals the role of circRNAs in hepatoblastoma. Dysregulated circRNAs have the potentials to be used as biomarkers for the screening of people with high-risk hepatoblastoma. However, based on these findings, much work is still required to uncover the mechanism of circRNA-mediated regulatory network in the pathogenesis of hepatoblastoma.

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Disclosure Statement

The authors declare that they have no Disclosure Statement.

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