Bioinformatics Analysis of Circular RNA Expression Profiles in HBx-Transfected HepG2 Cells by Transcriptional Sequencing

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Abstract

BACKGROUND

Circular RNA (CircRNA) and HBx genes separately play essential roles in the occurrence and development of hepatitis B (HBV)-related hepatocellular carcinoma (HCC). However, whether HBx expression in HCC is co-related to differential circRNA patterns remains unknown.

METHODS

HCC cell lines with HBx overexpression (HepG2 H6679) and empty vector control (HepG2 H5298) were successfully constructed. The high-throughput second-generation transcriptome sequencing technology (RNA-seq) was employed to sequence the two cell lines, and the selected circRNAs were verified by qPCR (quantitative real-time PCR). The differentially expressed circRNAs were analyzed. Bioinformatics analyses, including clustering, differential expression, GO analysis, and KEGG pathway, were performed. Target Scan and Miranda software were employed to predict miRNAs corresponding with circRNAs.

RESULTS

We identified 1120 circRNAs upregulated and 1447 circRNAs downregulated in HepG2 cell lines with HBx overexpression compared to its control. We selected 36 circRNAs with significant differences (also consistent with log2fold change absolute value ≥ 1.0 or $P \leq 0.05$) displayed by cluster analysis and then performed qPCR validation. Among them, 15 circRNAs (hsa_circ_0005603, hsa_circ_0004448, hsa_circ_0006845, hsa_circ_0064654, hsa_circ_0006460, hsa_circ_0045350, hsa_circ_0000824, hsa_circ_0005227, hsa_circ_0067991, hsa_circ_0064656, hsa_circ_0005224, circRNA11716, circRNA759, circRNA14848 and circRNA13751) are consistent with sequencing results. Hsa_circ_0005603 and hsa_circ_0006845 showed significant differences and were chosen for further study. GO analysis shows that many target genes are involved in biological processes, cellular components, and molecular functions. Nearly 193 target genes were enriched on KEGG pathways analysis. Actin cytoskeleton regulation, tight junction, and FoxO signaling pathway are among the top three pathways involved in most genes. We predicted that hsa_circ_0005603 might interact with micro-RNAs, including miR-182-5p, hsa-miR-27a-3p, hsa-miR-98-5p, and hsa-miR-198, that might thereby regulate downstream genes involved in tumor progression. Similarly, hsa_circ_0006845 was predicted to be referred to HBV-related HCC by acting as a sponge for hsa-miR-106a-3p and hsa-miR-198. Furthermore, we discovered two novel circular RNAs (circRNA11716 and circRNA13751) which might be involved in HCC occurrence.

CONCLUSION

In this study, we comprehensively explored the differentially expressed circRNAs in HepG2 cells with different HBx expression, and our results indicate that hsa_circ_0005603, hsa_circ_0006845, and novel circular RNAs (circRNA11716 and circRNA13751) might play an important role in HBV-related HCC, deserving further research.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the top ten tumors in China and the fourth leading cause of cancer-related deaths worldwide[1]. HCC pathogenesis remains unclear, and current effective treatment relies on surgical liver resection or transplantation. Despite improvements in clinical technology, the patient's prognosis remains poor, with a low 5-year survival rate and high tumor metastasis/recurrence rates[2, 3]. Research on the genetic level of HCC helps us explore its possible pathogenic mechanism. Growing evidence demonstrates that HBx gene and its expressed protein play an important role in the occurrence and development of HCC[4–6].

HBx is a protein composed of 154 amino acids encoded by the hepatitis B virus HBV X gene, with a molecular weight of about 17.5 kDa. Studies have shown that among proteins encoded by HBV genome, HBx is the only viral protein with multiple regulatory functions and is closely related to liver cancer[7]. HBx expression is dynamically distributed in cells and can directly or indirectly interact with the host protein to regulate transcription, signal transduction, protein degradation, etc., which eventually leads to liver cancer occurrence and development[5]. The clinical studies have shown that high hepatitis B virus HBV load is closely correlated with postoperative recurrence and metastasis of HCC and may be related to high expression of hepatitis B-related HBx[5, 8]. HBx has a wide range of transactivation effects, which can activate various regulatory genes in cells and promote HBV replication, drug resistance, tumor occurrence, and invasion/metastasis of HCC [4, 5].

CircRNA is a special non-coding RNA produced by the driving cycle or reverse splicing between the splice donor site of the downstream exon and the splice acceptor site of the upstream exon[9]. It has a single-stranded cyclic closed-loop structure, without a 5'-end cap and 3'-end poly(A) tail, resulting in high stability and resistance to RNA exonuclease degradation[9, 10]. Compared with the homologous linear subtype, the expression level of circRNA is more than 10 times, with a half-life of more than 48 hours[10, 11]. There is evidence that circRNA can be used as an intermediary to isolate RNA-binding proteins(RBP), a regulatory factor for nuclear transcription, regulate the expression of parental genes, and translate into polypeptides, and so on[12].

Simultaneously, circRNA indirectly regulates the expression of miRNA-targeted mRNA through competitive binding with miRNAs[13]. CircRNA is involved in cell division, differentiation and growth, and its potential clinical application value has attracted considerable attention.

Many studies have shown that circRNA is complicatedly involved in the formation and progression of HCC[14, 15]. However, no relevant report is found on whether HBx expression in HBV-related HCC is co-related to potentially differential circRNA patterns. In this study, based on HepG2 cells, an HBx overexpression cell line and its empty vector control group were constructed, and then high-throughput second-generation transcriptome sequencing (RNA-seq) was used to screen out circRNAs with differences. qPCR was employed to verify the selected circRNAs with meaningful differences, and miRNA prediction was performed. Furthermore, this study also carried out GO function and KEGG pathway analysis, screened circRNAs significantly-regulated, and conducted scientific research mapping. Finally, we speculate that certain circRNAs may be associated with HBx, and influence HCC occurrence and development by acting as miRNA sponges on downstream target genes.

2. Materials And Methods

2.1 Cell culture and related reagents

HepG2,293T cells were purchased from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cell lines were propagated with DMEM + 10% FBS + 1% P/S (37 ℃, 5% CO_2). The proteins were visualized using BCA Protein Assay Kit (HyClone-Pierce, Guangzhou, China) and ECL-PLUS/Kit (Amersham, UK). Antibodies used in this study were as follows: anti-HBx (Abcam, WB:1:2000), anti-Tubulin(Bioworld, WB:1:5000), and Goat Anti-mouse IgG (Beyotime, WB: 1:3000) were obtained from Shanghai, China.

2.2 Lentiviraltransfection

Lentiviruses for HBx overregulation and control lentiviruses were purchased from Heyuan biology technology co., ltd (Shanghai, China). To generate stable HBx overexpression cells, HepG2 cells were infected with H6679 (*pLOV-EF1a-blasticidin-CMV-EGFP-P2A-HBx-3FLAG*) or the corresponding H5298*(pLOV-EF1a-blasticidin-CMV-EGFP-P2A-3FLAG)* control lentivirus. Stable cells were selected with 5 μg/mL blasticidin treatment for about two weeks, and the expression of HBx was measured using Western-blot and qPCR. All the primer sequences are presented in Table 1.

Table 1
Sequences of oligonucleotides used for qPCR

Gene		Primer sequence
GAPDH	Sense	F: 5′ GTCTTCACCACCATGGAGAA3′
	Antisense	R 5′ TAAGCAGTTGGTGGTGCAG3′
hsa_circ_0005603	Sense	F: 5′ CAAGAGATGCTGGCTGTAGG3′
	Antisense	R 5′ TGCTGTAGATTTGAAAGGTGGA3′
hsa_circ_0006845	Sense	F: 5′ GAACAACTTGACTGGCTCCTT3′
	Antisense	R: 5′ GTTGGCCTGGCTGACTTATG3′
circRNA11716	Sense	F: 5′ CTGGTAGCCTGTCAACAATGT3′
	Antisense	R: 5′ TTCTTCTCCACTGCACTCTTAT3′
circRNA13751	Sense	F: 5′ GGCAGTGCAGGCATTACTAA3′
	Antisense	R: 5′ CCATTGCCAGATTGAGTGGT3′

2.3 RNA library construction and sequencing

The total RNA was isolated and purified using Trizol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's procedure. The RNA amount and purity of each sample were quantified using NanoDropND-1000 (NanoDrop, Wilmington, USA). The RNA integrity was assessed by Agilent 2100 with RIN number > 7.0. Approximately 5 μg of total RNA was used to deplete ribosomal RNA according to the manuscript of Ribo-Zero™ rRNA Removal Kit (Illumina, San Diego, USA). After removing ribosomal RNAs, the left RNAs were fragmented into small pieces using divalent cations under high temperature.

The cDNA was obtained using reverse transcription reagents (TAKARA, Beijing, China), OligodT (Sangon Biotech, Shanghai, China), and RNase-free items (Axygen, California, USA), followed by the synthesis of U-labeled DNA using E.coli DNA polymerase I, RNase H and dUTP. After heat-weakened UDG enzyme treatment, the ligated products are amplified with PCR by the following conditions: initial denaturation at 95 ℃ for 3 min; 8 cycles of denaturation at 98 ℃ for 15s, annealing at 60 ℃ for 15s, and extension at 72 ℃ for 30s; and then final extension at 72 ℃ for 5 min. The average insert size for the final cDNA library was 300 bp (± 50 bp). At last, we performed the paired-end sequencing on an IlluminaHiseq 4000 (LC Bio, China) following the vendor's recommended protocol.

2.4 Analysis of circRNA bioinformatics

Firstly, Cutadapt was used to remove the reads that contained adaptor contamination, low-quality bases, and undetermined bases. Then, sequence quality was verified using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). We used Bowtie2 and Hisat2 to map reads to the genome of species. The remaining reads (unmapped reads) were still mapped to the genome using tophat-fusion. Initially, CIRCExplorer and CIRI were used to denovo assemble the mapped reads to circular RNAs; then, back splicing reads were identified in unmapped reads by tophat-fusion. All samples were generated unique circular RNAs.

2.5 Measurement of circRNAs by qPCR

Based on circRNAs expression profiles, we selected 36 of the most significant circRNAs for further verification, including 27 upregulated (hsa_circ_0006460, hsa_circ_0045350, hsa_circ_0008440, hsa_circ_0077493, hsa_circ_0087641, hsa_circ_0002359, hsa_circ_0000824, hsa_circ_0005035, hsa_circ_0005227, hsa_circ_0067991, hsa_circ_0006156, hsa_circ_0005791, hsa_circ_0131242, hsa_circ_0008777, hsa_circ_0006357, hsa_circ_0064656, hsa_circ_0001165, circRNA764, circRNA16240, circRNA759, circRNA13751, circRNA14974, circRNA15571, circRNA14848, circRNA2238, circRNA14864 and circRNA9767) and 9 downregulated (hsa_circ_0004188, hsa_circ_0058174, hsa_circ_0005603, hsa_circ_0116930, hsa_circ_0004448, hsa_circ_0006845, hsa_circ_0064654, hsa_circ_0005224 and circRNA11716). The total RNA was extracted from HepG2-H6679 and HepG2-H5298 cell lines using Trizol Reagent (Invitrogen, Carlsbad, USA) and reversely transcribed into cDNA using M-MLV (TAKARA, Beijing, China). The mRNA content was normalized to the housekeeping gene GAPDH. All data were shown as fold change ($2^{-\Delta\Delta Ct}$).

2.6 Annotation for circRNA/miRNA interaction

The circRNA/miRNA interaction was predicted using Target Scan (www.targetscan.org/vert_71) and Miranda (www.microrna.org/microrna/home.do). All differentially expressed circRNAs were annotated in detail using circRNA/miRNA interaction network by Cytoscape v3.8.0.

2.7 GO and KEGG bioinformatic analysis

GO analysis was performed to explore the functional roles of target genes regarding biological processes (BP), cellular components (CC), and molecular functions (MF). Pathway analysis is a functional analysis that can map genes to the "Kyoto Encyclopedia of Genes and Genomes" (KEGG). The P-value (EASE-score, Fisher-P value, or Hypergeometric-P value) denotes the pathway's significance correlated to the conditions.

2.8 Statistical analysis

All data were analyzed using SPSS v.22.0 (SPSS, USA), and all results were presented as mean ± SD. Differences between the two groups were estimated using the Student's t-test, and log2fold-change ≥ 1.0 or $P \leq 0.05$ were considered to indicate a statistically significant difference.

3. Results

3.1 Construction of stable cell line HepG2-H6679 overexpressing HBx

The HepG2 cells originate from human HCC cells, which have a normal cell morphology of epithelial-like, adnexal growth. After the infection of HepG2 cells with lentivirus, the fluorescence images of the stable strains screened with blasticidin after 14 days showed that the fluorescence rate reached over 90% (Fig. 1A). To verify HBx expression, Western-blot and qPCR were used to detect the constructed 293T, and HepG2 cell lines, respectively, both of which were infected lentivirus H6679 and control H5298 (Fig. 1B-D), and target bands could be found at about 17 kDa (HepG2-H5298 vs. HepG2-H6679: 1.010 ± 0.177 vs. 2314.841 ± 18.570, P<0.001).

3.2 Different expression circRNAs between HepG2-H6679 and HepG2-H5298 cell lines

A total of 14788 circRNAs were found in HepG2-H6679 and HepG2-H5298. Among them, 1120 circRNAs were upregulated, and 1447 circRNAs were downregulated in HepG2-H6679 (Fig. 2A). To further obtain valuable circRNAs, we customize the screening rules based on the sequencing analysis results: a. the two groups are quite different and express more counts; b. the gene or maternal gene has relevant literature reports related to HBV; c. try to comply with statistical significance, that is, log2-foldchange absolute value ≥ 1 or P-value ≤ 0.05. We screened out 36 circRNAs and then applied a gene heatmap for judging the clustering pattern and gene regulation expression under different experimental conditions (Fig. 2B).

3.3 Verification of differentially expressed circRNAs

Thirty-six differentially expressed circRNAs were selected and used for qPCR verification. The final analysis showed that 15 circRNAs were consistent with the sequencing results. Among them, compared with the control group, hsa_circ_0006460, hsa_circ_0045350, hsa_circ_0000824, hsa_circ_0005227, hsa_circ_0067991, hsa_circ_0064656, circRNA759, circRNA14848 and circRNA13751 were upregulated. Hsa_circ_0005603, hsa_circ_0004448, hsa_circ_0006845, hsa_circ_0064654, hsa_circ_0005224, circRNA11716 were downregulated in the HepG2-HBx group. The verification results showed meaningful differences and considerable counts in both groups with the following four circRNAs: hsa_circ_0005603 and hsa_circ_0006845 (the former, HepG2-H5298 vs. HepG2-H6679: 1.004 ± 0.111 vs. 0.057 ± 0.003, P<0.001; the latter, HepG2-H5298 vs. HepG2-H6679: 1.008 ± 0.155 vs. 0.006 ± 0.0002, P<0.001), and two novel circular RNAs (circRNA11716 and circRNA13751) (the former, HepG2-H5298 vs. HepG2-H6679: 1.009 ± 0.159 vs. 0.235 ± 0.012, P<0.01; the latter, HepG2-H5298 vs. HepG2-H6679: 1.007 ± 0.142 vs. 1.726 ± 0.312, P<0.01). These two novel circular RNAs we found have never been reported, which deserved further research (Fig. 2C, Table 2).

Table 2
The information and characteristics of 15 cirRNAs we selected

Accession	regulation	log2FoldChange	pvalue	chr	start	end	strand	exon Count	circType	geneName
hsa_circ_0005603	Down	-1.25	0.13	Chr17	12054889	12113360	+	6	circRNA	MAK2P4
hsa_circ_0004448	Down	-1.20	0.17	chr12	68820325	68824651	+	3	circRNA	MDM2
hsa_circ_0006845	Down	-1.08	0.29	chr16	69695136	69695379	+	1	circRNA	NFAT5
hsa_circ_0064654	Down	-1.07	0.48	chr3	30644747	30674246	+	4	circRNA	TGFBR2
hsa_circ_0006460	Up	3.75	0.00	chr7	140776912	140794467	-	6	circRNA	BRAF
hsa_circ_0045350	Up	1.67	0.07	chr17	65549520	65558736	-	2	circRNA	AXIN2
hsa_circ_0000824	Up	1.12	0.17	chr18	6263947	6312056	-	4	circRNA	L3MBTL4
hsa_circ_0005227	Up	0.94	0.24	chr2	161179614	161204793	+	3	circRNA	TANK
hsa_circ_0067991	Up	1.02	0.27	chr3	172251260	172307501	+	5	circRNA	FNDC3B
hsa_circ_0064656	Up	0.50	0.55	chr3	30671638	30674246	+	2	circRNA	TGFBR2
hsa_circ_0005224	Down	-0.51	0.64	chr3	30644747	30650460	+	2	circRNA	TGFBR2
circRNA11716	Down	-1.20	0.25	chr2	171925537	171953001	+	2	circRNA	HAT1
circRNA759	Up	1.21	0.10	chr12	26481131	26655852	-	24	circRNA	ITPR2
circRNA14848	Up	0.95	0.27	chr10	97436418	97437750	-	3	circRNA	exosome component 1
circRNA13751	Up	1.17	0.12	chr9	96509673	96565483	-	13	circRNA	CDC14B

Accession: the ID number of circRNA in CircBase; regulation: The relationship between up and down circRNA in the comparison group and the control group (up or down); log2fold_change: log value, based on 2 fold change (fold change is the difference multiplier); pvalue: p value, hypothesis test from a statistical point of view, generally $P \leq 0.05$ is significant, $P \leq 0.01$ is extremely significant; chr: The number of the chromosome where the circRNA is located. For example, chr1 indicates that the host gene corresponding to the circRNA is located on chromosome 1; Start: The start site of circRNA on the chromosome; End: The termination site of circRNA on the chromosome; strand: circRNA is derived from the positive strand + or the negative strand - in the double strand of DNA; Exon count: circRNA consists of several exons, that is, the number of exons; circType: The classification of circRNA, circRNA composed of exons is labeled "circRNA", and circRNA composed of introns is labeled "ciRNA"; geneName: The gene name of the linear transcript corresponding to circRNA.

3.4 GO Enrichment analysis of differentially expressed circRNA-hosting gene

3.4.1 Differential expression circRNA-hosting gene enrichment analysis histogram

For clearly appreciating the three GO functions, we sort them in descending order according to the gene number annotated to GO term, and we select GO terms of Top25, Top15, and Top10 for drawing display (Fig. 2D). The top 3 processes were regulation of DNA-templated transcription, DNA-templated transcription, and transport in biological processes (BP), nucleus, cytoplasm, and cytosol in cellular components (CC), protein binding, metal ion binding, and nucleotide-binding in molecular functions (MF), respectively.

3.4.2 Differentially expressed circRNA-hosting gene GO enrichment analysis scatter plot

We use ggplot2 to plot the top20 GO term with significance (P-value) in the GO enrichment analysis results and display them as scatter plots (Fig. 3A). GO results demonstrate that the nucleus was associated with 809 genes linked to the largest number of genes. Nucleoplasm was associated with 519 genes linked to the second number of genes, and zinc ion binding was associated with 116 genes linked to the third number of genes.

3.5 Differentially expressed circRNA-hosting gene KEGG enrichment analysis

Similarly, according to the significance (P-value) of KEGG gene enrichment, the pathway term of Top 20 was taken to display the scatter plot (Fig. 3B). The results of KEGG shows that the Top 3 pathway terms with a better number of enriched genes were actin cytoskeleton regulation involved in 33 genes, tight junctions involved in 29 genes, and FoxO signaling pathway involved in 25 genes. Interestingly, we found that 23 genes are related to Hepatitis B, and they are successively as follows: circRNA15395 (ENSG00000171862); circRNA17387 (ENSG00000050748), circRNA6314 (E2F1), circRNA6586 (NFATC2), hsa_circ_0000413 (TBK1); hsa_circ_0001793 (IKBKB); hsa_circ_0002173 (CREB1); hsa_circ_0002968 (MAPK8); hsa_circ_0003077 (ENSG00000005339); hsa_circ_0003900 (DDB1); hsa_circ_0004431 (PIK3CB); hsa_circ_0004872 (MAPK1); hsa_circ_0005603 (MAP2K4); hsa_circ_0005927 (VDAC3); hsa_circ_0008798 (MAP2K1); hsa_circ_0008996 (MAP2K4); hsa_circ_0037654 (CREBBP); hsa_circ_0042094 (MAP2K4); hsa_circ_0043815 (STAT3); hsa_circ_0057144 (ATF2); hsa_circ_0060300 (SRC); hsa_circ_0072518 (MAP3K1); hsa_circ_0075805 (E2F3); hsa_circ_0098977 (TBK1); hsa_circ_0105404 (CREBBP); hsa_circ_0108590 (SMAD4); hsa_circ_0116638 (EP300); hsa_circ_0129195 (MAP3K1); hsa_circ_0129198 (MAP3K1). Previous studies and academic reports have shown that HBx expression is closely related to hepatitis B virus (HBV).

CircRNA, as a non-coding RNA, participates in various regulatory mechanisms, which also suggests that it may be involved in regulating HBx gene expression in HBV-related HCC.

3.6 Prediction of potential circRNA/miRNA interactions

The 15 selected circRNAs were implemented for miRNAs targeting prediction, and a visual interaction network can be shown in (Fig. 4). According to miRNA prediction energy and score and combined with previous miRNA researches, the miRNAs targetted by hsa_circ_0005603, hsa_circ_0006845, circRNA11716, and circRNA13751 were carefully picked out and annotated in detail with circRNA/miRNA (Table 3). It is interesting that hsa_circ_0005603 and hsa_circ_0006845 accidentally interact with hsa-miR-198 simultaneously.

Table 3
Annotation of the four circRNAs and miRNA response elements (MREs)

CircRNA	MREs
hsa_circ_0005603	hsa-miR-27a-3p;hsa-miR-27b-3p;hsa-miR-17-3p;**hsa-miR-198**;hsa-let-7a-5p;hsa-let-7d-5p;hsa-let-7e-5p;hsa-let-7g-5p;hsa-miR-182-5p;hsa-miR-98-5p;
hsa_circ_0006845	**hsa-miR-106a-3p**;**hsa-miR-198**
circRNA11716	hsa-miR-148a-5p
circRNA13751	hsa-miR-103a-2-5p;**hsa-miR-106a-3p**;hsa-miR-107; hsa-miR-124-5p; hsa-miR-128-3p; hsa-miR-132-3p; hsa-miR-181c-3p; hsa-miR-203a-5p; hsa-miR-212-3p; hsa-miR-217; hsa-miR-219a-1-3p

4. Discussion

Hepatitis B virus is closely related to HCC occurrence, but the specific mechanism remains unclear[5]. As a special kind of non-coding RNA, circular RNA recently gained prominence in tumors, including liver cancer. In this study, based on HBx overexpression in HepG2 cells, we comprehensively explored the differentially expressed circRNAs and found different expression patterns of circRNAs with significant differences. Our results first indicate that hsa_circ_0005603, hsa_circ_0006845, and novel circular RNAs (circRNA11716 and circRNA13751) might play an important role in HBV-related HCC.

It has been reported that HBx is associated with non-coding RNA, which promotes HCC development. For example, HBx can bind to estrogen receptor (ERα) to form a complex to inhibit the activity of LINC01352 promoter and eventually activate Wnt/β-catenin through LINC01352-miR-135b-APC axis, which is conducive to the growth and metastasis of HBV-related HCC[16]. HBx also can combine with lncRNA DLEU2 and be co-recruited by cccDNA to enable EZH2 to be replaced from viral chromatin, which promotes transcription and viral replication, simultaneously activates the transcription of EZH2 / PRC2 downstream genes and ultimately participates in HBV/ HBV-associated HCC[17]. CircRNA cSMARCA5 (also known as hsa_circ_0001445) is downregulated in hepatitis B-related HCC tissues and promotes TIMP3 expression by adsorbing miR-17-3p and miR-181b-5p. has_circ_0001445 inhibits cell proliferation in vitro experiments, and its downregulation is significantly related to tumor cell invasiveness and is considered to be a risk factor for overall survival and recurrence-free survival after hepatectomy[15]. Circ-MALAT1 (hsa_circ_0002082) is highly expressed in HCC tumor stem cells (CSCs) and forms a ternary complex with ribosomes and messenger RNA, which acts as a brake and delays the transcription of PAX5 messenger RNA and ultimately promotes the self-renewal of CSCs [18]. Therefore, we hypothesize that HBx may be associated with circRNAs and co-involved in HCC progression, although no similar reports are present to date.

This research found that 1120 circRNAs were upregulated and 1447 circRNAs were downregulated in HepG2withHBx. We verified the selected 36 circRNAs by QPCR and obtained 15 circRNAs consistent with the sequencing results. Among them, circRNA11716, circRNA13751, hsa_circ_0005603, and hsa_circ_0006845 have significant differences and considerable quantities. The circRNA11716 and circRNA13751 are novel biomarkers that both of their target miRNAs have not been reported by previous studies. Then, we predicted the miRNAs targeted by other circRNAs and found that hsa_circ_0005603 may interact with 10 miRNAs. It has been reported that 7 miRNAs (hsa-miR-27a-3p, hsa-miR-27b-3p; hsa-miR-17-3p; hsa-miR-198; hsa-miR-182-5p; hsa-miR-98-5p; hsa-let-7a-5p) are closely related to the biological characteristics of HCC. For instance, miR-182-5p is upregulated and activates AKT/FOXO3a/Wnt/β-catenin pathway by inhibiting expression of FOXO3a and degradation of catenin to enhance cell motility and invasion capacity of HCC. Overexpression of miR-182-5p allegorizes the poor prognosis of patients after hepatectomy and has great value as a diagnostic marker for HCC[19, 20]. The hsa-miR-27a-3p is downregulated and plays an antitumor role in HCC development by targeting the gene double-specific phosphatase 16 (DUSP16) and mediating inhibition of epithelial-mesenchymal transition (EMT)[21, 22]. miR-98-5p was found to reduce HBV DNA level and inhibit the proliferation and metastasis of HCC by targeting IGF2BP1 and nuclear factor-κB inducible kinase (NIK). The low expression of miR-98-5p is related to clinicopathological stage and survival rate of HBV-related HCC patients[23, 24]. Besides,we interestingly found that hsa_circ_0005603 and hsa_circ_0006845 share a common targetmiRNA, hsa-miR-198. Moreover, low expression of miR-198 in HCC is found to be significantly related to pathological characteristics, which can disrupt growth, migration, and invasion of HCC cells with various manners such as reducing the phosphorylation of p44/42 MAPK through targeting the HGF/c-MET pathway; mediate the upregulation of cells adhesion genes (E-cadherin and claudin-1), and so on[25–28]. Although hsa-let-7g-5p, has-let-7e-5p, hsa-let-7d-5p and hsa-miR-106a-3p have not been reported to be related to HCC, they still may possess potential values related to hsa_circ_0005603 or hsa_circ_0006845 and participate in the occurrence of HBV-related HCC, so they are worthy of our further research and exploration.

These circRNAs (especially hsa_circ_0005603, hsa_circ_0006845, circRNA11716, and circRNA13751) may represent potential biomarkers of HBx-related HCC. In the future, we will use a large number of tissue samples, combining in vitro and in vivo experiments to continue research, to verify the relationship between circRNAs and HBx/HBV, and reconfirm its biomarkers and functions.

Declarations

Ethics approval and consent to participate

The protocol was approved by the Ethics Committee of Hainan General Hospital affiliated to Hainan Medical University and was conducted in compliance with the Declaration of Helsinki.

Consent for publication

Consent to publish the paper was obtained in writing from all authors in this study.

Availability of data and material

The datasets analysed during the current study are accessible from the corresponding author upon reasonable request.

Competing interests

All authors declare no conflict of interests.

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Authors' contributions

LLZ participated in experimental design, experimental operation, data collection, and paper writing. WJC designed experiments, reviewed papers, provided fund support, and work guidance. CJC and CL performed experimental design, experimental operation, and financial support. CC and XDF contributed to experimental operation. LSX analyzed and interpreted the experimental data. LXX performed statistical analysis and prepared the figures. All authors read and approved the final manuscript.

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Figures

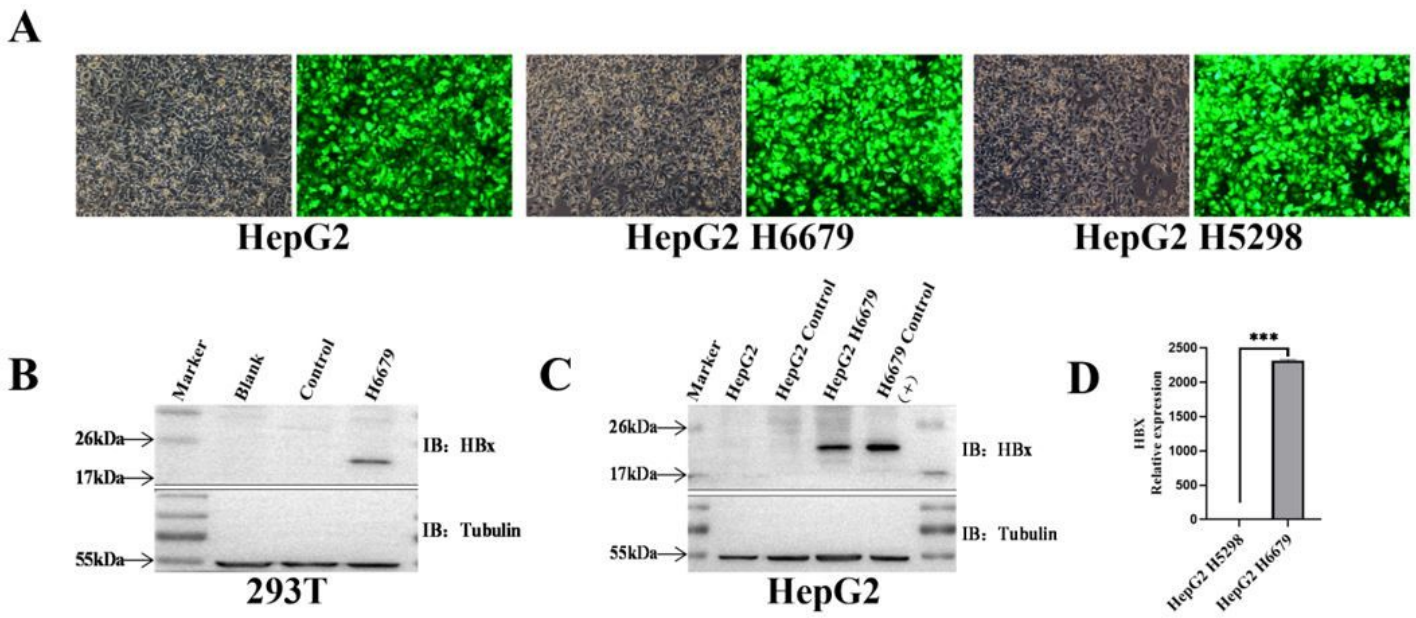

Figure 1

Construction of stable cell lines of HepG2-HBx overexpression and control. A Fluorescence photo of the stable strain after lentiviral infection of HepG2 cells with blasticidin drug screening for 14 days, as seen by a fluorescence rate of more than 90%; B WB verification after lentiviral transfection of 293 Tcell showing that the H6679 plasmid could express HBx; C Lentiviral infection of HepG2 cells, WB results indicating successful construction of stable HepG2-H6679 cell line, “H6679 Control(+)” is a positive control group of H6679 plasmid expression; D qPCR to detect HBx expression in the two stable cell lines.

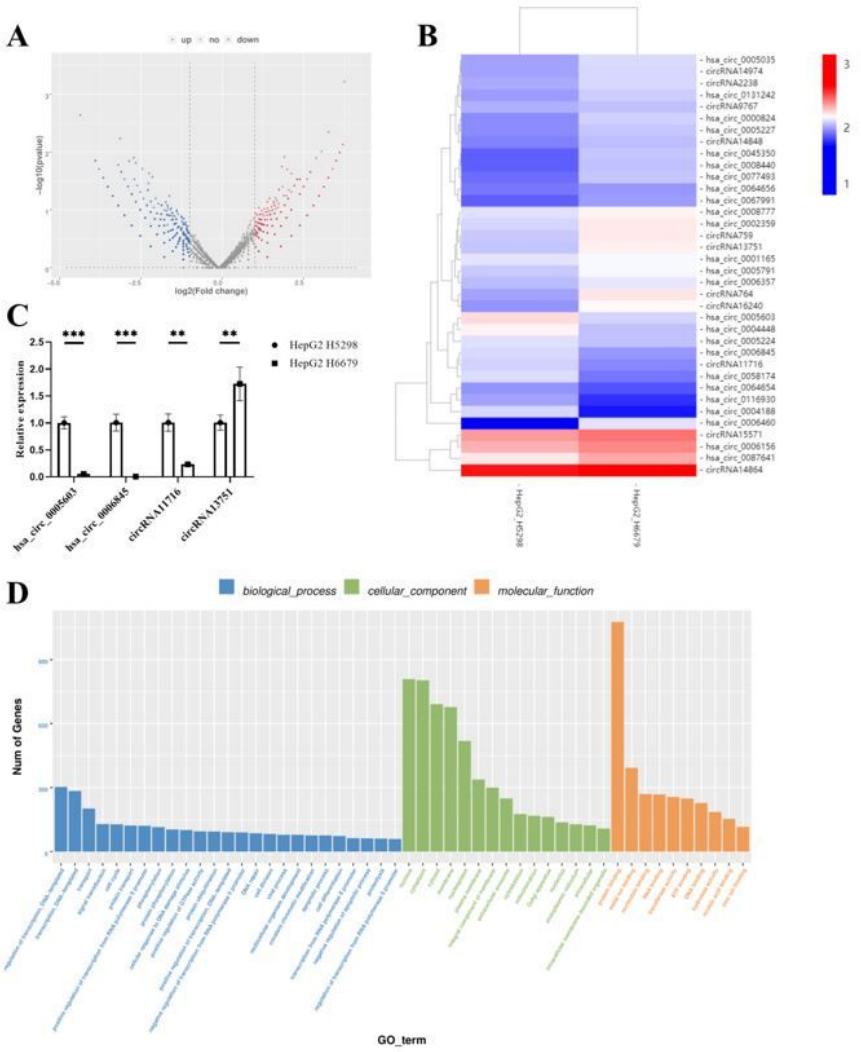

Figure 2

Differentially expressed CircRNAs and GO analysis. A The abscissa of the volcano graph represents the fold change of the differential expression of genes in the comparison group, and the ordinate represents the statistical significance of the difference in gene expression changes; B The heatmap shows the gene cluster analysis of 36 selected circRNAs; C The qPCR verified the expression levels of hsa_circ_0005603, hsa_circ_0006845circRNA11716, and circRNA13751 in the two stable cell lines. The measurement was repeated three times, * means P□0.05, ** means P□0.01, *** means P□0.001; D GO analysis histogram of circRNAs according to the values in the enrichment score under the theme of BP, CC, and MF.

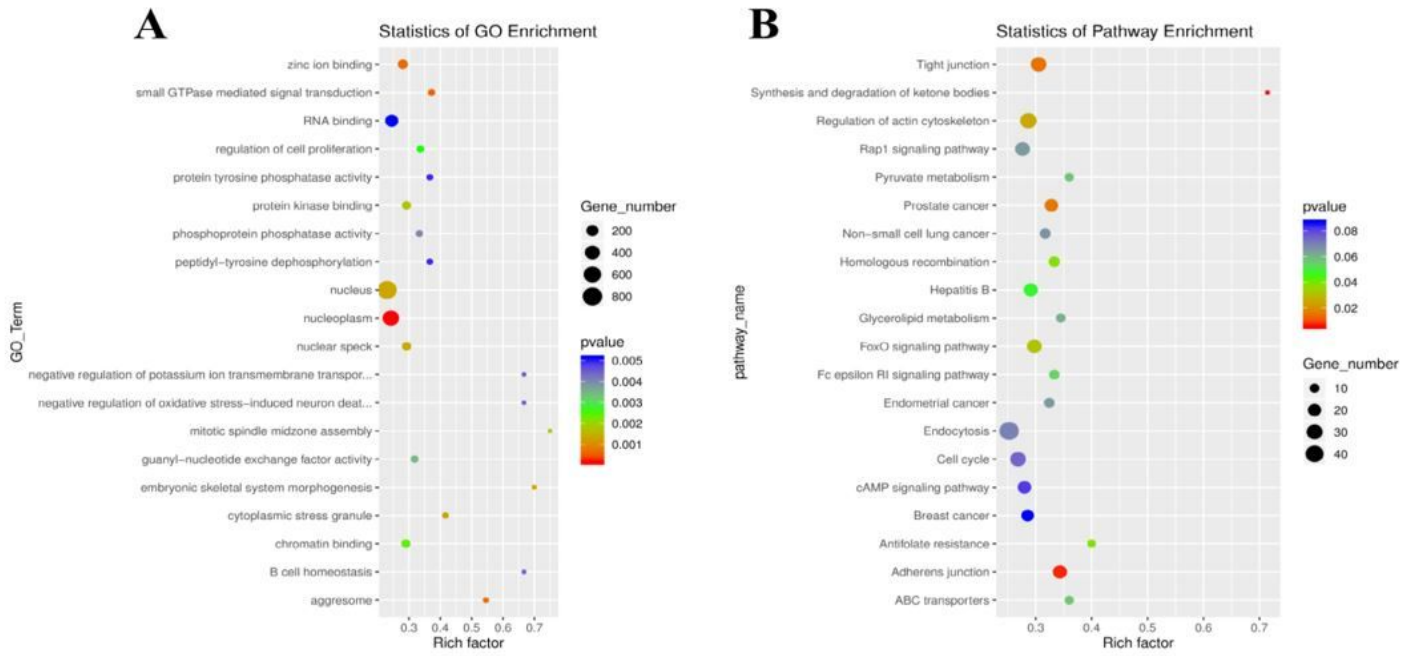

Figure 3

GO and KEGG pathway enrichment analysis. The dot plot shows gene rich factor value of the top 20 most significant enrichment pathways. Rich factor: the differential gene number/ the total gene number in a column of GO/KEGG.

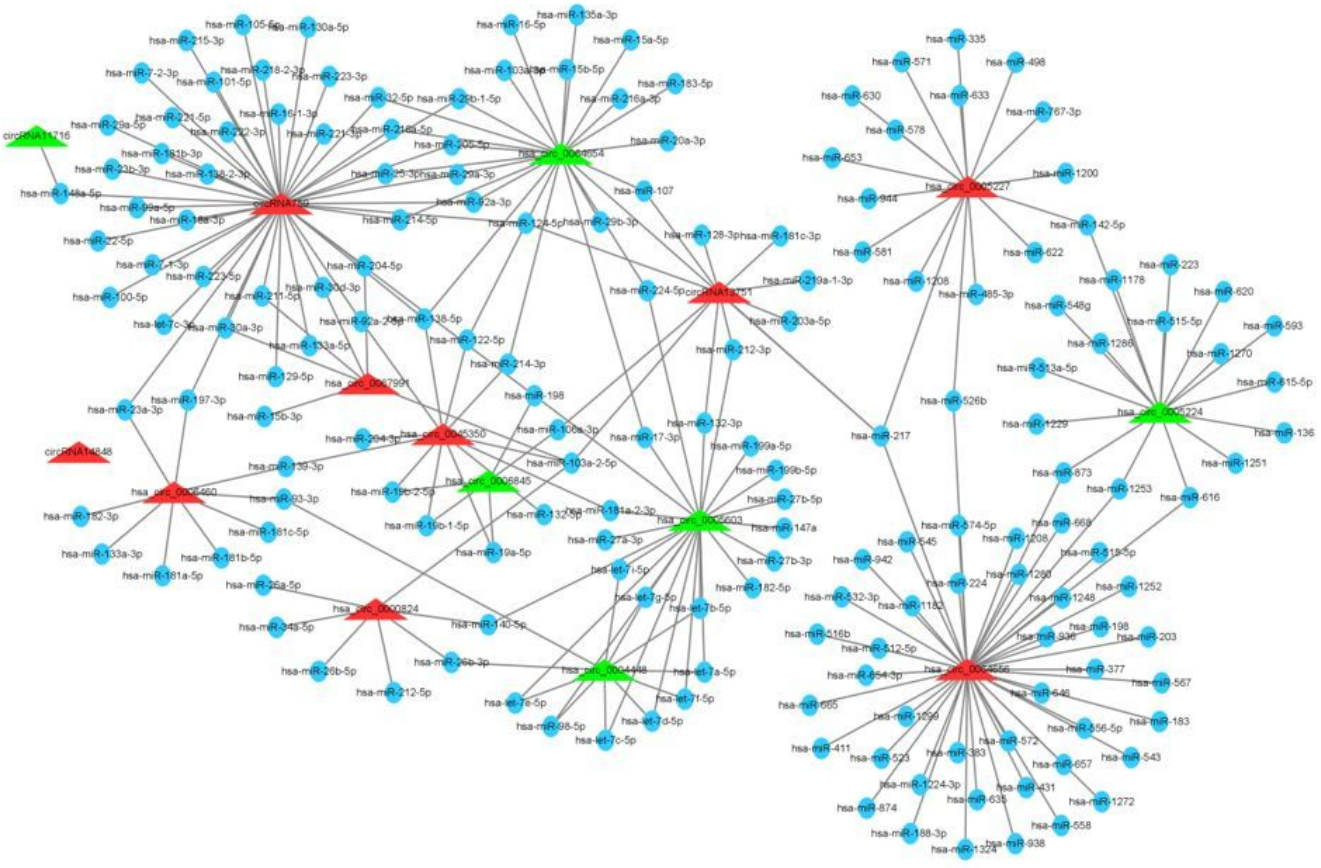

Figure 4

CircRNA/miRNA interactions network. The network showed the relationship of 15 circRNAs with target miRNAs. Bule nodes are target miRNAs, red nodes are upregulated circRNAs, and green nodes are downregulated circRNAs.