circEPS15 Overexpression in Hepatocellular Carcinoma Modulates Tumor Invasion and Migration

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Abstract

Background

Hepatocellular carcinoma (HCC) is one kind of the leading causes of cancer-related deaths in the world. Recent evidence indicates that circular RNAs (circRNAs) play important roles in tissue development, gene transcription, signal regulation and tumorigenesis. However, whether circRNAs are involved in HCC progression and encode functional proteins remains largely unknown.

Methods

circRNAs microarrays were performed using three pathologically diagnosed HCC samples and their paired adjacent normal liver tissues. Cell invasion, migration, cycle, and apoptosis post circRNA overexpression were measured using a transwell culture system, a wound healing assay, and flow cytometry. Full-length, mutated, and truncated sequences of circEPS15 with a FLAG tag were inserted into a circular expression vector. Western blotting was used to confirm circEPS15 expression and the requirement of internal ribosomal entry site (IRES) elements within the circRNA. The miRNA and mRNA expression profiles were obtained by analyzing data retrieved from The Cancer Genome Atlas (TCGA) database. We then constructed a ceRNA network of circEPS15, miRNAs, and mRNAs.

Results

The expression of circEPS15 was downregulated in HCC tissues, and the survival curves showed that low circEPS15 levels were associated with poor overall survival in HCC patients. Overexpression of circEPS15 suppressed tumor invasion and migration by inhibiting the TJP1/CDH2/VIM signaling pathway and retarded cell cycle progression, yet had no effect on cell apoptosis. ceRNA analysis and qRT-PCR results suggest a possible circRNA (circEPS15)-miRNA (miR-24-3p)-mRNA (CIDEA) network in HCC. The spanning junction open reading frame in circEPS15 driven by IRES encoded a novel protein.

Conclusions

Endogenous circEPS15 plays a novel role in repressing HCC through the ceRNA network and encoding a functional protein.

Background

Hepatocellular carcinoma (HCC) is a kind of the most common malignant tumors ranking fifth in the incidence of malignant tumors and third in mortality[1]. Intrahepatic metastasis and postoperative recurrence are the main causes of death in patients with HCC. Epithelial mesenchymal transition (EMT) is the process of transforming epithelial cells into mesenchymal cells with specific physiological or pathological conditional phenotypes[2]. In recent years, EMT has been found to be closely associated with the recurrence and metastasis of HCC and has gain significant attention. Elucidating the molecular

regulatory mechanism underlying EMT and the relationship between EMT and malignant tumors would assist in the search for new molecular targets [2, 3].

Circular RNA (circRNA) is different from traditional linear RNA. It has a 5' - 3' polar covalent closed loop structure and is relatively stable, abundant, and conserved in the eukaryotic transcriptome. Recent studies have shown that the expression of circRNAs is tissue- and cell-specific [4-6]. Evidence shows that circRNA is associated with the occurrence, development, prognosis, and prognosis of cancer [7-9]; however, the role and molecular mechanism of circRNAs in HCC has not been elucidated. The hepatitis B virus (HBV) is prevalent in China, and the incidence of primary HCC is also higher than that in developed countries such as Europe and the United States [10]. Therefore, it is urgent and important to understand the role of circRNA in the occurrence and development of HCC and provide an effective target for HCC diagnosis and treatment.

HCC is a complex molecular process driven by gene mutations accompanied by epigenetic modifications [11]. At present, there is no effective diagnosis or treatment strategy, thus it is urgent to find a potential effective diagnostic marker and therapeutic target. Non-coding RNAs, including small interfering RNA (siRNA) and long non-coding RNA (lncRNA), have been found to be scrambled in patients with HCC, possibly having potential clinical value [12]. However, the expression and function of circRNAs in HCC have not been elucidated.

Studies have shown that circRNA imbalance is associated with several human diseases and may be associated with the onset and progression of systemic lupus erythematosus, diabetes, and Alzheimer's disease [13-15]. Other studies have shown that circRNAs are involved in the occurrence and development of tumors, including colon, gastric, and cervical cancers [16-19]. Although circRNAs have received increased attention, the biological functions and mechanisms of several circRNAs have yet to be elucidated, and it remains unknown whether these circRNAs are transcribed.

The molecular mechanism of circRNA biological function primarily includes miRNA absorption through the sponge effect, which affects the biological function of the miRNA [20]. circRNA is also a biological regulator that binds to proteins [21], and can translate functional proteins thereby influencing glioma tumorigenesis [22]. However, whether circRNA plays a biological role in HCC and its underlying mechanism has not been elucidated.

miRNAs in circulation can be used as potential biomarkers for pancreatic cancer, Barrett's esophagus and esophageal adenocarcinoma [23, 24]. However, although a large number of clinical studies have been conducted to HBV-related HCC and hepatitis C virus (HCV)-related HCC [25, 26], the instability of miRNAs and difficulty in reproducing results under different experimental conditions make miRNAs a poor biomarker for clinical practice. In contrast, circRNA is more stable due to its closed-loop structure and tolerance to several RNA enzymes present in the environment. Moreover, due to its tissue-specific expression [27], circRNA could be an ideal biomarker for predicting the occurrence, progression, and prognosis of disease.

Further investigations into the mechanism of circRNA may elucidate its potential therapeutic use in the future. Additionally, due to its stability, circRNA can be used as a continuous cell regulator. For example, a specific circRNA can be synthesized, through genetic engineering, to play a specific biological role by binding to a specific miRNA, competing for binding ribosomal protein, or translating a small polypeptide. One study has shown that an overexpressed synthesized circRNA could absorb miRNA-122 through sponge-like interactions thereby affecting the HCV replication cycle by inhibiting its protein synthesis [28]. Therefore, synthetic circRNA has broad application in molecular medicine.

In this study, we investigated the cancer inhibition role of circRNA-100226 in HCC using paired human HCC and para-cancerous tissues. Using the human reference genome, we further estimated that the circRNA located at chr1:51868106–51874004 is derived from EPS15 (epidermal growth factor receptor pathway substrate 15), which is located on chromosome 1p32.3. Thus, we referred to circRNA-100226 as “circESP15.” We also overexpressed circEPS15 to further investigate the mechanisms of tumor growth.

Methods

1. Patients and specimens

The study was approved by the Ethic Committee of Third Hospital, Peking University. Written consent was obtained from all patients prior to surgery. A total of 71 HCC tissues and matched adjacent nontumor tissues were obtained from patients who underwent surgery in the Third Hospital, Peking University. None of the patients had received chemotherapy, radiotherapy, or any other anticancer therapy before surgery. The diagnoses were histologically confirmed by experienced pathologists. Paired tissue specimens (tumor and adjacent normal tissues) were collected from the patients, immediately frozen in liquid nitrogen, and stored at -80°C until use. Clinical information and follow-up data of the patients are listed in Table 1.

2. CircRNA microarray hybridization and data analysis

Total RNAs were digested with RNase R (20 U/μL, Epicentre, Madison, WI, USA) to remove linear RNAs and enrich circular RNAs. The enriched circular RNAs were amplified and transcribed into fluorescent cRNA utilizing a random priming method (Super RNA Labelling Kit; Arraystar, Rockville, MD, USA). The labeled cRNAs were hybridized onto the Human circRNA Array (8 × 15K, Arraystar). The slides were incubated for 17 h at 65°C in a hybridization oven (Agilent Technologies, Santa Clara, CA, USA). CircRNAs, with statistically significant fold-change differential expression between HCC and paired normal tissues ($FC \geq 2$ and $p \leq 0.05$) were identified by Volcano plot filtering. Hierarchical clustering was performed to determine the distinguishable expression pattern of circRNAs among samples.

3. Cell culture

The human HCC cell lines (BEL-7402, BEL-7404, Huh7, Hep3B, and HepG2) and normal hepatocyte cells HL-7702 were purchased from the Shanghai Institute of Cell Biology (Shanghai, China) with a certificate

of authenticity for each cell line. The samples were maintained in DMEM/RPMI1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 100 U/mL penicillin–streptomycin mixture (Gibco BRL, Grand Island, NY, USA) at 37°C in 5% CO₂. After culturing the cells for several months, all cell lines were evaluated for genetic identification prior to the experiments. Based on the appraisal reports provided by CoBioer Biosciences Co., Ltd. (Nanjing, China), no mutations or contaminations were found in our cell lines.

4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

All primers used for qRT-PCR were designed using primer 5.0 software (Premier, Canada) and synthesized by Tsing Ke Biotech (Chengdu, China). The cDNAs for circRNA, mRNA, or miRNA measurement were synthesized using random, oligo(dT)18, or stem-loop primers, respectively, using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed in triplicate using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) on the CFX connect real-time system (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the intrinsic control for circRNA and mRNA, while U6 was used as the endogenous control for miRNA. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta C_t}$ method.

5. circEPS15-overexpressing vector construction and transduction

The circEPS15-overexpressing plasmid was synthesized and cloned into the adenoviral vector GV138 (Genechem, Shanghai, China) with a Flag-tag. Full-length circEPS15 was serially diluted with XtremeGENE siRNA transfection reagent (Roche, Mannheim, Germany). For western blot analysis, cell proteins were prepared 72 h post-transfection.

6. Wound healing Assay

The cells were seeded into 6-well plates and transfected following the manufacturer's instructions. When the cell confluence reached approximately 80% at 48 h post-transfection, the cells were serum-starved for 12 h and scratched using the fine end of a 100-μL pipette tip. Wound healing was observed at different time points within the scratch line. Every 24 h, scratch lines were imaged using an inverted Leica phase-contrast microscope (DFC 300 FX, Wetzlar, Germany) under a 20× objective lens. Image processing and analysis in Java was performed to obtain the wound-healing assay results. Duplicate wells were examined for each condition, and each experiment was repeated three times.

7. Transwell assay

A cell invasion assay was performed in a transwell chamber (24-well type, 8 mm pore size, Corning, Inc., Corning, NY, USA). BD Matrigel Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ, USA) was used according to the manufacturer's instructions. Next, 0.5 mL of serum-free medium was added in the upper chambers; DMEM containing 10% FBS was added to the bottom chambers. Equal numbers (1×10^5) of cells were plated in the upper chambers of the quadruplicate wells and incubated at 37°C for 72 h.

The cells were then fixed with paraformaldehyde and stained with crystal violet to visualize the nuclei. Average values were calculated from the results of three independent experiments. Image processing and analysis in Java was performed to obtain the transwell assay results.

8. Cell cycle and apoptosis assay

To assess the cell cycle and apoptosis, 3×10^5 treated cells were seeded into 6-well plates and cultured for 48 h at 37°C. For cell cycle analysis, the cells were digested using trypsin (Hyclone, Logan, UT, USA), washed twice with phosphate-buffered saline (PBS), and fixed in 70% ethanol overnight at 4°C. The cells were centrifuged at $500 \times g$ for 5 min, washed twice with cold PBS, and centrifuged. After treating the cells with RNase A (0.1 mg/mL) and propidium iodide (PI, 0.05 mg/mL, 4A Biotech, Beijing, China)) for 30 min at 37°C, cell cycle analysis was performed using fluorescence-activated cell sorting flow cytometry (Beckman Coulter, Palo Alto, CA, USA). To analyze apoptosis, the cells were trypsinized followed by two PBS washes. The cells were stained using the Annexin V/PI detection kit (4A Biotech) for 5 min at 25°C. Apoptotic cells were measured using flow cytometry (Beckman Coulter). All experiments were repeated at least three times.

9. Western blot analysis

To detect protein, the cytoplasmic and nuclear protein extracts were prepared from the cells. The protein concentration of each sample was determined using a NanodropTM spectrophotometer (Thermo Fisher Scientific). Protein (100 µg) from each sample were subjected to SDS-PAGE (4% stacking and 10% separating gels) and then transferred overnight onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight with the following antibodies: poly clonal anti-human TJP1 antibody (1:200, Abgent, San Diego, CA, USA); anti-CDH2 antibody (1:1,000, Abcam, Cambridge, UK); anti- α VIM antibody (1:1,000, Cell Signaling Technology, Danvers, MA, USA); anti-EPS15 antibody (1:500, Abgent); anti-GAPDH antibody (1:1,000, Santa Cruz Biotechnology, Dallas, TX, USA); and anti-Flag antibody (1:1,000, Cell Signaling Technology). Next, the blots were incubated with peroxidase-conjugated goat anti-rabbit antibody or goat anti-mouse antibody (1:4,000, Millipore) for 1 h. Finally, the membranes were subjected to immunoblotting analysis using an ECL immunoblotting kit according to the manufacturer's recommendations (Beyotime Institute of Biotechnology, Shanghai, China).

10. IRES prediction

IRESfinder [29] was used to identify the IRES sequence of circRNAs. Each sequence of circRNA was evaluated by the sliding window approach, with window size of 174 nt and step size of 50 nt. The region with the highest score was considered as the IRES sequence of circRNA.

11. Statistical analyses

All statistical analyses were performed using SPSS version 21.0 (SPSS, Inc., Chicago, IL, USA) and Prism version 5.0 (GraphPad Software, La Jolla, CA, USA) software. Categorical variables were expressed as a

count or percentage and tested using χ^2 or Fisher's exact test, as deemed appropriate. Continuous data are shown as the mean $\pm$ standard deviation (SD) and were compared using Student's *t*-test, one-way analysis of variance, or Mann-Whitney test as deemed appropriate. Correlations were calculated using Pearson's correlation analysis. The median expression of target genes was used as the cut-off value to stratify patients into high and low expression groups. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. All tests were 2-sided, and results with $p < 0.05$ were considered statistically significant.

Results

The expression profiles circRNA in HCC

Two pairs of HCC tissues (T) and matched para-carcinoma normal tissues (C) were analyzed by circRNA microarray to explore the potential involvement of circRNAs in HCC. We found that the 608 circRNAs were upregulated in HCC tissue with fold change of > 2 ($p < 0.05$), while 737 circRNAs were downregulated using the same cutoff parameters (Fig. 1a). Using hierarchical clustering, we identified the top five upregulated and downregulated circRNAs in HCC tissues (Fig. 1b), and the variation in circRNA expression was summarized in the scatter and volcano plots as shown in Figure 1c-d. To verify the expression of these top five upregulated and downregulated circRNAs in 32 randomly selected hepatoma adjacent and their tissues, we used q-RT-PCR and junction primers. circEPS15 and circRNA-103595 were the top down- and up-regulated circRNAs in HCC tissues, respectively, which was consistent with our microarray analysis (Fig. 1e).

Correlation between circEPS15 expression and HCC patients' clinical characteristics

To further probe the role of circEPS15 in HCC, we analyzed the relation between circEPS15 expression in HCC tissues and the clinical pathological characteristics of HCC patients. As shown in Table 1, there was a significantly correlation between low expression level of circEPS15 in HCC tissues and poor tumor differentiation ($p = 0.017$), and vascular invasion ($p=0.011$). In addition, other characteristics of HCC, including the AFP level, tumor number, tumor size and BCLC stage, were also not associated with circEPS15 expression. These data indicate the importance of circEPS15 in the tumorigenesis and intrahepatic metastasis of HCC.

Prognostic and diagnostic value of circEPS15 and circRNA-103595

71 patients completed the clinical follow-up and obtained the available survival information., from which Receiver Operating Characteristic (ROC) analysis was performed to determine whether circEPS15 and circRNA-103595 had diagnostic significance. Results showed that the Area Under Curve (AUC) of circEPS15 and circRNA-103595 was 0.735 and 0.627, respectively, which confirmed that circEPS15 was diagnostically significant for HCC (Fig. 2a-b). Kaplan-Meier analysis of the correlation between circEPS15 and patient survival showed that low circEPS15 level was significantly associated with a poor overall survival (Fig. 2c).

Effects of circEPS15 on HCC tumor cell proliferation and cycle progression

Based on these results, we investigated the specific function of circEPS15 in HCC formation and development, and confirmed that the tumor cell lines HL-7402 and HL-7404 had low expression of circEPS15 compared to normal cell lines HL-7702 and L02. We constructed an adenovirus vector to overexpress circEPS15 in HL-7402 and HL-7404 cell lines. Following transfection, the expression of circEPS15 increased in HL-7402 cells but not in HL-7404 cells, indicating successful transfection and overexpression of circEPS15 in HL-7402 (Fig. 3a); therefore, we used HL-7402 for further study. We investigated the effect of circEPS15 overexpression on the cell cycle by measuring the percentage of HCC cells in different cell cycle stages (e.g. G1/G0, S, and G2/M) and found that circEPS15 overexpression increased the percentage of cells in G2/M compared to the vector control (Fig. 3b). In addition, no significant difference in apoptosis was observed between transfected cells and negative control (Fig. 3c). These results indicated that circEPS15 elicited cell cycle arrest but had not effects on apoptosis.

Overexpression of circEPS15 suppressed HCC cell migration and invasion by inhibiting the TJP1/CDH2/VIM signaling pathway

Since circEPS15 suppressed tumor growth, we subsequently investigated the effect of circEPS15 on HCC function, including migration and invasion. Wound healing and transwell assays indicated that circEPS15 significantly increased HCC cell migration and invasion compared to negative control (NC) (Fig. 3d-e). To investigate the molecular mechanism underlying the specific function of circEPS15 in HCC, we examined several proteins involved in the signaling pathways that affect cell migration and invasion using western blotting. Overexpression of circEPS15 reduced TJP1 and VIM protein expression but induced the expression of CDH2 (Fig. 3f). These results indicated that circEPS15 inhibited HCC cell migration and invasion by affecting the proteins involved in the TJP1/CDH2/VIM signaling pathway.

Prediction of circRNA-miRNA interactions and competing endogenous RNA (ceRNA) regulatory network analyses

circRNAs act as microRNA sponges associated with related miRNAs, and together they made up the circRNA-miRNA axis involved in disease pathogenesis. To determine the function of circEPS15, its target miRNAs were predicted by informatics analysis using TargetScan (http://www.targetscan.org/vert_72/) and miRanda (<http://www.microrna.org/microrna/home.do>). The top five miRNAs associated with each validated circRNA and the predicted circEPS15 interaction sites are shown in Fig. 4. To further elucidate the underlying mechanism of circEPS15, a circRNA-miRNA-mRNA regulatory network was constructed using Cytoscape (Fig. 5a). The network suggested that circRNAs could indirectly regulate miRNA target genes by competitively binding to miRNAs. To confirm that circEPS15 can regulate its target gene, 11 target genes were examined using qRT-PCR, which showed that there was a positive correlation between circEPS15 and *CED1A* (shown in Fig. 5B and 5C). These results suggest that circEPS15 may regulate *CED1A* through a ceRNA regulatory network, which affects the process of epithelial mesenchymal transition.

Evaluation of circEPS15 protein-coding ability

circEPS15's sequence was matched in circRNADB; therefore, we next analyzed the putative open reading frame (ORF) in circEPS15 (Fig. 6a). There was a potential spanning junction ORF in circEPS15 that could encode a 150-amino acid protein. Conservation analysis showed that this ORF was highly conserved among different species, suggesting that this ORF was translatable. To test the putative ORF transcription ability, a wild type (ATG) or mutation type (AAG) circEPS15 was cloned and inserted into the GV486 vector (Fig. 6b). To test the putative IRES activity in circEPS15, a full-length or truncated putative circEPS15 IRES was also cloned into the GV486 vector. To add a FLAG tag to the gene of interest, we moved the junction to the stop codon, with side flanking sequences (circEPS15-FLAG) (Fig. 6b). We transfected these plasmids into 293T cells and detected their potential translated products. As shown in Figure 6c, the FLAG antibody only detected a ~25 kDa protein in wild-type circEPS15-FLAG (OE5) and circEPS15 (ins_FLAG)-IRES1del (OE4) transfected cells but not in the mutated (AAG), IRES2 truncated, or IRES1 and IRES2 truncated circEPS15-FLAG transfected cells. The scores for the IRES prediction in these five sequences were consistent with the western blotting results, wherein OE4 and OE5 had the highest scores (0.75) by IRESFinder (Table 2). These results indicated that circEPS15 could encode a protein, the ORF was translatable, and ATG and IRES2 were essential for the 5'-cap independent translation of circEPS15.

Discussion

Because many studies have shown that a large number of circRNAs can be stably expressed in eukaryotic cells, several researchers have begun to pay attention to the role of circRNAs in various diseases. Investigating the role(s) of circRNAs in tumor is also becoming a hot research topic.

At present, research on the role of circRNAs in tumor is mainly focused on the discovery and identification of new target circRNAs, their influence on tumor biological behavior, and the molecular mechanism of circRNAs.

A variety of new circRNAs have been found due to the popularization and application of circRNA chips. Through RNA enzyme digestion and sequencing technology, we can identify the circular structure of newly found circRNA and confirm it as circRNA.

The role of circRNA on tumor biological behavior is becoming a new hotspot. In hepatocellular carcinoma, some circRNAs have been found and confirmed to be related to the biological behavior and prognosis of hepatocellular carcinoma. For example, circfbim1 can competitively bind to mir-346 through ceRNA mechanism, thus promoting the progress of hepatocellular carcinoma. This study indicates that circRNA exists in hepatocellular carcinoma and plays an important role in the occurrence and development of hepatocellular carcinoma. As a new type of RNA, it is of great significance to prove its existence and role in liver cancer, as it may increase awareness about liver cancer in people and provide a novel way(s) to treat the disease.

The Molecular mechanism of circRNA has always been an interesting as well as difficult area in this field. Interestingly, artificial circRNA has been proved to be translatable in eukaryotic cells [36], and the current evidence also shows that other types of noncoding RNAs can initiate protein synthesis, raising the question whether endogenous circRNA can encode proteins in mammalian cells [37-39]. At present, the molecular mechanisms of circRNA are: miRNA sponge adsorption, CeRNA mechanism, and the recently discovered and confirmed coding polypeptide effect in some circRNAs and lncRNA. For example, circfbxw7, found in glioma, can encode small peptides and inhibit the growth and replication of glioma; and peptides encoded by a segment of lncRNA can be stored in the muscle activity of SERCA. These findings greatly expand the possible mechanism of circRNA, and overturn the cognition that circRNA cannot be translated as non-coding RNA. As we all know, protein, as the basic component of life, is an important form of biological function.

In this study, we detected the expression level of circEPS15, and statistically analyzed the overall survival of corresponding liver cancer patients and selected the effective cut-off value, divided circEPS15 into high expression group and low expression group, and also found that this person from eps15 was related to the overall survival of patients. This conclusion suggests that circEPS15 can be used as a potential biomarker for prognosis analysis and monitoring of HCC patients.

In this study, we used microarray and qRT-PCR analysis, combined with in vivo experiments, to determine the relationship between the down-regulation of circEPS15 in HCC and the prognosis of HCC. The down-regulation of circEPS15 in HCC tissues was significantly correlated with the tumor differentiation and intravascular tumor thrombus, suggesting that circEPS15 has antitumor effect, and may play an important role in epithelial mesenchymal transition of HCC. This hypothesis has been verified in vivo and in vitro. In addition, we have demonstrated that circEPS15 can regulate CEDIA through the ceRNA regulatory network, and has the ability to encode proteins.

As we name it, circEPS15 is derived from its female parent gene eps15. Therefore, we need to understand eps15 and its functions before elucidating functions of circEPS15. Eps15 is transcribed from ch38.p13, which encodes a part of the EGFR pathway [40], exists in the pits of the reticular protein coating, and participates in the receptor-mediated endocytosis of epidermal growth factor (EGF) [41]. In addition, alternative splicing leads to multiple transcriptional variants that encode different subtypes [42]. In addition to affecting cell migration and cancer-related signal transduction, eps15 is also associated with poor prognosis in a variety of tumor types, including breast cancer, esophageal squamous cell carcinoma, and glioma [43-45]. Previous studies have shown that edh2 (eps15 homologous domain contains 2) may inhibit the migration and invasion of hepatocellular carcinoma through interaction with E-cadherin [46]. However, the underlying mechanism of eps15 inhibiting tumor remains unclear. Consistent with previous studies, expression of eps15 mRNA was significantly down-regulated in HCC tissues compared to that in matched non-tumor tissues, while the mRNA-eps15 expression level in HCC tissues was positively correlated with that of circEPS15. These observations suggest that the expression of circEPS15 in HCC may be mainly regulated by host gene amplification. Bai et al. [47] He et al. [48] it has been reported that circfbim1 and circgrfa1 can regulate the corresponding linear fbim1 and grfa1 mRNA transcriptional

expression through ceRNA mechanism. Therefore, circEPS15 may regulate the transcriptional expression of the corresponding linear eps15 mRNA through the above-mentioned ceRNA mechanism. This is only a hypothesis, which will be further tested, proved and discussed in the following research.

In this study, we found that circEPS15 could encode a protein in vitro by matching the RNA deep sequencing data with circnadb. In view of the key role of eps15 gene in tumorigenesis, we hypothesized that the same function of eps15 subtype, and the shorter and more stable translation protein of circEPS15 may be the key regulators of cell proliferation. As this study is limited to cultured cells, clinical significance of circEPS15 needs to be studied in a larger clinical cohort. In addition, this study confirmed the translatability of circEPS15, but the structure and function of the peptide encoded by circEPS15 need to be further verified by synthesizing specific antibodies, which are expected to be clarified in the following studies. As an important biological regulator, circRNAs have been found to encode polypeptides in gliomas. However, no translatable circRNAs and their products have been reported in HCC.

Conclusion

A novel circRNA named circEPS15 was found in HCC, and its expression level was related to the overall survival of HCC patients. Our observations of expression and activity of circEPS15 suggest that circEPS15 and its translatability may play some role in the progression of HCC and the clinical prognosis of patients.

Abbreviations

HCC: Hepatocellular carcinoma

circRNAs: Circular RNAs

IRES: Internal ribosomal entry site

TCGA: The Cancer Genome Atlas

RT-PCR: Real-time PCR

EMT: Epithelial mesenchymal transition

siRNA: Small interfering RNA

lncRNA: Long non-coding RNA

HCV: Hepatitis C virus

EPS15: Epidermal growth factor receptor pathway substrate 15

FC: Fold-change

cDNAs: Complementary DNAs

ROC: Receiver Operating Characteristic

AUC: Area Under Curve

NC: Negative control

EGF: Epidermal growth factor

EDH2: Eps15 homology domain-containing 2

Declarations

Ethics approval and consent to participate

This study was approved by Peking University Third Hospital Medical Science Research Ethics Committee. Written informed consent was obtained from all patients who provided HCC and precancerous samples and the principles of the Declaration of Helsinki were followed.

Consent for publication

Not Applicable.

Competing Interest

The authors have no conflict of interest.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

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Not applicable.

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Maolin Tian, Gang Li and Bin Jiang contribute equally to this work as co-first authors.

Author contributions

B.Y.T. and Y.C.H. performed the described studies, analyzed data, and prepared the manuscript. T.M.L., L.G. and J.B. provided clinical and experiments suggestions. T.M.L., L.G., J.B. and A.S. provided materials and assisted in experiments. X.D.R. and Y.C.H. advised on study design and prepared the manuscript. All authors have read and approved the manuscript.

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Tables

Due to technical limitations, table 1 and 2 is only available as a download in the Supplemental Files section.

Figures

Fig.1

Figure 1

Differential expression of circRNAs between human hepatoma and adjacent tissues Microarray analysis was conducted to compare circRNA expression differences between normal and hepatoma human cells.

a) Venn analysis of commonly upregulated and downregulated circRNAs indicating that some circRNAs have their specific expression characteristics in tumor heterogeneity. b) Heat map of differentially expressed circRNAs between three patients of normal and tumor tissues (significant fold change > 4). c) Scatter plots depicting the raw variations in the expression profile between the two tissues types. d) A volcano plot depicting fold change and p-value between hepatoma and adjacent tissues. The horizontal line represents $p = 0.05$ and the red points represent the circRNAs that are statistically different between the two cell types investigated. The green lines in a and b represent a 1.5-fold change in expression. e) Expression levels of circEPS15 and circRNA-103595 in 32 randomly selected paired hepatoma and adjacent tissue samples detected with junction primers ($p < 0.01$).

Fig.2

Figure 2

Profiling and characterization of circEPS15 as a circular RNA in human hepatoma and adjacent tissues a- b) Combined with the prognosis results from these patients, an ROC curve was constructed, and AUC of 0.735 and 0.627 for circEPS15 and circRNA-103595, respectively, was obtained, indicating that the expression of circEPS15 has a better predictive value for prognosis in HCC patients. c) Kaplan-Meier's survival curves depict the correlation between circEPS15 and the overall survival of HCC patients. The patients were stratified into two groups according to a cut-off value of 0.8796 for circEPS15. Patients with low circEPS15 expression had a shorter overall survival compared to patients with high circEPS15 expression. A total of 71 patients included in each experiment, and values indicate the mean $\pm$ SD of three independent experiments. The Student's two-tailed unpaired test and Log-rank test were used for statistical analysis of these data.

Fig.3

Figure 3

circEPS15 suppressed HCC cell proliferation, migration, and invasion a) Real-time PCR was used to detect circEPS15 expression in Bel-7402 and Bel-7404 cells transfected with circEPS15 overexpression adenovirus constructs or an empty vector control ($p < 0.01$). b-c) Flow cytometry was used to detect cell cycle and cell apoptosis progression in transfected Bel-7402 cells ($p < 0.01$). d) Migration of cells overexpressing circEPS15 or the negative control was measured in a wounding assay at 0 h and 48 h. The bar chart illustrates the relative migration distance of cells into the wounded region. The data are mean $\pm$ SEM of three independent experiments ($p < 0.01$, compared to 0 h). e) A trans-well assay was used to evaluate the invasion ability of cells overexpressing circEPS15 or the empty vector control) after

the putative ORF are shown in black, internal ribosomal entrance site (IRES) sequences are shown in yellow, and start (ATG) and stop (TGA) codons are shown in red. b) WT: Illustration showing how endogenous circEPS15 is formed; the circular junction is inside the ORF and forms the unique sequences (yellow). OE5: A FLAG tag was divided to both sides (light and dark green). Circularization of this vector formed the same circular RNA as the endogenous circEPS15, except with a FLAG tag added behind the ORF. OE1: The start codon was mutated from ATG to AAG to clarify the role of the start codon. OE2: IRES sequences (IRES1 and IRES2) in circEPS15 were truncated. OE3: IRES sequences (IRES2) in circEPS15 were truncated. OE4: IRES sequences (IRES1) in circEPS15 were truncated. Lower: A FLAG tag antibody was used to detect circEPS15 expression in 293T cells transfected with the vectors described above.

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