Ferroptosis Involved in the Progression of Hepatocellular Carcinoma Through Circ0097009/miR-1261/SLC7A11 Axis

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Research

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Abstract

Background: Circular RNAs (circRNAs) is a class of non-coding RNAs and have been demonstrated to play important roles in tumorigenesis. Nevertheless, how circRNAs regulate the progression of hepatocellular cancer (HCC) remains unclear.

Methods: CircRNA microarrays was performed in HCC tissues to screen circRNAs that are extinct expressed. Quantitative real-time PCR (qRT-PCR) was conducted in HCC cell lines, and tissues and circ0097009 were found to be significantly up-regulated. Then, we explored the functions of circ0097009 in HCC by a series of experiments including cell proliferation, invasion and mouse xenograft assay. Additionally, luciferase assay and RNA immunoprecipitation (RIP) assay was used to explore the interactions of circ0097009, miRNA-1261 and SLC7A11 (a key regulator in cancer cell ferroptosis) in HCC.

Results: Microarray analysis and qRT-PCR verified a circRNA circ0097009, which was significantly up-regulated in HCC tissues and cell lines. Knockdown of circ0097009 inhibited proliferation and invasion in HCC. Luciferase reporter assays showed that circ0097009 and SLC7A11 directly bind to miR-1261. Subsequent experiments showed that circ0097009 and SLC7A11 regulated the expression of each other by sponging miR-1261.

Conclusions: Circ0097009 act as a competing endogenous RNA (ceRNA) to regulate SLC7A11 expression, a key regulator in cancer cell ferroptosis through sponging miR-1261 in HCC. Circ0097009 may be used as a diagnostic biomarker and potential target for HCC therapy.

Background

Primary liver cancer is the sixth most commonly diagnosed cancer and the fourth leading cause of cancer-related death worldwide [1]. There were approximately 841,000 new cases and 782,000 deaths of liver cancer annually [1, 2]. Hepatocellular carcinoma (HCC) accounts for about 75%-85% of all liver cancer cases, and most of HCC are diagnosed at an advanced stage, which has lost opportunities to accept curative treatments [3, 4, 2]. Although target and immune therapies have developed rapidly in recent years, the prognosis of HCC remains poor [5–7]. There have been some studies reporting that many genes involved in the tumorigenesis of HCC; however, the underlying mechanisms are still largely unknown [8–10]. Therefore, it urges us to search for novel molecular targets to develop more effective therapeutic strategies for HCC [11].

Circular RNAs (circRNAs), a class of non-coding RNAs, were found to be widely expressed in mammals [12, 13]. A bunch of circRNAs have been identified, but their potential functions are poorly understood [13, 14]. Currently, few studies are exploring the role of circRNAs in HCC [15–19]. Yao et al. found that circZKSCAN1 suppresses HCC cell growth, invasion and migration [16]. Han et al. reported that circMTO1 play the role of the sponge of miR-9 to inhibit HCC progression [15]. Revealing the role that circRNAs played in HCC will be critical to deeply understand the molecular mechanisms of HCC progression and find new biomarkers or therapeutic targets for HCC [20].

It is reported that circRNAs could function as competitive endogenous RNAs (ceRNAs) to coregulate other RNAs by competing for shared miRNAs [14]. Kong et al. found that cirPLK1 inhibited triple-negative breast cancer progression via sponging to miR-296-5p [21]. circSMARCA5 involved in the growth and metastasis of HCC by functioning as ceRNA for miR-17-3p and miR-181b-5p [18]. All these findings suggested that circRNAs could function as miRNA sponges to regulate cancer occurrence and development.

In this study, we performed microarrays to analyze the expression profiles of circRNAs in HCC tissues. A significantly up-regulated circRNA, circ0097009, was detected by quantitative real-time PCR in HCC tissues and cell lines. We further investigated the functions of circ0097009 in HCC and found that circ0097009 knockdown could inhibit cell proliferation and invasion. Additionally, luciferase reporter and RNA immunoprecipitation (RIP) assay showed that circ0097009 could bind to miR-1261. Furthermore, the catalytic subunit solute carrier family 7 member 11 (SLC7A11), a key regulator of cancer ferroptosis, was also a direct target of miR-1261. Therefore, our findings indicated that circ0097009 might act as a ceRNA to regulate the expression of SLC7A11 by sponging to miR-1261. circ0097009 may be used as a potential target in HCC therapy.

Methods

Cell lines and culture

Human normal liver cell line LO2 and HCC cell lines HepG2, 7402 and 97H were obtained from American Type Culture Collection (ATCC, USA). The cells were cultured in DMEM (Invitrogen, USA) with 10% FBS (GIBCO, Brazil) at 37 °C with 5% CO₂. All cell lines are passaged in our laboratory for less than six months after resuscitation of frozen aliquots and re-authenticated by DNA fingerprinting every six months.

Quantitative RT-PCR analysis (qRT-PCR)

Total RNA of cells and tissues was isolated by TRIzol reagent (Life Technologies, USA). cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio Inc, China), and qRT-PCR was performed using SYBR Premix Ex Taq (Takara Bio Inc.) with CFX96 Real-time PCR system (Bio-Rad, USA). The abundance of target transcripts was evaluated using house-keeping genes U6 or β -actin as control. The relative fold-change in expression for a control sample was calculated by the $2^{-\Delta\Delta C_t}$ method.

RNase R digestion experiment

The RNA extracted from 97H cells was evenly divided into two groups: treated by RNase R group (Epicentre Technologies, USA) and treated by the buffer control group. In RNase R group, 2 μ g of total RNA was incubated with RNase R (3 U/ μ g) for 20 min at 37 °C. β -Actin served as an internal control.

Actinomycin D assay

Cells (1 x 10⁵) were seeded into 6-well plates and treated with actinomycin D (2 mg/L; Sigma, USA). All the treated cells were collected at 8 h, 16 h, 24 h respectively for qRT-PCR analysis of circ0097009 and

SLC7A11 mRNA.

CCK8 assay and Transwell assay

Cell proliferation was evaluated by CCK-8 assay (Dojindo Laboratories, Japan). Cells (1×10^3) were seeded into 96-well plates and incubated at 37 °C for 24 h before transfection. CCK-8 solution (10 μ l) was added to each well 48 h after transfection. After two h of incubation at 37 °C, the absorbance at 450 nm was tested using a microtiter plate reader (Bio-Tek EPOCH2, USA). For transwell assay, cells (at a density of 1×10^4) were seeded into migration chamber (BD Biosciences, USA), and medium (containing 10% FBS) was subsequently added into the lower chamber as an attractant. The cells were fixed by methanol after 24 h incubation, dyed by 0.1% crystal violet, and then counted under a microscope. Triplicate independent experiments were carried out.

Mouse xenograft model

All animal studies are following the guidelines of the Institutional Animal Experimental Ethics Committee of Sun Yat-Sen University Cancer Center. 4-week-old female BALB/c nude mice were subcutaneously inoculated with 2×10^6 cells (5 mice per group). Then, intratumoral injection (40 μ L si-circ0097009 or si-NC) was conducted every four days. Tumour weights were measured after four weeks when the mice were euthanized. For lung metastases, cells (1×10^5) were intravenously injected into mouse tail veins (6 mice per group). Eight weeks later, the lungs of anaesthetized mice were removed, and the numbers of metastatic lung nodules were visually counted and verified by H&E-stained sections under a microscope.

Luciferase reporter assay

Luciferase reporter vector with the full length of the 3'-UTR of SLC7A11 or circ0097009 were constructed. Then we generated the mutant luciferase reporter vectors with QIAGEN XL-site directed Mutagenesis Kit (QIAGEN, California, USA). HepG2 cells were seeded into 96-well plates and co-transfected with a luciferase reporter vector and miR-1261 mimics or miR-1261 LNA. After 48 h of incubation, the luciferase activities were quantified with a dual-luciferase reporter assay (Promega, USA). Cells (5×10^3) were plated and co-transfected with the constructed vectors and miR-1261 mimics for incubation (48 h). Then, the relative luciferase activity was assessed by a dual-luciferase reporter assay system (Promega).

RIP assay

HepG2 cells are transfected with MS2bs-circ0097009, MS2bs-circ0097009 mt or MS2bs-Rluc with MS2bp-GFP by Lipofectamine 2000. Forty-eight hours later, RIP was performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA). After purification of RNA complexes, the miR-1261 level was measured. The Ago2-RIP assay was performed with an anti-Ago2 antibody (Millipore). After purification of RNAs, the levels of circ0097009, SLC7A11, and miR-1261 were quantified.

Western blots

Total proteins were extracted, separated by SDS-PAGE (10%) and subsequently transferred to PVDF membranes (Millipore, USA). The membranes were then blocked with 5% skim milk for one h at room temperature. Subsequently, the membranes were incubated with primary anti-SLC7A11 (1:100, Abcam, USA) or anti-GAPDH (1:1000, Affinity, USA) antibody. HRP-labelled secondary antibody (CST) was used and detected by chemiluminescence.

Assessment of GSH/GSSG ratio

GSH/GSSG Quantification Kit II (Dojindo, Shanghai, China) was used to measure the intracellular ratio of glutathione (GSH) and oxidized glutathione (GSSG) levels. Sample preparation, standard solution and detection of concentration were performed according to the manufacturer's protocols. The relative levels were calculated on a microplate reader (Molecular Devices Flexstation3, USA).

Results

Circ0097009 is up-regulated in HCC cell lines and tissues

CircRNA microarray analyses on three pairs of HCC tissues (T) and matched para-carcinoma normal tissues (N) showed that 6024 circRNAs downregulated with fold change greater than 2, $P < 0.05$ in HCC tissue, while 10,720 circRNAs up-regulated by the same cutoff, as we previously described [17]. Among the top five up-regulated circRNAs, we analyzed the expression level of circ0097009 in three HCC cell lines. It was found that circ0097009 was up-regulated in HCC cells (Fig. 1A) and tissues (Fig. 1B). Then, an RNase R digestion experiment was performed to confirm the circular characteristics of circ0097009 (Fig. 1C). Besides, an actinomycin D assay was carried out, suggesting that the circular transcript circ0097009 was more stable than the linear transcript SLC5A8 in 97H cells (Fig. 1D).

Knockdown of circ0097009 inhibits the tumour growth in HCC

To evaluate the biological functions of circ0097009 in HCC, we used RNA interference to knock down the expression of circ0097009. The qRT-PCR analysis showed that the inhibition was successful (Fig. 2A). CCK-8 assay and colony formation assay revealed that downregulation of circ0097009 significantly suppressed cell growth (Fig. 2B and Fig. 2C). Then xenograft experiments were carried out to investigate the function of circ0097009 in tumour growth in vivo. We observed that inhibition of circ0097009 significantly suppressed tumour growth (Fig. 2D).

Downregulation of circ0097009 inhibits the invasion and metastasis of HCC

To assess the role of circ0097009 in the invasion of HCC cells, we conducted a transwell assay. It was found that silencing the expression of circ0097009 could significantly impair the capacity of invasion in HepG2, 7402 and 97H cells (Fig. 3A). To further investigate the function of circ0097009 in tumour invasion and metastasis in vivo, xenograft models were established, and the result showed that inhibition of circ0097009 could reduce the number of lung metastases (Fig. 3B), indicating that circ0097009 play an important role in the metastatic ability of HCC.

Circ0097009 functions as a sponge of miR-1261

CircRNAs may function as a ceRNA to regulate miRNAs, leading to the liberation of corresponding miRNA-targeted genes. Therefore, we explored whether circ0097009 could act as a miRNA sponge and predicted the potential molecular target of circ0097009 based on the circRNA interactome (<https://circinteractome.nia.nih.gov>). We found that miR-1261 has potential binding site within the circ0097009 sequence (Fig. 4A). qRT-PCR showed that miR-1261 was downregulated in HCC cell lines (Fig. 4B). Subsequently, we performed luciferase reporter assay and found that after co-transfection with miR-1261 mimics, the luciferase activity was inhibited; however, for the mutant luciferase reporter, there was no such effect (Fig. 4C). To further validate the direct binding between circ0097009 and miR-1261, MS2bp-MS2bs based RIP assay was conducted, and the results suggested that miR-1261 was primarily enriched in the MS2bs-circ0097009 group (Fig. 4D), indicating that a direct interaction between circ0097009 and miR-1261. Therefore, our data confirmed that circ0097009 functionally interacts with miR-1261 and serves as a sponge for miR-1261 in HCC.

Knockdown of circ0097009 promotes ferroptosis in HCC

To further predict potential downstream targets of miR-1261, we used the TargetScan algorithm and SLC7A11 was identified as the candidate target oncogene (Fig. 5A). To validate the predicted targeted interaction, the expression levels of SLC7A11 was detected in HCC cell lines and found to be up-regulated (Fig. 5B). Subsequently, to confirm whether miR-1261 could directly bind to SLC7A11 mRNA, we performed a RIP assay and found that the luciferase activity decreased after transfection with the wild-type luciferase reporter of SLC7A11 and miR-1261 mimics, whereas no such effect was observed in the mutant luciferase reporter of SLC7A11 (Fig. 5C). Moreover, miR-1261 mimics suppressed the expression of SLC7A11 in HepG2, 7402 and 97H cell lines, suggesting that SLC7A11 is regulated by miR-1261 (Fig. 5D). Western blotting showed that with the knockdown of circ0097009 and miR-1261, SLC7A11 expression was downregulated in HepG2, 7402 and 97H cell lines (Fig. 5E). Additionally, RIP assays on Ago2 were conducted, and the results revealed that circ0097009, SLC7A11 and miR-1261 were primarily enriched in the Ago2 complex; moreover, inhibition of circ0097009 dramatically increased SLC7A11 enrichment in the Ago2 fraction and reduced circ0097009 enrichment in Ago2 complexes (Fig. 5F), indicating that circ0097009 competes with SLC7A11 for binding to miR-1261. H&E-stained sections of dissected xenograft tumours also confirmed that knockdown of circ0097009 decreased the expression levels of SLC7A11 (Fig. 5H). Interestingly, SLC7A11, a consist of system Xc⁻, is identified as a key regulator in cancer cell ferroptosis [22]. Based on the evidence above, it was concluded that knockdown of circ0097009 promotes ferroptosis via circ0097009/miR-1261/SLC7A11 axis. Moreover, we found that knockdown of circ0097009 lead to a decrease in the GSH/GSSG ratio and deletion of glutathione peroxidase 4 (GPX4) (Fig. 5G). The reduction in the GSH/GSSG ratio causes the deactivation of GPX4, which is another key regulator in ferroptosis and reduce lipid peroxides [23]. Therefore, knockdown of circ0097009 promotes ferroptosis in HCC might go via two independent pathways system Xc⁻ and GPX4.

Discussion

A variety of miRNAs and lncRNAs have been reported to regulate HCC progression. However, currently, few studies are investigating the role of circRNAs in HCC, a family of non-coding RNAs with covalently closed, single-stranded transcripts [19]. In this study, we identified the expression profile of circRNAs in HCC and found that circ0097009 was up-regulated in HCC tissues and cell lines. A series of experiments *in vitro* and *in vivo* showed that knockdown of circ0097009 inhibited cell growth and invasion in HCC. These results revealed that circ0097009 plays a vital role in HCC progression, indicating that it might serve as a diagnostic biomarker and therapeutic target for HCC.

Recently, numerous studies revealed that pseudogenes, lncRNAs (long non-coding RNAs) and circRNAs could interact with or coregulate each other by competing for miRNA binding [14, 24], which was called ceRNA mechanism. CircRNAs have been shown to act as ceRNA by sponging to miRNA to regulate gene expression in cancer [14, 25]. Here, we performed bioinformatics analysis, and the outcomes showed that circ0097009 contained an MRE of miR-1261. Functional assays were also conducted to confirm the direct relationship between circ0097009 and miR-1261. Previous studies found that miR-1261 play a regulatory role in a variety of cancers. He et al. first report that miR-1261 promote invasion and migration of prostate cancer cells by binding to lncRNA prostate cancer antigen 3 [26]. MiR-1261 has been demonstrated to regulate the development of papillary thyroid cancer and glioma [27, 28]. In liver cancer, Zhang et al. found that miR-1261 acts as a sponge for lncRNA BCAR4 to promote tumour progression by up-regulating ANAPC11 [29]. In this study, we found that miR-1261 could target both circ0097009 and SLC7A11, suggesting that circ0097009 might function as a miR-1261 sponge to regulate SLC7A11 expression through ceRNA mechanism. There are several lines of evidence implicating that circ0097009 functions as a ceRNA to SLC7A11 in HCC as a sponge of miR-1261. First, as mentioned above, bioinformatics analyses showed that the 3'UTR of SLC7A11 and circ0097009 contain binding sites for miR-1261. Second, luciferase reporter assays and MS2bp-MS2bs based RIP assay verified this prediction. Third, knockdown of circ0097009 reduced SLC7A11 expression. Finally, inhibition of miR-1261 reversed the effect of circ0097009 knockdown. All these findings reveal that circ0097009 and SLC7A11 are ceRNAs regulated by miR-1261.

Ferroptosis is a different form of programmed cell death from apoptosis, necroptosis and autophagy [30]. Iron accumulation and lipid peroxidation lead to accumulation of reactive oxygen species (ROS), which is the key characteristic of ferroptosis [31]. In recent years, inducing ferroptosis has been regarded as a promising therapeutic approach for cancer [32, 33]. The SLC7A11 and solute carrier family 3 member 2 (SLC3A2) are the two key members of System Xc⁻, which is a heterodimeric cystine/glutamate antiporter and the upstream molecule in the process of ferroptosis. As the key regulator in cancer cell ferroptosis, SLC7A11 could be regulated at the transcriptional level, and a reduction SLC7A11 would induce ferroptosis [34]. Our findings showed that inhibition of circ0097009 decreased the expression levels of SLC7A11, suggesting that circ0097009 involved in the progression of HCC may respond via the mechanism of ferroptosis by regulating SLC7A11.

Moreover, we also found that knockdown of circ0097009 leads to a decrease in the GSH/GSSG ratio and deletion of GPX4. GPX4 is an antioxidant enzyme and another key regulator of ferroptosis [23]. It is

reported that overexpression of GPX4 cause resistance to ROS-induced tumour cell death and silencing GPX4 sensitizes tumour cells to ROS-induced cell death [35]. Therefore, based on our findings above, we concluded that circ0097009 promotes ferroptosis in HCC might go via two independent pathways system Xc- and GPX4.

Conclusion

The study proved that circ0097009 is up-regulated in HCC and knockdown of circ0097009 inhibits HCC growth and invasion. Moreover, circ0097009 functions as a ceRNA to regulate SLC7A11 expression by sponging to miR-1261. circ0097009/miR-1261/SLC7A11 axis mediates HCC progression through regulating ferroptosis. So, circ0097009 may serve as a diagnostic biomarker and a potential target for HCC therapy.

List Of Abbreviations

- ATCC, American Type Culture Collection
- circRNAs, circular RNAs
- ceRNAs, competitive endogenous RNAs
- GPX4, glutathione peroxidase 4
- GSH, glutathione
- GSSG, oxidized glutathione
- HCC, hepatocellular carcinoma
- qRT-PCR, quantitative RT-PCR
- RIP, RNA immunoprecipitation
- ROS, reactive oxygen species
- SLC3A2, solute carrier family 3 member 2
- SLC7A11, solute carrier family 7 member 11

Declarations

- **Ethics approval and consent to participate**

This study was approved by the Ethics Committee of Sun Yat-Sen University Cancer Center Health Authority and was performed according to the ethical standards of the Declaration of Helsinki. Patients' consents were obtained before the study conduct. All animal studies were approved and performed according to the guidelines of the IACUC of Sun Yat-Sen University Cancer Center.

- **Consent for publication**

The consent for publication was obtained by all authors.

- **Availability of data and materials**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

- **Competing interests**

The authors declare that there is no conflict of interest.

- **Funding**

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- **Authors' contributions**

NL, HLT and MZ designed the experiments. NL, YZ and YNK conducted the experiments. NL, YZ and YNK analyzed and interpreted the data. NL, YZ and YNK were the main contributors in writing the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Circ0097009 is up-regulated in HCC cell lines and tissues (A) The expression level of circ0097009 in normal and HCC cell lines. (B) The expression level of circ0097009 expression level in HCC tissues and matched normal tissues. (C) RNase R digestion experiment was performed to confirm the circular characteristics of circ0097009. (D) Stability of the circular transcript circ0097009 and linear transcript SLC5A8 (SLC711A) in 97h cells assessed by actinomycin D assays.

Figure 2

Knockdown of circ0097009 inhibits the tumour growth in HCC (A) Si-circ0097009 successfully knocked down circ0097009. (B) Cell proliferation was evaluated by CCK-8 assay in HepG2,7402, 97H cell lines. (c) Colony formation number in HepG2,7402, 97H cell lines. (D) Tumour volume was measured every 4 days for 4 weeks. (E) The expression status of Ki67 in H&E stained sections of dissected xenograft tumours.

Figure 3

Downregulation of circ0097009 inhibits the invasion and metastasis of HCC (A) Transwell assay was performed to assess the invasive ability of HCC cells. (B) The number of metastatic lung nodules was quantified, and H&E-stained sections of metastatic lung nodule were showed.

Figure 4

Circ0097009 functions as a sponge of miR-1261 (A) The predicted direct binding sites of miR-1261 within the circ0097009 sequence. (B) The expression level of miR-1261 in the LO2 cell line and HCC cell lines. (C) The relative luciferase activity of HepG2 cells co-transfected with the miR-1261 mimics and the wild-type or mutant luciferase reporter of circ0097009. (D) MS2-based RIP assay in HepG2 cells transfected with MS2bs-circ0097009, MS2bs-circ0097009-mt or MS2bs-Rluc.

Figure 5

Knockdown of circ0097009 promotes ferroptosis in HCC (A) The predicted direct binding sites of miR-1261 within the SLC7A11 sequence. (B) The expression level of SLC7A11 in the LO2 cell line and HCC cell lines. (C) The relative luciferase activity of HepG2 cells co-transfected with miR-1261 mimics and the wild-type or mutant luciferase reporter of SLC7A11. (D) The expression level of SLC7A11 after transfection with miR-1261 mimics. (E) Western blotting was performed to detect the expression levels of SLC7A11, GPX4, and GADPH. (F) RIP assay detected the relative enrichment of circ0097009, SLC7A11, and miR-1261 in the Ago2 fraction (left); the relative enrichment was detected by an Ago2-related RIP assay (right). (G) The expression status of SLC7A11 in H&E stained sections of dissected xenograft tumours. (H) GSH/GSSG ratio was detected by the GSH/GSSG Quantification Kit.