Kalifu *et al. Egyptian Liver Journal* (2024) 14:44
https://doi.org/10.1186/s43066-024-00345-4

Egyptian Liver Journal

ORIGINAL RESEARCH ARTICLE

Open Access

Silencing circ-RNA-049637 influences hydatid outer cyst wall formation by liver fibrosis

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Abstract

Introduction and objectives In diseases characterized by fibrosis, hepatic stellate cells (HSCs) are mesenchymal cells that play an important role in liver fibrosis. circRNAs are involved in regulating hydatid exocyst formation through miRNA sponge adsorption. The mechanisms of hepatic cystic hydatid outer cyst formation, HSC, and liver fibrosis are unclear.

Materials and methods Based on our sequencing data, we validated the mechanism by which circRNA-049637 regulated hepatic cystic hydatid growth and promoted outer fibrocystic wall formation.

Results Our results revealed that circRNA_049637 silencing promoted the proliferation of LX-2 human HSCs, affected the cell cycle, and increased the mRNA and protein expression levels of liver fibrosis-related indicators such as α-SMA, COL1A1, COL3A1, and TGFβRII.

Conclusions CircRNA_049637 may induce the formation of hepatic hydatid cysts by promoting hepatic fibrosis via HSC activation.

Keywords Hepatic cystic hydatid, Liver fibrosis, LX-2, circRNA-049637

Introduction

Hepatic cystic hydatid disease is the formation of parasitic cystic lesions caused by *Echinococcus granulosus* invading the liver through the portal system [1]. Chronic damage to the liver by *E. granulosa* is caused by various factors, including direct erosive stimulation, mechanical compression, and toxic injury, eventually leading to liver fibrosis and the formation of a fibrous outer capsule [2]. Chronic liver disease progressively advances from a persistent inflammatory response to liver fibrosis, which is a highly integrated and dynamic aggregation of molecules, cells, and tissues. It has been reported that parasitic infection can stimulate and activate hepatic stellate cells (HSCs), resulting in the massive formation of collagen fibers and excessive deposition of extracellular matrix (ECM). This process may be affected by certain cytokines [3–5]. It has also been reported that HSCs are activated and metastasize when hepatic parenchymal cells are damaged, resulting in the deposition of ECM components [6, 7]. When HSCs are continuously activated, they cause cell proliferation and contraction, matrix degradation, fiber formation, production of inflammatory factors and cytokines, chemotaxis, and reduction of retinol, resulting in excessive deposition of ECM, which in turn leads to liver fibrosis [8, 9]. Hence, to treat liver fibrosis, it is crucial to explore the molecular mechanisms underlying HSC changes.

CircRNAs are a type of non-coding RNA that express a wide range of closed circular structures in living organisms [10]. Some circRNA molecules can use the corpus cavernosum to competitively bind to miRNA, thereby

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regulating the transcription and expression of target genes. In recent years, in-depth research on non-coding RNAs has been carried out to verify that circRNAs play a very important role in the progression of many diseases, such as preeclampsia [11], osteoarthritis [12], arteriosclerosis [11, 13], and other diseases, and various malignant tumors have widely abnormally expressed circRNAs in the blood, tissues, or cells. Recently, its role in liver fibrosis has become a popular research topic [14]. Although there are many basic studies on liver diseases, micro miRNAs are widely known; however, as a type of non-coding RNA, miRNAs account for a small part, and much of the information is on lncRNAs and circRNAs [15]. At present, relevant studies have shown that liver fibrosis is the key link in the formation of hepatic hydatid [16]; however, there are no reports on how circRNA regulates the growth of hepatic cystic hydatid and promotes the formation of the outer fibrous cyst wall.

Our previous omics results suggested that circRNA_049637 was significantly differentially expressed in hepatic cystic hydatid fibrous outer cyst tissues and normal liver tissues [17]. Therefore, we conducted a preliminary cell function study on circRNA_049637, analyzed the role of stellate cells in liver fibrosis, and detected changes in liver fibrosis-related indicators to explore related regulatory mechanisms.

Materials and methods

Cell culture and transfection

LX2 human HSCs were purchased from Yagi Bio Company, and the cell culture conditions were 90% DMEM + 10% FBS, 37 °C, 5% CO_2, and saturated humidity.

To determine the appropriate infection conditions, the preliminary experiments were divided into four groups. Group M (normal culture medium, the changes that occurred under normal culture conditions when cells were infected with virus), Group A (normal medium combined with HiTransG A group), Group P (normal medium combined with HiTransG P group), and control. The cell suspension at a density of 5×10^4 cells per mL was placed in a 96-well plate and cultured at 37 °C for 24 h until the cell confluence reached approximately 30%. The virus was diluted to three different titers; namely, 1×10^8 TU, 1×10^7 TU, and 1×10^6 TU per mL. The virus and the corresponding infection enhancer were mixed uniformly, and the conventional medium was changed after 12 h. The appropriate titer was determined based on the transfection efficiency 3 days after infection.

To verify the effect of lentivirus silencing, MOI = 10 and infection time 72 h were selected and divided into a control group, negative control (NC) group, LV-hsa_circ_0049637-1,2, and 3 groups (infected with LV-hsa_circ_0049637-shRNA, shRNA2, and shRNA3).

Cell counting kit-8 assay

There were five groups of cells, including the control, NC, and LV-hsa_circRNA_0049637-shRNA1 groups (optimal MOI = 100, A co-infection solution, infection time 72 h). After the intervention of five duplicate wells in each group, 10 μL of the medium and CCK-8 solution were added to each well. After incubation for 2 h, the optical density (OD) at 450 nm was measured using a microplate reader.

Flow cytometry

The experimental groups were the same as those previously described. After the intervention, the cells were washed with PBS and resuspended. Then, the cells were incubated at 4 °C for 30 min in the dark. To detect the red fluorescence of the cells, the excitation wavelength of the flow cytometer was set to 488 nm.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from cells using the TRIzol kit (Invitrogen). RNA reverse transcriptase M-MLV was used to reverse-transcribe RNA to cDNA. RT-qPCR was performed using the SYBR Green method. The conditions of the 20 μL reaction system were pre-denaturation at 95 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, and extension at 70 °C for 1 min, with a total of 32 cycles, and an extension of 20 min at 72 °C. The $2^{-\Delta\Delta Ct}$ method was used for the data analysis. Table 1 shows the primer information.

Western blot

We separated the proteins using SDS-PAGE. The separated proteins were then transferred to PVDF membranes using the semi-dry method at 80 mV for 30–45 min. Primary antibodies were added to α-smooth muscle actin (α-SMA, 1:1000, ab108424, Abcam), COL1A1 (1:1000, ab254341, Abcam), COL3A1 (1:1000, ab4717, Abcam), transforming growth factor β TGF-βRII (1:1000, ab75602, Abcam), and β-actin (1:5000, ab8227, Abcam) overnight at 4 °C after blocking with 5% bovine serum albumin for 1 h. After three washes in TBST (5 min each), the corresponding secondary antibodies (Beyotime, Shanghai,

Table 1 Primer information table for fluorescence quantitative detection

Name	Sequence (5′ to 3′)	Size bp
hsa_circRNA_049637 F1	ATTAAATTTTGTGTCTCCGCG	68
hsa_circRNA_049637 R1	CCTTTAAAACGACCCTCCG	

China) were added and incubated for 1 h. Later, the membrane was rinsed three times (5 min each). A chemiluminescence reagent was added for color development. Protein bands were assessed using the Bio-Rad Gel Dol EZ Imager (Hercules, CA, USA) and Image J software (NIH, Bethesda, Maryland, USA) to analyze the protein bands and gray values, respectively.

Statistical analysis

SPSS 21.0 (IBM Corp., Armonk, New York, USA) and GraphPad Prism 8.0.1 were used to process all data in this study. Mean ± standard deviation was used to show the measured data. The *t*-test was used to compare the two groups. One-way analysis of variance (ANOVA) or two-way analysis of variance was used to analyze the data with $p < 0.05$ set as the threshold of significant difference.

Results

Construction and validation of hsa_circRNA_0049637 gene-silencing cell line

Based on the basic results of previous research (https://doi.org/10.1002/jcla.23687.), hsa_circRNA_049637 has further research potential. The hydatid outer cyst tissue and normal liver tissue of patients who underwent hepatic cystic hydatid surgery were collected for PCR verification ($n = 21$). The results suggested that, compared with normal tissue, hsa_circRNA_049637 in the outer cyst tissue of hepatic hydatids showed a decreasing trend (Fig. 1A, $p = 0.0026$). The cck-8 results of human HSCs (LX2) showed that the cells grew well, and the cells that entered the log phase after 3 days of culture were selected for subsequent experiments (Fig. 1B).

The virus was then transfected according to different gradient MOI values (1, 10, and 100). Three days later, the fluorescence distribution of the cells in the different groups was observed using a fluorescence microscope (Fig. 1C). Figure 1C shows that, compared with the control group, when the MOI value is 100, the infection efficiency can exceed 80% and can be used as an ideal experimental titer. Five groups of cell circRNAs were verified by PCR (control, LV-shRNA-NC, LV-shRNA-circ_049637-1, LV-shRNA-circ_049637-2, and LV-shRNA-circ_049637-3 groups). PCR results revealed that the expression of circ-04963–3 in the circ-049637 group was significantly decreased ($P < 0.05$, Fig. 1D) compared with that in the control and LV-shRNA-NC groups, indicating that the LX2 cell line with silenced circ_049637 was successfully established.

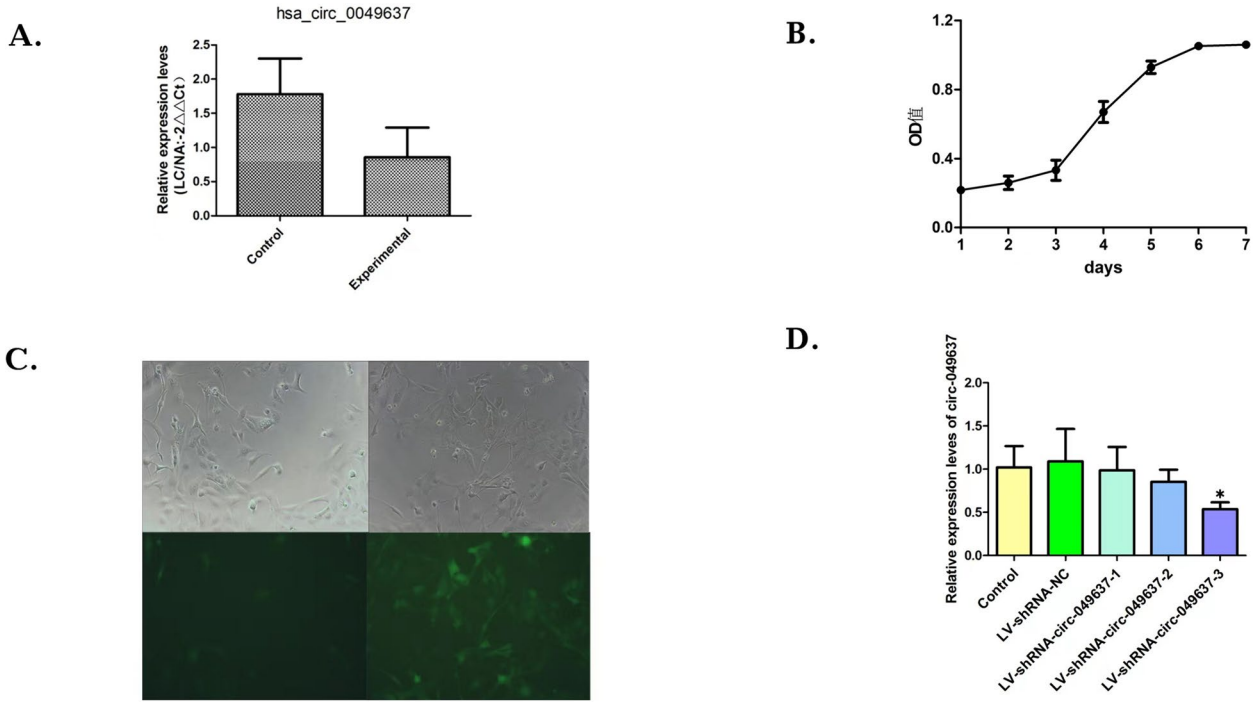

Fig. 1 Construction and validation of hsa_circRNA_0049637 gene silencing cell line. **A** Relative expression of circRNA_049637 in cystic echinococcosis pericystic tissue and normal liver tissue. **B** LX2 cell growth curve. **C** Transfection effects of different MOI and enhancement fluid. **D** Histogram of circRNA-049637 gene expression in each group

Effects of silencing hsa_circRNA_0049637 gene on cell proliferation and cycle

The proliferation ability of LX2 cells treated with LV-shRNA was detected using CCK-8. The CCK-8 assay revealed that LX2 cells proliferated significantly after circRNA-049637 was silenced compared with the control and LV-shRNA-NC groups ($P<0.05$, Fig. 2A), indicating that low expression of circRNA-049637 promoted the proliferation of LX2 cells.

Because there was no difference between the control and LV-shRNA-NC groups in the proportion of cell growth in different cycles, it indicates that the lentiviral vector does not cause cell cycle changes. This result revealed that the main reason for the cycle changes was the silencing of circRNA_049637. Flow cytometry results revealed that compared to the control group, the ratio of G0/G1 phase in the LV-shRNA-circ_049637 group decreased significantly from 60.9 to 45.15%, and the ratio in the S phase increased from 23.9 to 38.4%, indicating that silencing circRNA_049637 accelerates cell replication, and a DNA-doubling to DNA-doubling process causes HSC activation and fibrosis (Fig. 2B).

qRT-PCR validation of silencing hsa_circRNA_0049637 gene on liver fibrosis

To evaluate whether excessive deposition or fibrosis occurred in the ECM, we determined the gene and protein levels of COL3A1, COL1A1, α-SMA, and TGF-β. RT-qPCR results revealed that the expression of α-SMA ($P<0.05$), COL3A1 ($P<0.05$), COL1A1 ($P<0.05$), and TGF-βRII ($P<0.05$) mRNA was significantly increased in the circRNA-049637-silenced group compared with the control group. Compared with LV-shRNA-NC, α-SMA ($P<0.05$), COL3A1 ($P<0.05$), and TGF-βRII mRNA expression ($P<0.05$) were significantly increased. COL3A1 expression was not significantly different between the two groups ($P>0.05$) (Fig. 3A–D).

Western blot validation of silencing hsa_circRNA_0049637 gene on liver fibrosis

To further verify the above results, Western blot (WB) results revealed that the protein expression of α-SMA, COL1A1, COL3A1, and TGF-βRII in the circRNA-049637-silenced group were significantly increased and had statistical differences compared to the NC and blank control groups. The results of WB and PCR were consistent (Fig. 4A–E).

Discussion

In diseases characterized by fibrosis, HSCs are mesenchymal cells that play an important role in liver fibrosis [9, 18]. After HSC activation, it transforms into fibroblasts and strongly expresses α-smooth muscle actin (α-SMA), thereby promoting the deposition of ECM substances, including collagen (COL) 1A1, COL3A1, fn1a (fibrils), Laminin 1a, and laminin at the site of injury [19]. Fibrosis process regulation is achieved by the secretion of active liver fibrosis mediators, mainly TGF-β, platelet-derived growth factor, and connective tissue growth factor [20, 21]. The activation behavior of HSCs induced by persistent stimulation is generally possible through seven changes: chemotaxis, increased reproduction, contraction, fibrosis, cytokine secretion, interstitial degradation, and loss of vitamin A [22]. Research on the relationship

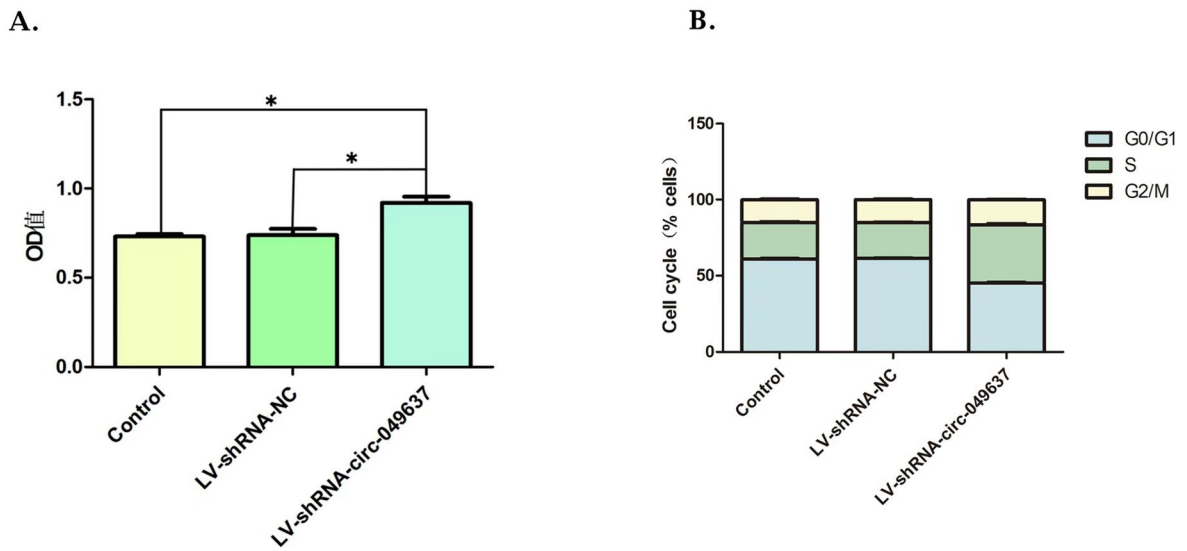

Fig. 2 Effects of silencing hsa_circRNA_0049637 gene on cell proliferation and cycle. **A** Histogram of cell proliferation. **B** Histogram of cell cycle

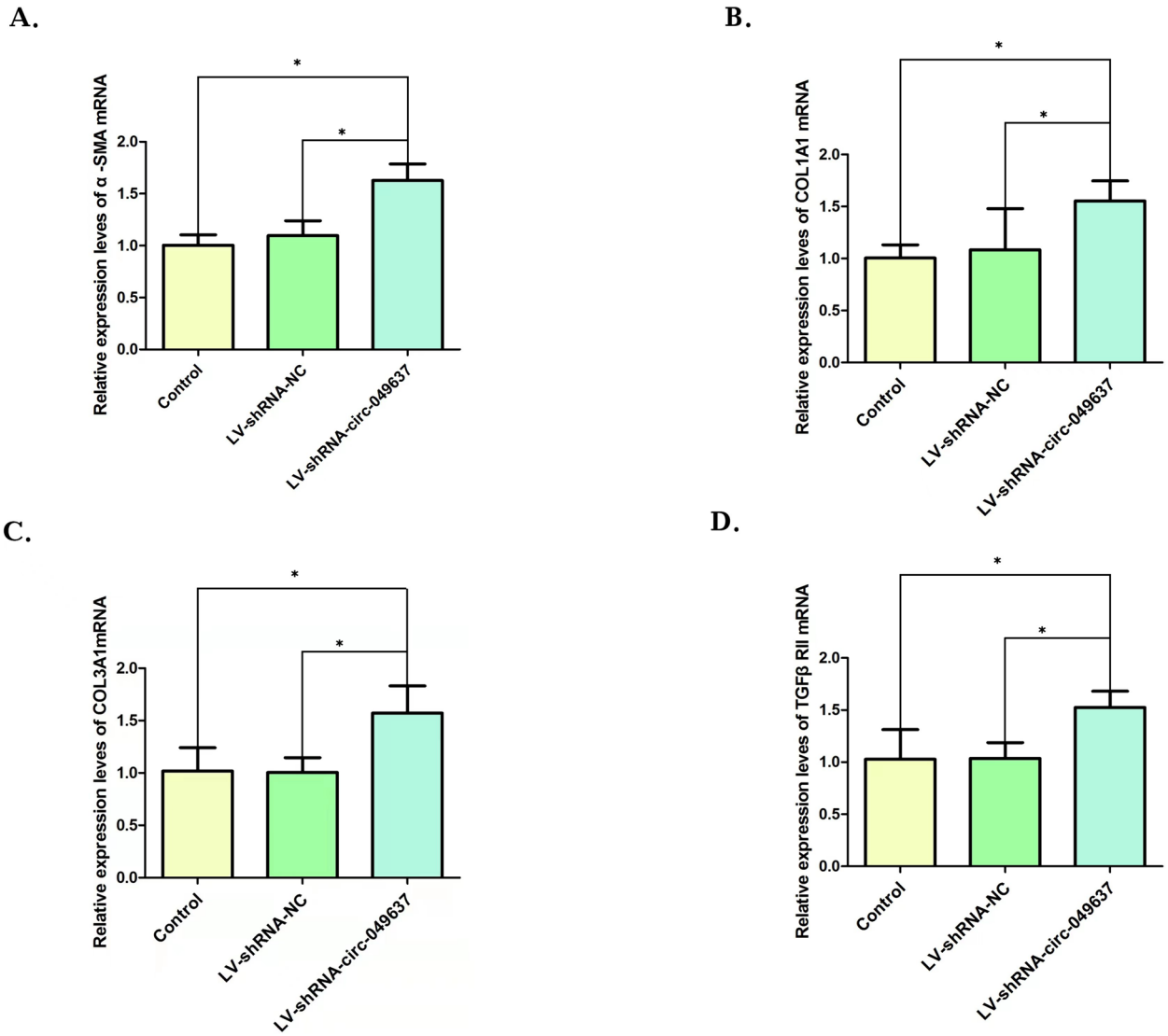

Fig. 3 qRT-PCR validation of silencing hsa_circRNA_0049637 gene on liver fibrosis. **A** Level of α-SMA. **B** Level of COL1A1. **C** Level of COL3A1. **D** Level of TGF-β

between the formation of hepatic cystic hydatid outer cysts and HSC and liver fibrosis may be an important breakthrough point. The liver stellate cell line required for the experiment was obtained from Professor Xu Lieming of Shanghai University of Traditional Chinese Medicine, and it is the most used cell line in current liver research. Therefore, we selected LX-2 cells for this part of the cell experiment.

Because circRNAs participate in regulation through miRNA spongy adsorption [23], we can speculate that circRNAs regulate the formation of hydatid exocysts through their effects on stellate cells. In this study, the proliferation rate of LX2 human HSCs was significantly increased after circRNA_049637 silencing, indicating that low expression of circRNA_049637 may promote the activation of HSCs. The results of flow cytometry showed that the LV-shRNA-circ_049637 group was compared with the control group. The ratio of G0/G1 and S phases was significantly decreased and increased, respectively, indicating that silencing circRNA_049637 accelerated cell replication, doubling the DNA to double the DNA process, causing HSC activation and fibrosis. During liver fibrosis, the activation of HSCs and the promotion of ECM production are key components [24, 25]. Detection of ECM proteins in HSCs affected by the target RNA is an important means to infer the effect of target RNA on HSCs; therefore, we explored ECM-related mRNA and protein expression.

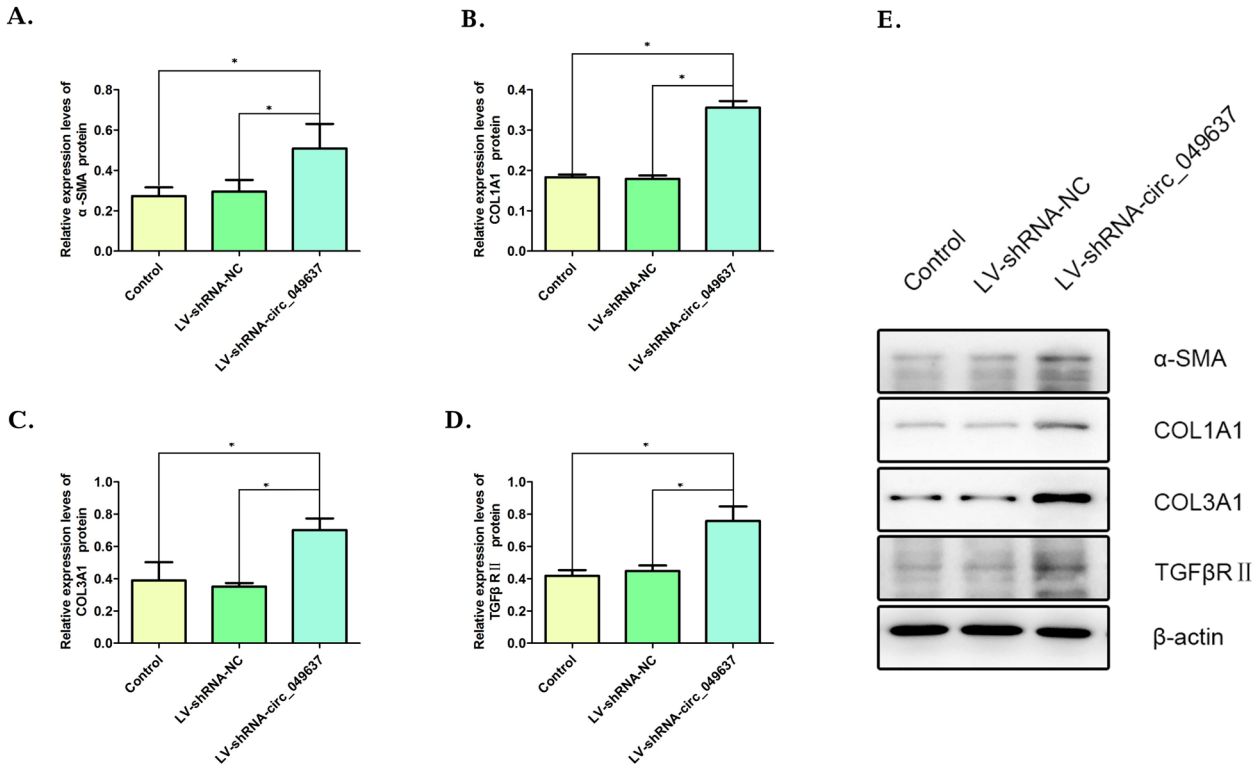

Fig. 4 Expression level analysis of proteins in different groups. **A** Level of α-SMA. **B** Level of COL1A1. **C** Level of COL3A1. **D** Level of TGF-β. **E** Expression levels of α-SMA, COL1A1, COL3A1, and TGF-β were analyzed by Western blotting

In this study, the expression of COL1A1 and COL3A1 in circRNA_049637-silenced LX2 cells was analyzed to investigate the role of circular RNA in the formation of hepatic hydatid cysts. The results revealed that both COL1A1 and COL3A1 were highly expressed in circRNA_049637-silenced cells, suggesting that circRNA_049637 participates in ECM formation.

Moreover, TGF-β is an important cytokine that promotes fibrosis, stimulates and induces T cells, and regulates their levels [26]. Furthermore, this cytokine plays a role in reducing cytotoxic effector immune responses and balancing immunogenicity in chronic diseases and various physiological conditions. This multifunctional cytokine plays a central role in tissue recovery and fibrogenesis [27], and existing research suggests that inhibition of TGF-β receptor expression, especially TGFβRII, can interfere with TGF-β/Smad signaling [28]. Signal transduction inhibits HSC activation or collagen deposition; therefore, detecting the expression of TβRII is an important indicator for studying the regulation of liver fibrosis [29]. In this study, we examined the expression of TβRII in LX2 cells after circ_049637 silencing. Expression levels have a negative regulatory effect. It is further speculated that the expression level of TβRII increases after the low expression of circRNA_049637, and the TGF-β/Smad pathway is reduced and inhibited, resulting in accelerated liver fibrosis and activation of HSCs.

Based on the sequencing results, we selected circRNA_049637 for preliminary functional exploration, selected HSCs closely related to hepatic fibrosis as transfected cells, and explored the imaging of LX human HSCs and the detection of hepatic fibrosis-related factors by circRNA_049637 to provide preliminary evidence of the formation mechanism of 049637 in the fibrous outer cyst wall of hepatic hydatids. This study has various shortcomings, and further research is needed on its mechanism. In follow-up work, we plan to further explore the expression of downstream ceRNAs and miRNAs and analyze related signaling pathways to reveal a clearer and more complete mechanism.

Authors' contributions

XC conceived and supervised the study. BK, YM, and Z-GM designed the study and prepared the manuscript. G-LT and J-GW carried out data analysis and performed the experiments. CM made manuscript revisions.

Funding

This work was supported by grants from the Regional Collaborative Innovation Special Project of Xinjiang Autonomous Region, China (Grant No.2022E02133).

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants and animals, and ethical approval is not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 17 August 2023 Accepted: 18 May 2024
Published online: 10 June 2024

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