

ORIGINAL ARTICLE

Effects of CircRNA-ITCH on proliferation and apoptosis of hepatocellular carcinoma cells through inhibiting Wnt/ β -catenin signaling pathway

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Summary

Purpose: To explore the effects of circular ribonucleic acid (circRNA)-E3 ubiquitin protein ligase (ITCH) on the proliferation and apoptosis of hepatocellular carcinoma (HCC) cells and its possible molecular mechanism.

Methods: To study the role of circRNA-ITCH in the occurrence and development of HCC. The relative expression level of circRNA-ITCH was detected via quantitative polymerase chain reaction (qPCR) in four kinds of HCC Huh-7, U251, HB611 and SMMC-7721 cell lines, and human normal liver L-02 cell line. Moreover, the effect of overexpression of circRNA-ITCH on the TCF luciferase activity was detected using β -catenin/TCF-responsive luciferase reporter assay. Finally, the influences of overexpression of circRNA-ITCH on the protein levels of β -catenin and Wnt3a and the mRNA levels of *c-myc* and *cyclinD1* were determined using Western blotting and qPCR, respectively.

Results: Compared with that in human normal liver L-02 cell line, the expression of circRNA-ITCH was significantly

down-regulated in HCC Huh-7, U251, HB611 and SMMC-7721 cell lines ($p < 0.05$). According to the results of CCK-8 assay, colony formation assay and flow cytometry, the overexpression of circRNA-ITCH could obviously inhibit cell proliferation, suppress colony formation ability and induce apoptosis ($p < 0.05$). The results of dual-luciferase reporter assay revealed that there was a significant interaction between circRNA-ITCH and miR-7 or miR-214 ($p < 0.05$). CircRNA-ITCH was involved in the regulating of Wnt/ β -catenin signal transduction pathway and inhibited the expressions of *c-myc* and *cyclinD1*.

Conclusions: CircRNA-ITCH affects the proliferation and apoptosis of HCC cells through inhibiting the Wnt/ β -catenin signal transduction pathway, thereby exerting a carcinogenic effect in the occurrence and development of HCC. The research results provide a new therapeutic target for HCC.

Key words: hepatocellular carcinoma, circRNA-ITCH, Wnt/ β -catenin, proliferation, apoptosis

Introduction

Hepatocellular carcinoma (HCC) is the most common subtype of primary liver cancer, and its mortality rate ranks 2nd among cancer-related deaths in the world [1,2]. According to cancer statistics in China, there are about 466,100 newly-diagnosed cases and 422,100 deaths of HCC every year [3]. The important features of HCC are inva-

siveness, poor prognosis and limited treatment options. Currently, surgery is still the most common treatment means for HCC, but most HCC cases cannot be treated by surgical treatment due to multifocal development and distant metastasis [4]. Although new diagnostic and therapeutic strategies have been developed, the prognosis of HCC patients

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remains poor [5]. Therefore, it is imperative to explore the molecular mechanism of the occurrence and development of HCC for identifying potential novel therapeutic targets.

Circular ribonucleic acids (circRNAs) are a type of RNAs with a closed-loop structure, different from traditional linear RNAs. So far, exploring the function of circRNAs has become a global hotspot. Previous studies have shown that circRNAs can act as the miRNA sponge and transcriptional regulator. In addition, they can be translated into proteins or interact with RNA-binding proteins [6]. As a new subgroup of non-coding transcripts, circRNAs are widely expressed in mammalian cells, which can regulate gene expression at the transcriptional or post-transcriptional level. According to previous studies, circRNAs can act as oncogenes or tumor suppressors in human cancers [7-9]. Li et al [7] found that circHIAT1 serves as a suppressor in clear cell renal cell carcinoma through inhibiting metastasis. It is reported that some circRNAs have biological functions. DLGAP4 is directly associated with early-onset cerebellar ataxia, and circDLGAP4 can be used as a new clinical therapeutic target for acute ischemic stroke [10,11]. In terms of the mechanism, circRNAs can regulate the mRNA expression through miRNA sponge, thereby regulating the occurrence and development of tumors [12,13].

The abnormal activation of the Wnt/ β -catenin pathway plays a key role in the occurrence, development and metastasis of HCC [14]. E3 ubiquitin protein ligase (ITCH) inhibits the Wnt/ β -catenin signal transduction in cancer through promoting the ubiquitination and degradation of phosphorylated Dsh homolog 2 (Dvl2) [15]. CircRNA-ITCH shares some miRNA binding sites with the ITCH 3'-untranslated region (UTR). As the miRNA sponge, circRNA-ITCH increases the level of ITCH, thereby indirectly inhibiting the activation of the Wnt/ β -catenin pathway. These effects ultimately lead to growth inhibition on tumor cells. However, the function and role of circRNA-ITCH in HCC have not been reported yet. In this paper, therefore, the biological functions of circRNA-ITCH in HCC and its potential mechanism were mainly explored.

Methods

Materials

Human HCC Huh-7, U251, HB611 and SMMC-7721 cell lines and human normal liver L-02 cell line (Cell Bank of Chinese Academy of Sciences, Shanghai, China), high-glucose Dulbecco's modified Eagle medium (DMEM) (HyClone, South Logan, UT, USA), fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), psiCHECK-2 plasmids and pcDNA3.1(+) plasmids (preserved by our

laboratory), TRIzol reagent (Invitrogen, Carlsbad, CA, USA), transfection reagent (Biontix, München, Germany), reverse transcription kit and SYBR Green PCR kit (TaKaRa, Tokyo, Japan), cell counting kit-8 (CCK-8) kit (Dojindo, Kumamoto, Japan), apoptosis assay kit (Life technologies, Gaithersburg, MD, USA), β -catenin antibody and Wnt3 α antibody (Santa Cruz, CA, USA), β -actin antibody (MBL Biotech, Beijing, China), Thermal Cycler CFX6 System real-time quantitative PCR instrument (Bio-Rad, Hercules, CA, USA), and FACScan flow cytometer (BD, Franklin Lakes, NJ, USA).

Cell culture and transfection

The cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin sulfate in a humidified incubator with 5% CO₂ at 37°C. Huh-7 cells were used for *in vitro* experiments, and the medium was replaced once every 3 days. The cDNA of circRNA-ITCH was synthesized and cloned into pcDNA3.1(+) vectors by Nanjing GenScript Biotech Co., Ltd. (Nanjing, China), named pcDNA3.1-circRNA-ITCH. It was confirmed by enzyme digestion and sequencing that the sequences inserted were completely correct. The pcDNA3.1(+) empty vectors were used as the negative control. Huh-7 cells in the logarithmic phase were transfected according to the instructions of the transfection kit (Biontix, München, Germany), followed by further experiments.

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted from the cultured cells using TRIzol reagent. Then 1 mg of total RNA was synthesized into complementary deoxyribose nucleic acid (cDNA) according to the instructions of the reverse transcription kit (TaKaRa, Tokyo, Japan), followed by qRT-PCR on the Thermal Cycler CFX6 System using the SYBR Green PCR master mix kit under the following conditions: denaturation at 95°C for 10 min, denaturation at 95°C for 30 s, annealing at 60°C for 30 s, for a total of 40 cycles, and extension at 72°C for 1 min. The relative RNA expression was calculated using the 2^{- $\Delta\Delta$ Ct} method and normalized to GAPDH. The qRT-PCR was performed for at least 3 times. The primers used were as follows: circRNA-ITCH: F: 5'-GCAGAGGC-CAACACTGGAA-3', R: 5'-TCCTTGAAGCTGACTACGCT-GAG-3'. C-myc: F: 5'-TTCGGGTAGTGGAAAACCAG-3', R: 5'-CAGCAGCTCGAATTTCTTCC-3'. CyclinD1: F: 5'-GAG-GAGCAGCTCGCAA-3', R: 5'-CTGTCAAGGTCCGGC-CAGCG-3'. glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5'-CCATGACCCCTTCATTGACC-3', R: 5'-TTGATTTTGGAGGGATCTCG-3'.

Cell proliferation analysis

In the cell proliferation assay, the transfected cells were inoculated into a 96-well plate (2 \times 10³/well). At 24, 48, 72 and 96 h after inoculation, the cell viability was detected using the CCK-8 reagent. Ten μ L of CCK-8 reagent were added into each well, followed by incubation at 37°C in the dark for 2 h. Finally, the absorbance of each well was measured at 450 nm using a microplate reader.

Colony formation assay

In the colony formation assay, the transfected cells were inoculated into a 6-well plate (400/well) and cultured in DMEM containing 10% FBS, and the medium was replaced once every 4 days. After 2 weeks, the cells were fixed with methanol and stained with 0.1% crystal violet, and the colonies formed were imaged and counted.

Detection of apoptosis via flow cytometry

After transfection, the cells were collected and washed twice with ice-cold phosphate buffered saline (PBS). After the cells were fixed with 70% cold ethanol for 1 h on ice, they were resuspended in the mixture of 100 μ L of binding buffer, 5 μ L of Annexin V-FITC and 5 μ L of propidium iodide (PI), followed by incubation at room temperature for 15 min in the dark. Finally, the stained cells were detected via flow cytometry.

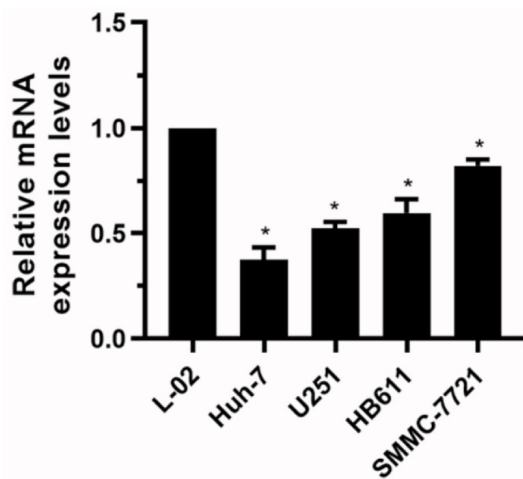


Figure 1. Relative expression level of circRNA-ITCH detected via qRT-PCR in four HCC cell lines (Huh-7, U251, HB611 and SMMC-7721), and human normal liver L-02 cell lines (* p <0.05).

Western blotting

The cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) on ice to separate the total protein. The protein concentration was measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). After separation via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), sealed with 5% skim milk powder prepared using tris buffered saline-tween (TBST), and incubated with the primary antibody at 4°C overnight. After the membrane was washed with TBST for 3 times, the protein was incubated again with the secondary antibody at room temperature for 2 h. The membrane was washed again with TBST for 3 times. Finally, the protein bands were visualized using the enhanced chemiluminescence reagent.

Luciferase activity assay

The miR-7 or miR-214 binding site sequences corresponding to the ITCH 3'-untranslated region (3'-UTR) were synthesized and inserted into the psiCHECK-2 empty plasmids. One day before transfection, Huh-7 cells were inoculated in the 24-well plate (10⁵/well), and transiently co-transfected with miR-7 mimics or miR-214 mimics and ITCH 3'-UTR luciferase reporter plasmids successfully constructed according to the instructions of the transfection reagent. After 48 h, the firefly and Renilla luciferase activities were determined through dual-luciferase reporter assay. Three replicates were set in each sample.

Statistics

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for all statistical analyses, and p <0.05 suggested statistically significant difference. The data were expressed as mean \pm standard deviation (\bar{x} \pm s). Differences of data were analyzed between the two groups using Student's t-test or chi-square test.

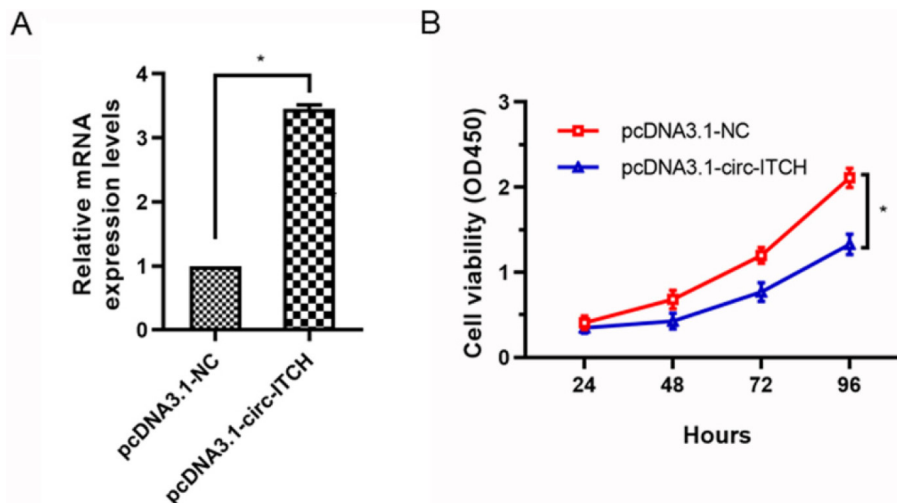


Figure 2. A: CircRNA-ITCH overexpression effect determined using qRT-PCR (* p <0.05), and **B:** effect of overexpression of circRNA-ITCH on Huh-7 cell proliferation (* p <0.05).

Results

CircRNA-ITCH expression was down-regulated in human HCC cell lines

To study whether the expression of circRNA-ITCH was changed in HCC, the relative expression level of circRNA-ITCH was detected in four kinds of HCC (Huh-7, U251, HB611 and SMMC-7721 cell lines), and human normal liver L-02 cell line. As shown in Figure 1, the expression of circRNA-ITCH was significantly decreased in the four kinds of HCC cells compared with that in human normal liver cells, indicating that the circRNA-ITCH expression is down-regulated in human HCC cell lines, which may play a role as a cancer suppressor gene, and its down-regulated expression may be closely related to the development of HCC.

Effect of overexpression of circRNA-ITCH on HCC cell proliferation

Considering the lowest expression of circRNA-ITCH in Huh-7 cells, circRNA-ITCH expression

plasmids were transfected to detect the effect of overexpression of circRNA-ITCH on Huh-7 cell proliferation. First, the circRNA-ITCH overexpression effect was determined using qRT-PCR (Figure 2A), and then the effect of overexpression of circRNA-ITCH on Huh-7 cell proliferation was determined using CCK-8 assay (Figure 2B). It was found that overexpression of circRNA-ITCH could significantly reduce the proliferation rate of Huh-7 cells.

Effect of overexpression of circRNA-ITCH on colony formation ability of HCC cells

The effect of overexpression of circRNA-ITCH on HCC Huh-7 cells was further verified through colony formation assay. As shown in Figure 3, the overexpression of circRNA-ITCH could clearly inhibit the colony formation of Huh-7 cells.

Effect of overexpression of circRNA-ITCH on apoptosis of HCC cells

The effect of overexpression of circRNA-ITCH on apoptosis of HCC Huh-7 cells was further an-

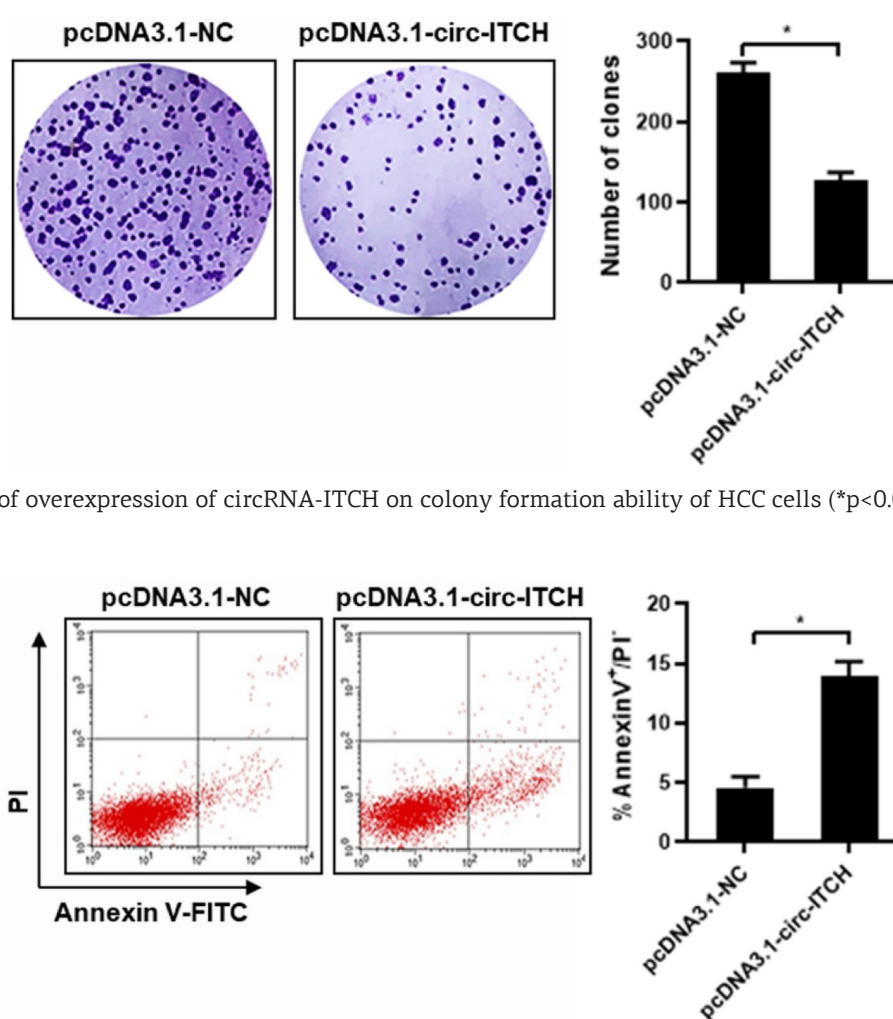


Figure 3. Effect of overexpression of circRNA-ITCH on colony formation ability of HCC cells (* $p < 0.05$).

Figure 4. Effect of overexpression of circRNA-ITCH on apoptosis of HCC cells (* $p < 0.05$).

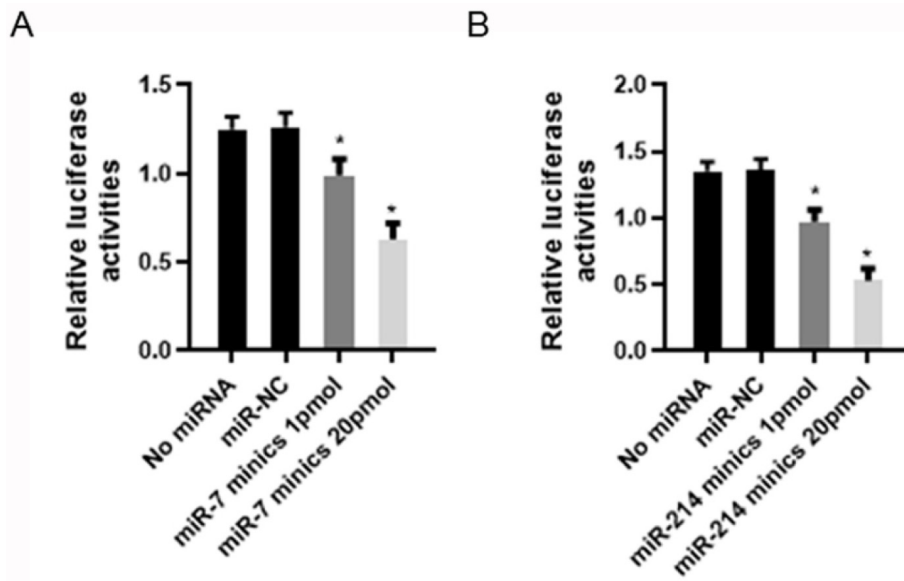


Figure 5. A: Interaction between circRNA-ITCH and miR-7 detected using luciferase activity assay (*p<0.05), **B:** interaction between circRNA-ITCH and miR-214 detected using luciferase activity assay (*p<0.05).

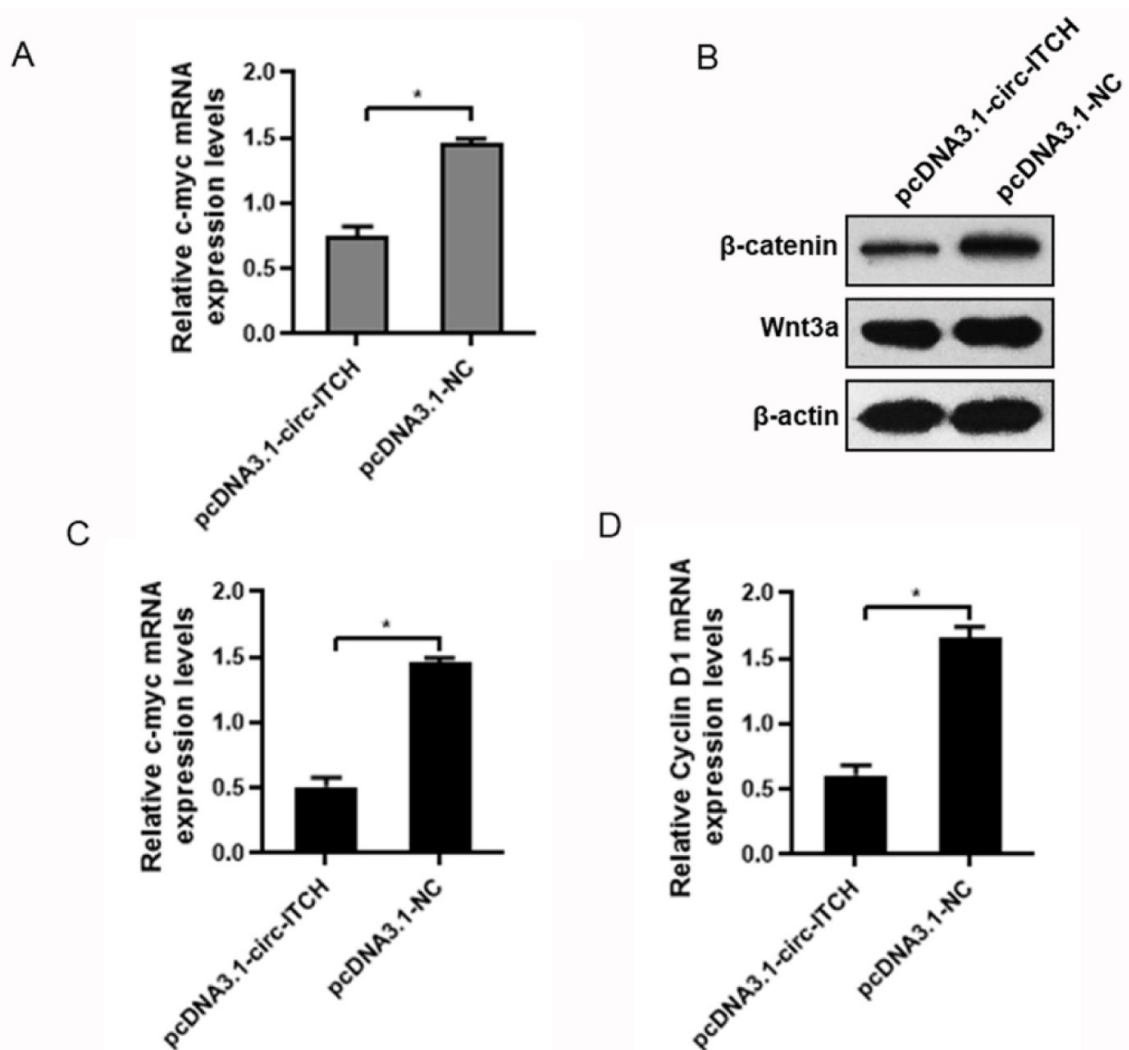


Figure 6. CircRNA-ITCH participates in regulating the Wnt/β-catenin signal transduction pathway *in vivo*. **A:** TCF activity determined using dual-luciferase reporter assay. **B:** β-catenin and Wnt3a levels determined using Western blotting. **C:** Expression of c-myc detected using qPCR. **D:** Expression of cyclinD1 detected using qRT-PCR. *p<0.05.

alyzed via flow cytometry. The results showed that the apoptosis rate of cells transfected with circRNA-ITCH expression plasmids was evidently higher than that in cells transfected with empty plasmids (Figure 4). Combined with the above results, it is concluded that the overexpression of circRNA-ITCH inhibits cell proliferation and induces apoptosis.

CircRNA-ITCH interacted with miR-7 or miR-214

It is reported in an article [16] that miR-7 or miR-214 can bind to the ITCH 3'-UTR, so the ITCH binding sequences of miR-7 or miR-214 were inserted into the psiCHECK-2 vectors. The Huh-7 cells were transiently co-transfected with miR-7 mimics or miR-214 mimics and ITCH 3'-UTR luciferase reporter plasmids were successfully constructed, and the luciferase activity was detected after 48 h. The results revealed that the luciferase activity in cells transfected with miR-7 mimics or miR-214 mimics significantly declined compared with that in cells transfected with empty normal control (NC) ($p < 0.05$) (Figure 5).

CircRNA-ITCH participated in regulating the Wnt/ β -catenin signal transduction pathway in vivo

ITCH promotes the ubiquitination and degradation of Dvl2, thereby inhibiting the classical Wnt signal transduction. To further verify whether circRNA-ITCH regulates the Wnt/ β -catenin signal transduction pathway in HCC cells, β -catenin/TCF-responsive luciferase reporter assay was performed. It was found that the overexpression of circRNA-ITCH inhibited the TCF luciferase activity (Figure 6A). The levels of β -catenin and Wnt3a in cells with circRNA-ITCH overexpression were detected using Western blotting. As shown in Figure 6B, the β -catenin protein level obviously declined, while that of Wnt3a did not change. Then, the expressions of c-myc and cyclinD1, two target genes of the Wnt/ β -catenin signaling pathway, in cells transfected with circRNA-ITCH were detected using qRT-PCR, which showed that their expressions were also suppressed (Figure 6C/D).

Discussion

HCC is a heterogeneous disease with complex etiology. The HCC molecular basis of protein-coding genes is mainly studied under the background of tumor occurrence, development and invasion. CircRNAs are a kind of novel non-coding RNAs whose functions remain to be fully known, and they serve as cancer driver genes. Understanding their dysregulation and regulation mechanisms

can facilitate the development of new diagnostic or therapeutic strategies.

With the development of next-generation sequencing, especially RNA sequencing technique, more than 30,000 circRNAs have been detected [17]. According to emerging research, circRNAs, as potential diagnostic and predictive biomarkers, play important roles in the occurrence and development of a variety of cancers. It has been reported that circRNAs play crucial roles in many aspects of malignant tumors, including cell cycle, apoptosis, angiogenesis, invasion and metastasis [6,17]. Further exploring the function of circRNAs as molecular markers in the occurrence of cancer has potential application prospects, such as early tumor diagnosis, therapeutic target screening, prognosis prediction and tumor targeted therapy. Li et al [18] found that circRNA-ITCH inhibits esophageal squamous cell carcinoma through suppressing the Wnt/ β -catenin pathway. In addition, similar mechanism was also found by Wan et al [19] in lung cancer. However, there have been no reports on the function and role of circRNA-ITCH in HCC. In the present study, it is hypothesized that circRNA-ITCH may be involved in the carcinogenesis of HCC. Therefore, the biological functions of circRNA-ITCH in HCC and its potential mechanism were mainly explored in this paper. It was found that the circRNA-ITCH expression was down-regulated in HCC cell lines, indicating that circRNA-ITCH may act as a cancer suppressor gene, and its down-regulated expression may be closely related to the occurrence and development of HCC. Then, the effects of overexpression of circRNA-ITCH on the proliferation and apoptosis of HCC cells were determined using CCK-8 assay, colony formation assay and flow cytometry which showed that the overexpression of circRNA-ITCH inhibited the proliferation and promoted the apoptosis of HCC cells. The above findings suggest that circRNA-ITCH plays an important role in regulating the proliferation and apoptosis of HCC cells and may become a biomarker for the diagnosis and risk prediction of HCC.

The abnormal regulation of the Wnt signaling pathway has become a ubiquitous research topic in cancer biology, and it is critical for the pathogenesis and progression of human HCC. CircRNA-ITCH serves as the miRNA sponge and increases the level of ITCH, which involves the Wnt/ β -catenin signal transduction pathway. According to previous studies, ITCH can facilitate the ubiquitination and degradation of phosphorylated Dvl2, thereby inhibiting the classical Wnt signal transduction [15]. Besides, whether the single gene regulates the Wnt/ β -catenin signal transduction pathway has been detected using the β -catenin/TCF-responsive

luciferase reporter assay. It was confirmed by this study and other studies [19] that the overexpression of circRNA-ITCH obviously inhibits the relative transcriptional activity of TCF.

The oncogenes c-myc and cyclinD1 are effector proteins of the nuclear division signal, which can trigger and regulate the transcription of genes related to proliferation, and they are often overexpressed in several human tumors, including HCC [20]. The results of this study showed that the expressions of c-myc and cyclinD1 significantly declined in cells transfected with circRNA-ITCH. Therefore, it is believed that circRNA-ITCH has an inhibitory effect on the classical Wnt pathway. CircRNA-ITCH exerts an anti-tumor effect through controlling the miRNA activity, as well as an in-

hibitory effect on the classical Wnt pathway, thus suppressing the expressions of c-myc and cyclinD1.

Conclusions

In conclusion, this study demonstrated that circRNA-ITCH inhibits the proliferation of HCC by inhibiting the Wnt/ β -catenin signal transduction pathway, which helps understand the molecular mechanism of circRNA-ITCH in the occurrence and development of HCC, and may provide a theoretical basis for new therapeutic strategies for HCC.

Conflict of interests

The authors declare no conflict of interests.

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