Research Article

Effects of circFOXO3 on the Proliferation and Invasion of Liver Cancer Cells by Regulating PI3K/Akt Pathway

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Objective. Hepatocellular carcinoma is a malignant disease occurring in the liver and is one of the main causes of death in cancer patients. Tumor cells are the main components of tumors and have a strong proliferative capacity. They are easily transferred to other parts of the body and can produce harmful substances that destroy the normal organ structure and endanger human life and health. In this study, we investigate the effect of circFOXO3 on the proliferation and invasion of hepatocellular carcinoma cells and its possible mechanism.

Methods. Human hepatocellular carcinoma cells BEL-7404, Hep G2, Hep 3B2.1–7, HuH-7, Li-7, and human normal hepatocytes HHL-5 were selected, and the expression level of circFOXO3 in the cell lines was determined by qRT-PCR. The cell line with low circFOXO3 expression level (HuH-7 cells) was used for follow-up experiments. HuH-7 liver cancer cells were divided into the control group (normal cultured), circFOXO3-NC group (transfected with circFOXO3 negative control), circFOXO3 mimic group (transfected with circFOXO3 mimic), PI3K activator group (20 μmol/L PI3K activator 740Y-P), and circFOXO3 mimic + PI3K activator group (transfected with circFOXO3 mimic + treated with PI3K activator 740Y-P). The qRT-PCR method was used to determine the expression level of circFOXO3 in HuH-7 liver cancer cells in each group, WB was used to detect the expression of apoptosis, invasion, and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway related proteins in HuH-7 liver cancer cells in each group, the CCK-8 method was used to determine the viability of HuH-7 liver cancer cells in each group, flow cytometry was used to determine the apoptotic ability of HuH-7 liver cancer cells in each group, the transwell chamber experiment was used to determine the invasion ability of HuH-7 liver cancer cells in each group, and the scratch test was used to determine the migration ability of HuH-7 liver cancer cells in each group.

Results. circFOXO3 showed low expression in liver cancer cells; compared with the control group, the circFOXO3 expression and apoptosis rate of HuH-7 liver cancer cells in the circFOXO3 mimic group were significantly increased ($P < 0.05$) and the PI3K/Akt pathway-related protein expression, cell viability, invasion, and migration abilities were significantly reduced ($P < 0.05$); the apoptosis rate of HuH-7 liver cancer cells in the PI3K activator group was significantly reduced ($P < 0.05$) and the PI3K/Akt pathway related protein expression, cell viability, invasion and migration abilities were significantly increased ($P < 0.05$). Compared with the circFOXO3 mimic group, the apoptosis rate of HuH-7 liver cancer cells in the circFOXO3 mimic + PI3K activator group was significantly reduced ($P < 0.05$) and the PI3K/Akt pathway related protein expression, cell viability, invasion and migration abilities were significantly increased ($P < 0.05$).

Conclusion. Highly expressed circFOXO3 can inhibit the proliferation and invasion of HuH-7 liver cancer cells, which may be achieved by inhibiting the PI3K/Akt pathway.

1. Introduction

Liver cancer is a malignant tumor disease occurring in the liver, and it is one of the main causes leading to the death of cancer patients. Virus infection, alcohol, environment, dietary habits, and other factors are all related to the occurrence of liver cancer. Patients with liver cancer often suffer from adverse symptoms such as pain, emaciation, hepatomegaly, and fatigue. Complications such as liver failure, rupture bleeding, and gastrointestinal bleeding will occur in the later stage, which seriously affects the life safety of patients [1, 2]. Tumor cells are the main components of tumors, which have strong proliferation ability. They are easy to transfer to other parts of the body and will produce harmful...
substances damaging the normal organ structure and endangering human life and health. Therefore, the study of the possible mechanism of tumor cell proliferation and invasion plays an important role in inhibiting the occurrence and development of cancer clinically [3].

Circular RNA (circRNA) is a special kind of noncoding RNA, which is abundant and stable in eukaryotes and participates in many biological activities such as protein synthesis, gene expression, and posttranscriptional modification. circFOXO3 is a member of circRNA family, which is closely related to tumor occurrence, cell survival, and apoptosis [4]. Recent studies have shown that the low expression level of circFOXO3 in breast cancer [5] and esophageal squamous cell carcinoma [6] is closely related to the occurrence and development of tumor diseases. The phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) signaling pathway plays an important role in the proliferation and migration of various tumor cells [7]. Some studies have found that downregulating the expression level of PI3K/Akt pathway-related proteins can significantly reduce the proliferation and migration ability of hepatoma cells [8].

Therefore, highly expressed circFOXO3 can inhibit the proliferation and invasion of HuH-7 liver cancer cells, which can be achieved by inhibiting the PI3K/Akt pathway. The Akt pathway is achieved. In this study, by measuring the transfection of circFOXO3 mimicking liver cancer cells, we explored the effect of circFOXO3 on the proliferation and migration of liver cancer cells and its possible mechanism, thus providing new ideas for clinical remission and treatment of liver cancer. Therefore, highly expressed circFOXO3 can inhibit the proliferation and invasion of HuH-7 liver cancer cells, which can be achieved by inhibiting the PI3K/Akt pathway. The Akt pathway is achieved. In this study, by measuring the transfection of circFOXO3 mimicking liver cancer cells, we explored the effect of circFOXO3 on the proliferation and migration of liver cancer cells and its possible mechanism, thus providing new ideas for clinical remission and treatment of liver cancer.

2. Materials

2.1. Cells. Human hepatoma cells BEL-7404, Hep G2, Hep 3B2.1–7, HuH-7, Li-7, and normal human hepatocytes were thawed quickly and cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% streptomycin at 37°C in a 5% CO2 incubator, as shown in Figure 1.

2.2. Main Reagents and Instruments. 740 Y-P (PI3K activator, item number: HY-P0175) was purchased from Med-ChemExpress; RNA extraction kit and reverse transcription kit (item number: R1200, T2240) were purchased from Beijing Solarbio Technology Co., Ltd.; protein extraction kit (RIPA lysate) and protein concentration determination kit (item number: P0013B, P0012S) were purchased from Shanghai Beyotime Biotechnology; Entranster TM-R4000 RNA transfection reagent (item number: 4000–3) was purchased from Beijing Engreen Biosystem Co., Ltd.; Caspase-3, Bcl-2, Bax, MMP-2, MMP-9, PI3K, p-PI3K, Akt, p-Akt, GAPDH primary antibody, and goat anti-rabbit IgG secondary antibody (item number: ab184787, ab194583, ab32503, ab181286, ab76003, ab32089, ab27854, ab8805, ab38449, ab9485, and ab150077) were purchased from British Abcam.

Cell incubators (model: BC-180/160S) were purchased from Shanghai Boxun Medical Bioinstrumentation Co., Ltd.; PCR instrument (model: PikoReal) was purchased from Thermo Scientific, USA; cell counters (model: Nucleo-Counter NC-250 TM) were purchased from ChemoMetc, Denmark; multifunctional microplate readers (model: SpectraMax iD3) were purchased from Molecular Devices; inverted microscopes (model: Ti2 Ts2) were purchased from Nikon, Japan; Gel imagers (model: SmartGel6000) were purchased from Beijing Saizhi Pioneering Technology Co., Ltd.; flow cytometers (model: CytoFLEX) were purchased from Beckman Coulter, Inc., USA.

3. Methods

3.1. Cell Culture. Human hepatocarcinoma cells BEL-7404, Hep G2, Hep 3B2.1–7, HuH-7, Li-7, and normal human hepatocytes were thawed quickly and cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% streptomycin at 37°C in a 5% CO2 incubator, as shown in Figure 1.

3.2. Assay of circFOXO3 mRNA Expression Level in Hepatoma Cells. Human hepatoma cells BEL-7404, Hep G2, Hep 3B2.1–7, HuH-7, Li-7 and normal human liver cell strains were collected. Total RNA was extracted from the cells by using an RNA extraction kit. cDNA was obtained by using reverse transcription RNA and amplified by PCR. Upstream primer of circFOXO3: 5′-GTGGGGAACCTT-CACTGGTGCTAAG-3′; downstream primer: 5′-GGGT TGATGATCCACCAAGACTCTT-3′; using U6 as internal reference, upstream primer: 5′-CTCGCTTCGGCAGCAC A-3′, downstream primer: 5′-AACGCTTCAGAATTT GCCGT-3′. The expression level of circFOXO3 in human hepatoma cells and normal human hepatocytes was calculated by the 2−∆∆CT algorithm, as shown in Figure 2.

3.3. Grouping and Transfection of HuH-7 Hepatoma Cells. The HuH-7 hepatoma cells were divided into the control group (normal culture), circFOXO3-NC group (negative control transfected with circFOXO3), circFOXO3 mimic group (mimetic transfected with circFOXO3), PI3K activator group (20 µmol/L PI3K activator 740 Y-P) [9], and circFOXO3 mimic+PI3K activator group (treatment of mimetic transfected with circFOXO3+PI3K activator 740 Y-P). The assay of the expression level of circFOXO3 in HuH-7 hepatocarcinoma cells in each group was the same as that in Section 2.2.

3.4. Assay of Vitality and Apoptosis Ability of HuH-7 Hepatoma Cells in Each Group. The transfected HuH-7 hepatoma cells in each group were prepared into cell suspension and
incubator for 24 h, the residual HuH-7 hepatoma cells in the upper chamber were wiped off. After that, the chamber was fixed with paraformaldehyde and stained with crystal violet. After drying, the cells were observed under a microscope and the number of invasive cells was calculated.

A scratch test was adopted to measure the cell migration ability. The transfected HuH-7 hepatoma cells were inoculated in 6-well plates and cultured in the incubator for 24 h. Vertical scratches were made on the bottom of the well plate with a gun head. Then, the 6-well plates were cultured in an incubator for another 24 h. The scratch healing rate was calculated. The higher the scratch healing rate is, the stronger the cell migration ability is. Scratch healing rate (%) = (0 h scratch width − 24 h scratch width)/0 h scratch width × 100%.

3.6. Assay of PI3K/Akt Pathway-Related, Apoptosis-Related, and Invasion-Related Proteins in HuH-7 Hepatoma Cells of Each Group. The transfected HuH-7 hepatoma cells of each group were taken. The total protein in the cells to be detected was extracted by the protein extraction kit and the protein content was determined. The protein was separated by SDS polyacrylamide gel electrophoresis. The separated protein was transferred to the PVDF membrane by the wet transfer method and then sealed by adding 5% skimmed milk powder for 1 h. After incubation at low temperature overnight, horseradish peroxidase labeled goat anti-rabbit IgG secondary antibody was added for incubation for another 2 h. Color development was carried out with an enhanced chemiluminescence reagent. Relative expression levels of PI3K/Akt pathway-related, apoptosis-related, and invasion-related proteins in HuH-7 hepatoma cells of each group were analyzed by Image J software.

3.7. Statistical Analysis. The experimental data were statistically analyzed by SPSS 22.0 software, and the measurement data were expressed by mean ± standard deviation (±s). One-way ANOVA was used for comparison between the two groups, and the LSD-t test was used for further comparison between the two groups. P < 0.05 indicated that the difference was statistically significant.

4. Results

4.1. Comparison of circFOXO3 mRNA Expression Level in Hepatoma Cells. Compared with human normal hepatocyte HHL-5, the expression level of circFOXO3 mRNA in human hepatoma cells BEL-7404, Hep G2, Hep 3B2.1–7, HuH-7, and Li-7 significantly decreased (P < 0.05). Among them, the expression level of circFOXO3 in HuH-7 cells was low, so HuH-7 cells were selected for subsequent experiments. There was no significant difference between the circFOXO3-NC group and the PI3K activator group. HuH-7 liver cancer cell viability and Bcl-2 protein expression level were significantly increased. The expression levels of asparaginase-3 and Bax proteins were significantly decreased. The viability and Bcl-2 protein expression levels of HuH-7 liver cancer cells in the circFOXO3 simulation group were significantly decreased, while the apoptosis rate, Caspase-3, and Bax
protein expression were significantly increased \( (P < 0.05) \), as given in Table 1.

4.2. Comparison of circFOXO3 Expression Level in HuH-7 Hepatoma Cells in Each Group. Compared with the control group, there was no significant difference between the circFOXO3-NC group and the PI3K activator group \( (P > 0.05) \), but the expression level of circFOXO3 in the circFOXO3 mimic group and circFOXO3 mimic + PI3K activator group increased significantly \( (P < 0.05) \), as given in Table 2.

4.3. Comparison of Vitality and Apoptosis Ability of HuH-7 Hepatoma Cells in Each Group. Compared with the control group, the circFOXO3-NC group had no significant difference \( (P > 0.05) \). In the PI3K activator group, the vitality and Bcl-2 protein expression level of HuH-7 hepatoma cells increased significantly \( (P < 0.05) \), while the apoptosis rate and Caspase-3 and Bax protein expression levels of hepatoma cells decreased significantly \( (P < 0.05) \). The vitality and Bcl-2 protein expression level of HuH-7 hepatoma cells decreased significantly \( (P < 0.05) \), while the apoptosis rate, Caspase-3, and Bax protein expression increased significantly \( (P < 0.05) \) in the circFOXO3 mimic group. Compared with the circFOXO3 mimic group, the vitality and Bcl-2 protein expression level of HuH-7 hepatoma cells in the circFOXO3 mimic + PI3K activator group significantly increased \( (P < 0.05) \), while the apoptosis rate, Caspase-3, and Bax protein expression level of hepatoma cells significantly decreased \( (P < 0.05) \), as given in Table 3.

4.4. Comparison of PI3K/Akt Pathway-Related Protein Expression in HuH-7 Hepatoma Cells in Each Group.
Compared with the control group, there was no significant difference in the circFOXO3-NC group ($P > 0.05$). The expression levels of p-PI3K and p-Akt protein in HuH-7 hepatoma cells in the PI3K activator group significantly increased ($P < 0.05$), while the expression levels of p-PI3K and p-Akt protein in HuH-7 hepatoma cells in the circFOXO3 mimic group significantly decreased ($P < 0.05$). Compared with the circFOXO3 mimic group, the expression levels of p-PI3K and p-Akt protein in HuH-7 hepatoma cells in the circFOXO3 mimic + PI3K activator group significantly increased ($P < 0.05$), as given in Table 4.

### 4.5. Comparison of Invasion and Migration Ability of HuH-7 Hepatoma Cells in Each Group

Compared with the control group, the circFOXO3-NC group had no significant difference ($P > 0.05$). The invasion and migration ability and MMP-2 and MMP-9 protein expression levels of HuH-7 hepatoma cells in the PI3K activator group significantly increased ($P < 0.05$), while the invasion and migration ability of MMP-2 and MMP-9 protein expression levels of HuH-7 hepatoma cells in the circFOXO3 mimic group significantly increased ($P < 0.05$). Compared with the circFOXO3 mimic group, the invasion and migration ability and MMP-2 and MMP-9 protein expression levels of HuH-7 hepatoma cells in the circFOXO3 mimic + PI3K activator group significantly increased ($P < 0.05$), as given in Table 5.

### 5. Discussion

Patients with liver cancer are often treated by surgical resection, radiotherapy and chemotherapy, chemotherapy, and biotherapy, but the overall mortality remains high [10, 11]. Malignant proliferation, strong invasion, and migration ability of hepatoma cells are the premise and foundation of tumor diffusion and metastasis, as well as one of the main reasons for further development of tumor disease [12]. Therefore, the exploration of the related mechanism of proliferation and invasion of hepatoma cells has a positive effect on clinical inhibition and alleviation of liver cancer. Widely existing in eukaryotes, circRNA has certain regulatory effects on protein synthesis, gene expression, transcription modification, and other cellular biological functions and plays an important role in the occurrence and development of various tumor diseases. It is one of the latest research hotspots in the field of RNA [13]. circFOXO3, a member of the circRNA family, has low expression in nonsmall cell lung cancer [14] and esophageal squamous cell carcinoma [15], closely related to biological activities such as tumor cell proliferation and migration. Wang [16] and other studies found that the overexpression of circFOXO3 can inhibit the proliferation of esophageal squamous cell carcinoma and promote the apoptosis of esophageal squamous cell carcinoma. The results of this study showed that the expression level of circFOXO3 in hepatoma cells BEL-7404, Hep G2, Hep 3B2.1–7, HuH-7, and Li-7 decreased significantly, which suggested that circFOXO3 was closely related to the occurrence and development of liver cancer. Besides, the expression level of circFOXO3 in HuH-7 hepatoma cells was low. Thus, HuH-7 hepatoma cells were selected in a follow-up study for the transfection experiment. The results showed that the activity, invasion, and migration ability of hepatoma cell HuH-7 decreased significantly and the apoptosis rate increased significantly after increasing the expression level of circFOXO3 in hepatoma cells, which indicated that the highly expressed circFOXO3 could inhibit the proliferation and migration abilities of hepatoma cells and had a certain anticancer effect. The results were consistent with the above research.

The PI3K/Akt signaling pathway is closely related to cell proliferation, apoptosis, metastasis, and other activities. Akt is an important downstream signaling molecule of PI3K, and PI3K mainly participates in the regulation of cell proliferation, apoptosis, and other signal transduction by activating Akt [17]. Recent studies have found that the PI3K/Akt signaling pathway plays an important role in the proliferation and migration of hepatoma cells [18]. Inhibition of the PI3K/Akt signaling pathway and reduction of phosphorylation of PI3K and Akt can inhibit the proliferation and migration of hepatoma cells and promote the apoptosis of hepatoma cells [18–20]. The expression level of PI3K/Akt pathway-related proteins in HCC cells in the circFOXO3 mimic + PI3K activator group was significantly higher than that in the circFOXO3 mimic group, suggesting that the overexpression level of circFOXO3 could inhibit the proliferation and migration of HCC cells by inhibiting the PI3K/Akt pathway.

To sum up, increasing the expression level of circFOXO3 could inhibit the proliferation and migration of HCC cells, which may be achieved by inhibiting the PI3K/Akt pathway. However, the mechanism of proliferation and migration of
Table 4: Expression level of PI3K/Akt pathway-related proteins in HuH-7 hepatoma cells in each group (\( \bar{x} \pm s, n = 3 \)).

| Group                          | p-PI3K/PI3K | p-Akt/Akt |
|--------------------------------|-------------|-----------|
| Control group                  | 1.02 ± 0.15 | 1.01 ± 0.14 |
| circFOXO3-NC group             | 0.97 ± 0.14 | 0.98 ± 0.15 |
| circFOXO3 mimic group          | 0.24 ± 0.03\(^{ab}\) | 0.26 ± 0.03\(^{ab}\) |
| PI3K activator group           | 1.54 ± 0.25\(^{abc}\) | 1.55 ± 0.24\(^{abc}\) |
| circFOXO3 mimic + PI3K activator group | 0.59 ± 0.08\(^{abcd}\) | 0.63 ± 0.06\(^{abcd}\) |

Compared with the control group, \(^{a}P < 0.05\); compared with the circFOXO3-NC group, \(^{b}P < 0.05\); compared with the circFOXO3 mimic group, \(^{c}P < 0.05\); compared with the PI3K activator group, \(^{d}P < 0.05\).

Table 5: Comparison of invasion and migration abilities of HuH-7 cells in different groups.

| Group                          | Number of invasive cells (number) | Scratch healing rate (%) | Expression level of invasion-related proteins |
|--------------------------------|----------------------------------|---------------------------|----------------------------------------------|
| Control group                  | 115.48 ± 10.03                   | 60.43 ± 7.69              | MMP-2/GAPDH: 0.98 ± 0.09, MMP-9/GAPDH: 0.97 ± 0.14 |
| circFOXO3-NC group             | 115.06 ± 10.01                   | 61.06 ± 7.35              |                                               |
| circFOXO3 mimic group          | 60.10 ± 5.50\(^{ab}\)           | 34.67 ± 3.78\(^{ab}\)     |                                               |
| PI3K activator group           | 150.26 ± 15.27\(^{abc}\)        | 85.44 ± 13.05\(^{abc}\)   |                                               |
| circFOXO3 mimic + PI3K activator group | 84.85 ± 8.15\(^{abcd}\)      | 47.48 ± 4.26\(^{abcd}\)   |                                               |

Compared with the control group, \(^{a}P < 0.05\); compared with the circFOXO3-NC group, \(^{b}P < 0.05\); compared with the circFOXO3 mimic group, \(^{c}P < 0.05\); compared with the PI3K activator group, \(^{d}P < 0.05\).

HCC cells is complex, and circFOXO3 may inhibit the proliferation and migration of HCC cells in other ways. Therefore, further research is needed.

Data Availability

The data generated or analyzed during this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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