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RESEARCH PAPER

Regulatory Role of SFN Gene in Hepatocellular Carcinoma and Its Mechanism

Ying Hui, Hao Zeng, Yi Feng, Wenzhou Qin, Peisheng Chen, Lifang Huang, Wenfu Zhong, Liwen Lin, Hui Lv, and Xue Qin

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Abstract Purpose: This study aims to explore the differential expression of SFN gene and its regulatory role in different hepatocarcinoma cells, and the impact on hepatocarcinoma. Materials and Methods: High and low SFN expression cells were screened by qRT-PCR and western blotting methods. SFN over expression and interference vectors were constructed. Cell viability was detected by CCK8 kit, cell cycle and apoptosis were detected by flow cytometry. Cell invasion and migration were detected. CCNB1 and CDK1 expression levels were detected by qRT-PCR and Western blotting methods. Results: The high SFN expression BEL7402 cells and the low SFN expression Hep3B cells were screened from Hep3B, HepG2, and BEL7402 cells. The activity of Hep3B cells overexpression vector SFN-pcDNA3.1(+) decreased and apoptosis increased, the ratio of G0/G1 decreased and the ratio of S phase increased. The activity of BEL7402 cells transfected with SFN siRNA decreased and apoptosis increased, the ratio of G0/G1 decreased and the ratio of G2/M increased. Interference and overexpression vectors have little effect on the invasion and migration of the two cells. The expression of CDK1 in Hep3B cells decreased significantly, the expression of CDK1 and CCNB1 in BEL7402 cells increased significantly. Conclusions: The differentially expressed SFN gene can regulate the growth of the two hepatocarcinoma cells, high expression of SFN gene can inhibit their growth. The mechanism may be achieved by regulating CCNB1 and CDK1 expression.

Keywords: SFN, cell screening, flow cytometry, CCNB1, CDK1, hepatocarcinoma

Ying Hui*†, Hao Zeng†, Yi Feng, Wenzhou Qin, Peisheng Chen, Lifang Huang, Wenfu Zhong, Liwen Lin, Hui Lv
Clinical Laboratory, Guigang People's Hospital, Guigang, Guangxi 537100, China
Tel: +86-13768155977
E-mail: hunyin112@163.com

Xue Qin*
Clinical Laboratory, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi 530021, China
E-mail: qinxiaoxue@126.com

†Equal contributors

1. Introduction

Primary liver cancer (PLC) is one of the two most common malignant tumors in the world, of which more than 90% are hepatocellular carcinoma (HCC) [1]. The incidence of liver cancer in the world is increasing year by year [2]. There are about 380,000 people die of liver cancer every year in China, which accounting for more than 50% of the world [3].

Surgical treatment is the main treatment of HCC. However, most of the patients could not be treated by surgery due to the location, size, number of tumors, or extrahepatic metastasis. There are some defects in non-surgical treatment, such as small molecule targeted drug treatment, selection of specific inhibitors, or chemoembolization agents [4]. Immunotherapy is considered to be a more promising treatment, but it is still in the exploratory stage, and the effect fluctuates greatly due to individual differences [5].

Stratifin (SFN, 14-3-3σ) is one of the highly conserved 14-3-3 proteins, its gene is located at 1p35, which is responsible for the regulation of G2 cell cycle checkpoint and plays an important role in DNA damage repair [6]. The expression of SFN is regulated by p53 and p21 genes, which are highly expressed in many normal tissues, and less expressed in some cancer tissues [7,8], but little research has been done in HCC. SFN is considered to be a tumor

suppressor protein, which can regulate cell proliferation, apoptosis and differentiation, and is closely related to tumor [9]. It was shown that SFN could promote the apoptosis of tumor cells, reduce the activity of cancer cells and inhibit their proliferation [10]. However, its specific mechanism is still unclear.

In this paper, SFN gene was screened out by high-throughput sequencing and comparison [11]. The effects of SFN on the function of hepatoma cells and related protein pathways were studied, and the relationship between SFN and hepatoma and its mechanism were explored.

2. Materials and Methods

2.1. Experimental cells

Human hepatoma cells HepG2 (BNCC338070), Hep3B (BNCC341747), and BEL-7402 (BNCC100924) were purchased from BeNa Culture Collection (Beijing, China). HepG2 and Hep3B cells were cultured in DMEM medium containing 10% FBS and 1% penicillin-streptomycin (Hyclone, Logan, UT, USA) at 37°C with 5% CO_2. BEL7402 cells were cultured in R1640 medium containing 10% FBS and 1% penicillin-streptomycin (Hyclone, Logan, UT, USA) at 37°C with 5% CO_2. SFN expression levels in three kinds of cells were detected by qRT-PCR and western blotting methods. Two kinds of cells with high and low SFN expression were screened for subsequent experiments.

2.2. Experimental reagents and instruments

DMEM Medium (KGM12800S-500, NanJing KeyGen Biotech Co.,Ltd., NanJing, China); Trypsin EDTA digestive solution (T1300, Solarbio, Beijing, China); Lipofectamine® 3000 (18882752, Invitrogen, Carlsbad, CA, USA); Trizon Reagent (CW0580S, Beijing ComWin Biotech Co.,Ltd., Beijing, China); Ultrapure RNA extraction kit (CW0581M, Beijing ComWin Biotech Co., Ltd., Beijing, China); HiFiScript cDNA synthesis Kit (CW2569M, Beijing ComWin Biotech Co., Ltd., Beijing, China); UltraSYBR Mix (CW0957M, Beijing ComWin Biotech Co., Ltd., Beijing, China); RIPA Cell lysis buffer (C1053, Beijing applygen Co., Ltd., Beijing, China); BCA Protein Assay Kit (CW0014S, Beijing ComWin Biotech Co., Ltd., Beijing, China); PVDF membrane (IPVH00010, Millipore, Billerica, MA, USA); Mouse Monoclonal Anti-GAPDH (TA-08, Beijing ZSGB-BIO Co., Ltd., Beijing, China); Horseradish Enzyme Labeled Goat Anti-Rat IgG (H+L) (ZB-2305, Beijing ZSGB-BIO Co., Ltd., Beijing, China); Mouse Monoclonal Anti-14-3-3 sigma (SFN) (ab14123, Abcam, Cambridge, UK, 1/1000); Anti-Cyclin B1 antibody (ab181593, Abcam, Cambridge, UK); Anti CDK1 antibody (ab18, Abcam, Cambridge, UK); Cell Cycle Staining Kit (CCS102, MultiSciences(Lianke)Biotech Co., Ltd., Hangzhou, China); Annexin V-FITC/PI Apoptosis Kit (AP101-100-kit, MultiSciences(Lianke)Biotech Co., Ltd., Hangzhou, China); Cell Counting Kit-8 (C0038, Shanghai Beyotime Biotechnology, Shanghai, China); Crystal Violet Staining Solution (G1061, Solarbio, Beijing, China); fluorescent quantitative PCR instrument (CFX Connect™, Bio-Rad Shanghai Laboratories, Shanghai, China); Microplate Reader (RT-6100, Rayto, Shenzhen, China); NovoCyte™ Flow cytometry (NovoCyte 2060R, ACEA Hangzhou Biosciences Inc., Hangzhou, China); Protein vertical electrophoresis instrument (DYY-6C, Beijing 61 instrument factory, Beijing, China); Microscope (CX41 OLYMPUS, Tokyo, Japan); Ultra High Sensitivity Chemiluminescence Imaging System (Chemi DocTM XRS+, Bio-Rad Shanghai Laboratories, Shanghai, China).

2.3. RNA extraction and qRT-PCR

Total RNA was extracted using Ultrapure RNA kit according to the manufacturer's protocol. Total RNA (1 μg) was subjected to reverse transcription using HiFiScript cDNA synthesis Kit. Real-time PCR were performed using SYNBR Green PCR Master Mix. At the end of each reaction, a melting curve analysis was performed to confirm the absence of primer dimmers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. Quantification of target genes

Table 1. Primers used in this study

Primer name	Primer sequence (5′-3′)	Primer length (bp)	Product length (bp)	Annealing temperature (°C)
SFN F	GGTGACTACTACCGCTACCTGG	22	116	61.4
SFN R	GGCATCTCCTTCTTGCTGACG	21		
CCNB1 F	TTGAGGAAGAGCAAGCAGTC	20	157	56.9
CCNB1 R	AACCGATCAATAATGGAGACAG	22		
CDK1 F	AGGATGTGCTTATGCAGGATTC	22	1.1	57.5
CDK1 R	CATGTACTGACCAGGAGGG	19		
GAPDH F	CAATGACCCCTTCATTGACC	20	106	57.2
GAPDH R	GAGAAGCTTCCCGTTCTCAG	20		

mRNA was performed using the$2^{-\Delta\Delta}$Ct method. Primers' sequences were listed in Table 1.

2.4. Western blotting method

Cells in different groups were harvested and lysed with RIPA Cell lysis buffer. The supernatant was collected after they were centrifuged at 4°C (10,000 rpm) for 5 min. Total proteins were extracted and protein concentration was determined using BCA. Proteins (50 μg per lane) were separated using 12% SDS-PAGE. Proteins were then electrotransferred to a PVDF membrane. The PVDF membrane was rinsed with TBS for 10-15 min, and placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder. It was incubated at 4°C overnight following the addition of an appropriate dilution of primary antibodies. The membrane was then rinsed with TBST for three times and incubated at room temperature for 1 h with horseradish peroxidase-labeled secondary antibody. Protein bands were detected using an enhanced chemiluminescence kit (orb90505, Biorbyt Ltd, Cambridge, UK) and quantified as the ratio to GAPDH. Quantification was performed using Imagequant LAS4000 (GE Healthcare, Chicago, IL, USA).

2.5. Cell transfection and group

The SFN overexpression vector SFN-pcDNA3.1(+) was constructed. SFN-siRNA and SFN-pcDNA3.1(+) transfection were performed with Lipofectamine 3000 kit according to the kit's manual. Hep3B cells with low SFN expression were used to transfect the SFN-pcDNA3.1(+). They were divided into blank control group (Control); overexpression of empty pcDNA3.1(+) group (NC) and overexpression of SFN-pcDNA3.1(+) group (SFN). BEL7402 with high SFN expression were used to transfect the SFN-siRNA. They were divided into blank control group (Control); interference with siRNA empty group (NC) and interference with SFN-siRNA group (SFN siRNA).

2.6. CCK8 test

Cell suspension (100 μL, 10^4-10^5 cells/well) was seeded in a 96-well plate and the plate was incubated in a humidified incubator at 37°C with 5% CO_2 for 24 h. Each well of the plate was added 10 μL of CCK-8 solution and incubated for 2 h. The absorbance at 450 nm was measured using a microplate reader.

2.7. Apoptosis analysis

Apoptosis analysis was performed using AnnexinV-FITC Analysis Kit (Beyotime, Shanghai, China) according to manual. Cells in each group were collected, digested with trypsin without EDTA, and washed twice with ice-cold PBS. Collect 1 × 10^6 cells/mL by centrifugation and resuspend cells in 300 μL 1× Binding Buffer. Add 3 μL Annexin V-FITC and 5 μL PI-PE according to the manufacturer's protocol. After 10 min of incubation at 4°C refrigerator in the dark, precooled 1 × binding buffer (200 μL) was added. The apoptosis rate was detected using flow cytometry. The light source is 488 nm argon ion laser. FITC emits green fluorescence and PI emits red fluorescence. Apoptotic cells could produce red fluorescence, while living cells had no red fluorescence. Therefore, on the scatter plot of bivariate flow cytometry, the lower left quadrant showed living cells as (FITC-/PI-), the upper right quadrant showed necrotic cells (FITC+/PI+), and the lower right quadrant showed apoptotic cells (FITC+/PI-). The experiment was repeated three times.

2.8. Detection of cell cycle

Cell suspension was centrifuged at 2500 rpm for 3 min and the supernatant was discarded. They were fixed with 1 mL absolute ethanol for more than 2 h at 4°C and centrifuged with 5,000 rpm for 3 min, the supernatant was discarded. They were washed with PBS and stained with 1 mL DNA staining solution at 4°C for 1 h. The cells were detected and analyzed by flow cytometry.

2.9. Cell migration

When the cell fusion degree reached more than 90%, the culture medium was discarded and the cells were washed with PBS. After trypsin digestion, cells were centrifuged and the supernatant was discarded, they were inoculated into a new plate and cultured at 37°C with 5% CO_2. When the cell fusion degree reached 70%, the cells were transfected and photographed with lines. They were photographed again after 24 h and the cell migration rate of each group was calculated.

2.10. Cell invasion

The cells in different groups were washed with serum-free medium three times and digested with trypsin. A cell suspension of 1.5 × 10^4 cells in 200 μL was added into each well. Complete medium (500 μL) was added to the invading lower chamber, the total volume of cells in the upper chamber and serum-free medium is about 300 μL. They were incubated at 37°C in 5% CO_2 incubator for 48 h. Then, the filters were fixed and stained with 0.1% crystal violet (dissolved in methanol). The cells of the inner chamber were removed by using a cotton swab. After photographing, the dye solution was removed from each well, prepared 33% acetic acid (1 mL) was added into each well to dissolve the dye solution in the cells. They were mixed and 200 μL solution was taken from each well and placed in the 96 well plate. The absorbance value of each

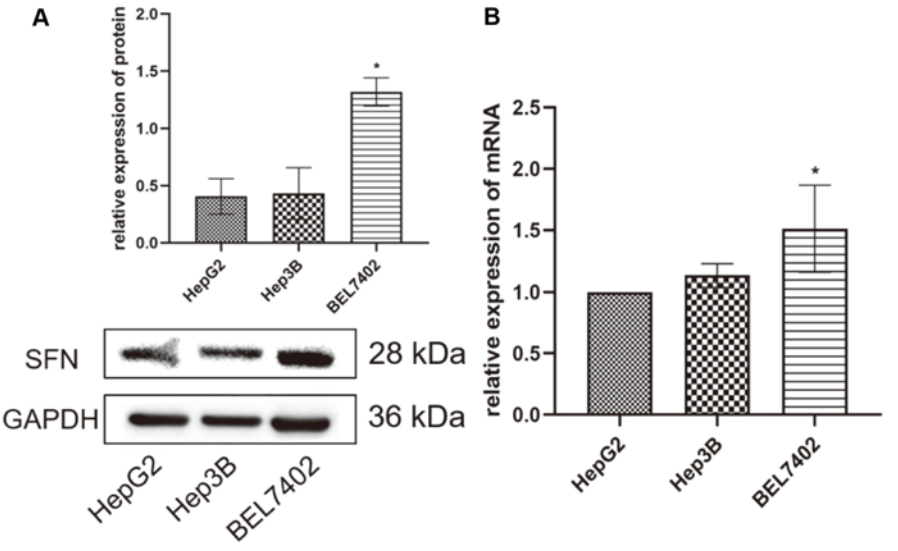

Fig. 1. SFN gene expression in Hep3B, HepG2, and BEL7402 cells (N = 3, error bars indicate S.D.). (A) Western blotting results of SFN protein expression in different cells; (B) qRT-PCR results of SFN mRNA expression in different cells. SFN expression was the highest in BEL7402 cells. *$p < 0.05$ vs HepG2 cells.

well was measured, the wavelength was 570 nm, and the invasiveness of each group was calculated.

2.11. Statistical analysis

The data were analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation (SD). The differences among groups were evaluated by t-test. $P < 0.05$ was considered to be significant.

3. Results

3.1. Cell screening

The qRT-PCR and western blotting results of SFN gene expression in Hep3B, HepG2, and BEL7402 cells were shown in Fig. 1. SFN expression was the highest in the BEL7402 cells and was lower in the Hep3B cells and HepG2 cells. Therefore, BEL7402 cells with high SFN expression and Hep3B cells with low SFN expression were selected for subsequent experiments.

3.2. Effects of SFN overexpression and SFN siRNA on cell viability, apoptosis, and cell cycle

As shown in Fig. 2A, the viability decreased, the apoptosis increased, the ratio of cell cycle G0/G1 decreased, and the ratio of S phase increased in the SFN overexpression group of Hep3B cells compared with that of other groups. In BEL 7402 transfected with SFN siRNA cells, their viability decreased, apoptotic rate increased, cell cycle G0/G1 ratio decreased, and G2/M phase ratio increased compared with that of other groups (Fig. 2B).

3.3. Effects of SFN overexpression and SFN siRNA on cell migration and invasion

Cell migration detection results were shown in Fig. 3A. It was found that the migration rate of Hep3B cells transfected with SFN-pcDNA3.1(+) decreased significantly compared with that of other groups, while that of BEL7402 cells transfected with SFN siRNA did not show significant difference. The invasion results were shown in Fig. 3B. There was no significant difference in the invasion rate among Hep3B cells transfected with SFN-pcDNA3.1(+) and BEL7402 cells transfected with SFN siRNA.

3.4. Expression of CCNB1 and CDK1

The expression levels of CCNB1 and CDK1 were detected by qRT-PCR and Western blotting methods. It was found that the expression of CCNB1 mRNA and protein in Hep3B cells transfected with SFN-pcDNA3.1(+) was not different from that of the control group, but the expression of CDK1 was significantly decreased (Fig. 4A). Compared with the control group, the mRNA and protein expression of CCNB1 and CDK1 in BEL7402 cells transfected with SFN siRNA were significantly increased (Fig. 4B).

4. Discussion

HCC is known as malignant liver cancer. The clinical manifestations of patients with HCC vary greatly because of its complexity, which can be asymptomatic, right upper

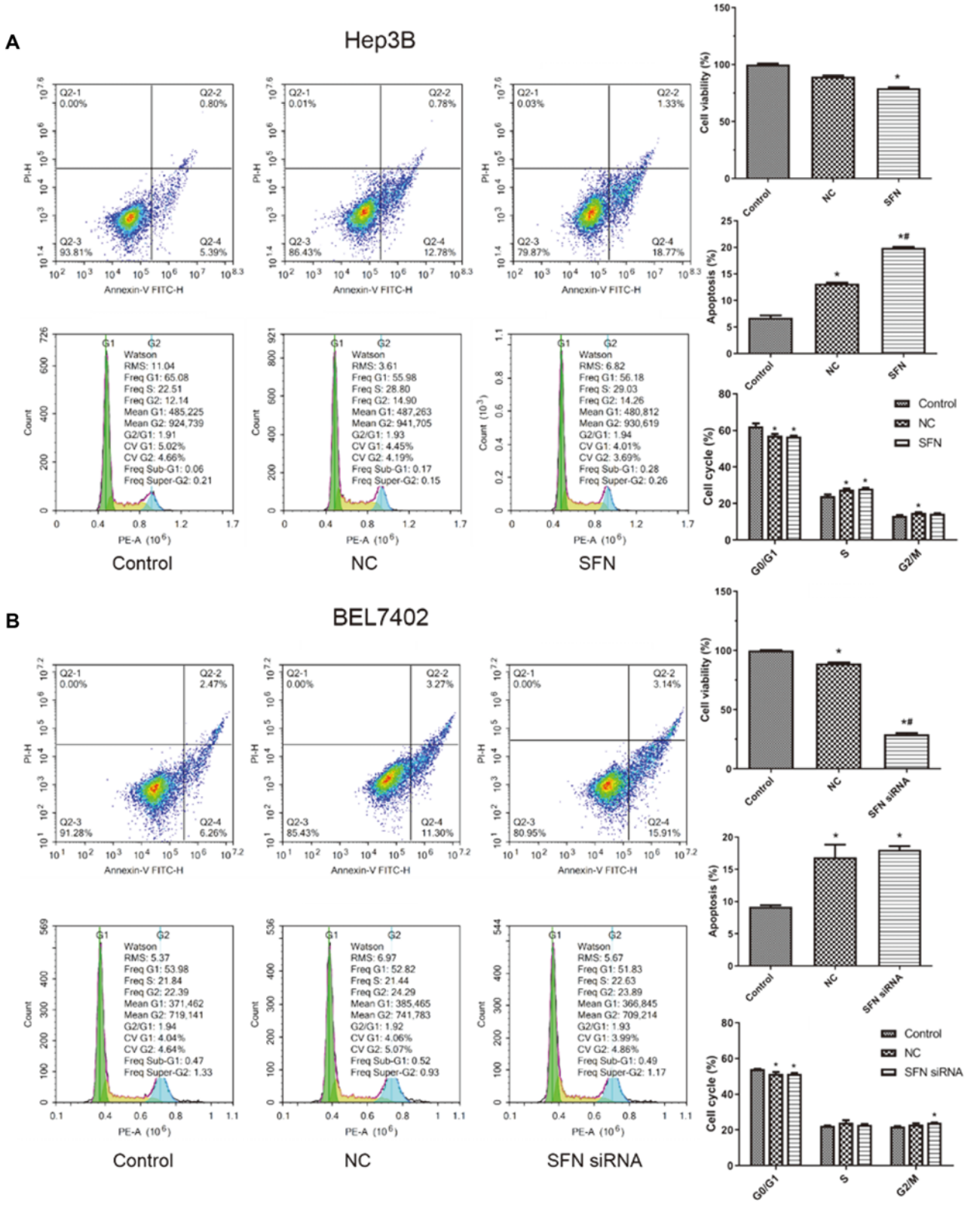

Fig. 2. Effects of SFN overexpression and siRNA on cell viability, apoptosis and cell cycle (N = 3, error bars indicate S.D.). (A) Hep3B cells with low SFN expression transfected by SFN-pcDNA3.1(+). The viability decreased, apoptotic rate increased, cell cycle G0/G1 ratio decreased, and the ratio of S phase increased in SFN group compared with that of other groups; (B) BEL7402 with high SFN expression transfected by SFN-siRNA. The viability decreased, apoptotic rate increased, cell cycle G0/G1 ratio decreased, and G2/M phase ratio increased in SFN siRNA cells compared with that of other groups. *$p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. NC; NC: empty vector.

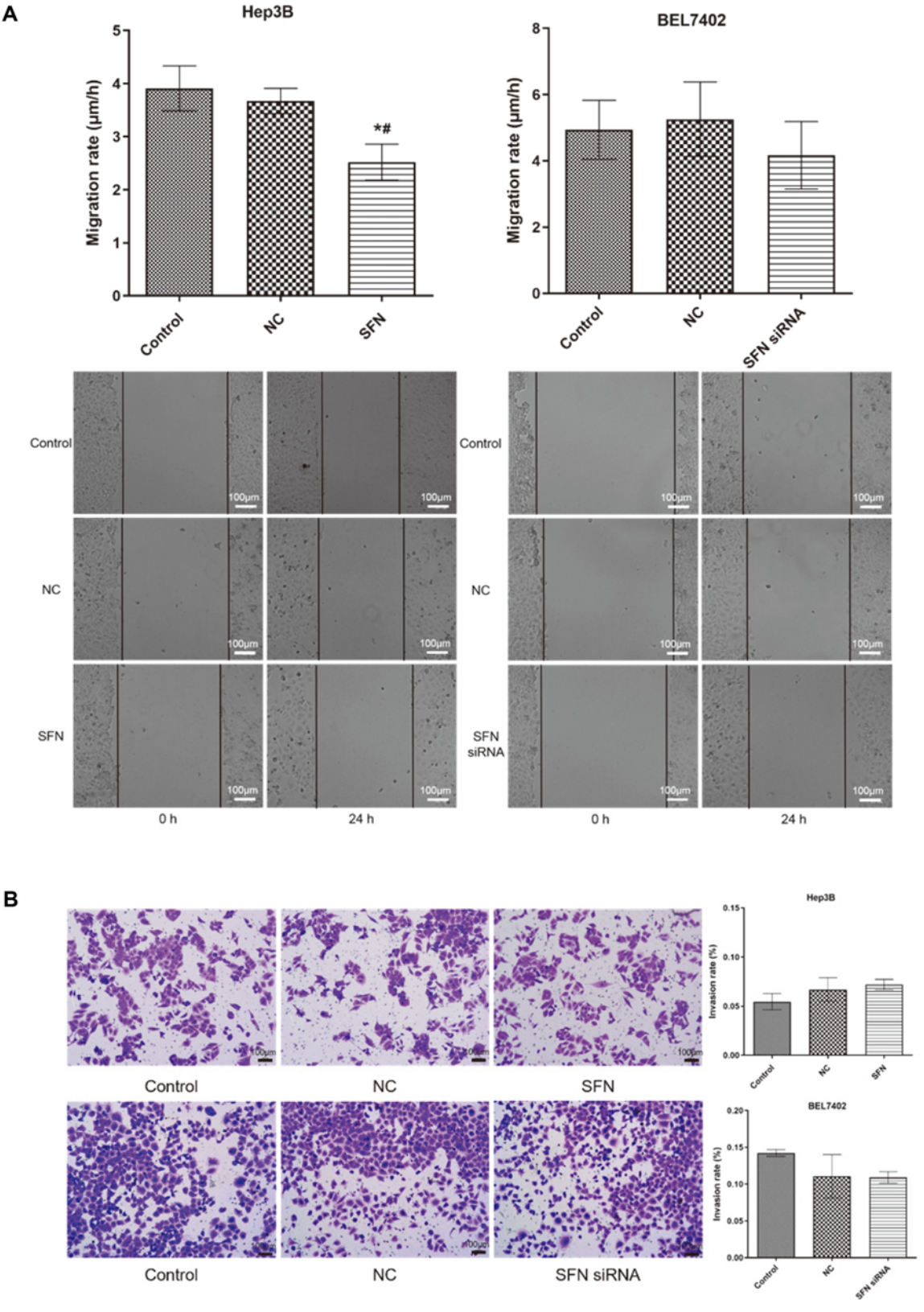

Fig. 3. Effects of SFN overexpression and SFN siRNA on cell migration and invasion (N = 3, error bars indicate S.D.). (A) Cell migration rate in different groups; (B) Cell invasion rate in different groups. The migration rate of Hep3B cells transfected with SFN-pcDNA3.1(+) decreased significantly, and there was no significant difference in the invasion rate among groups. *$p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. NC; NC: empty vector.

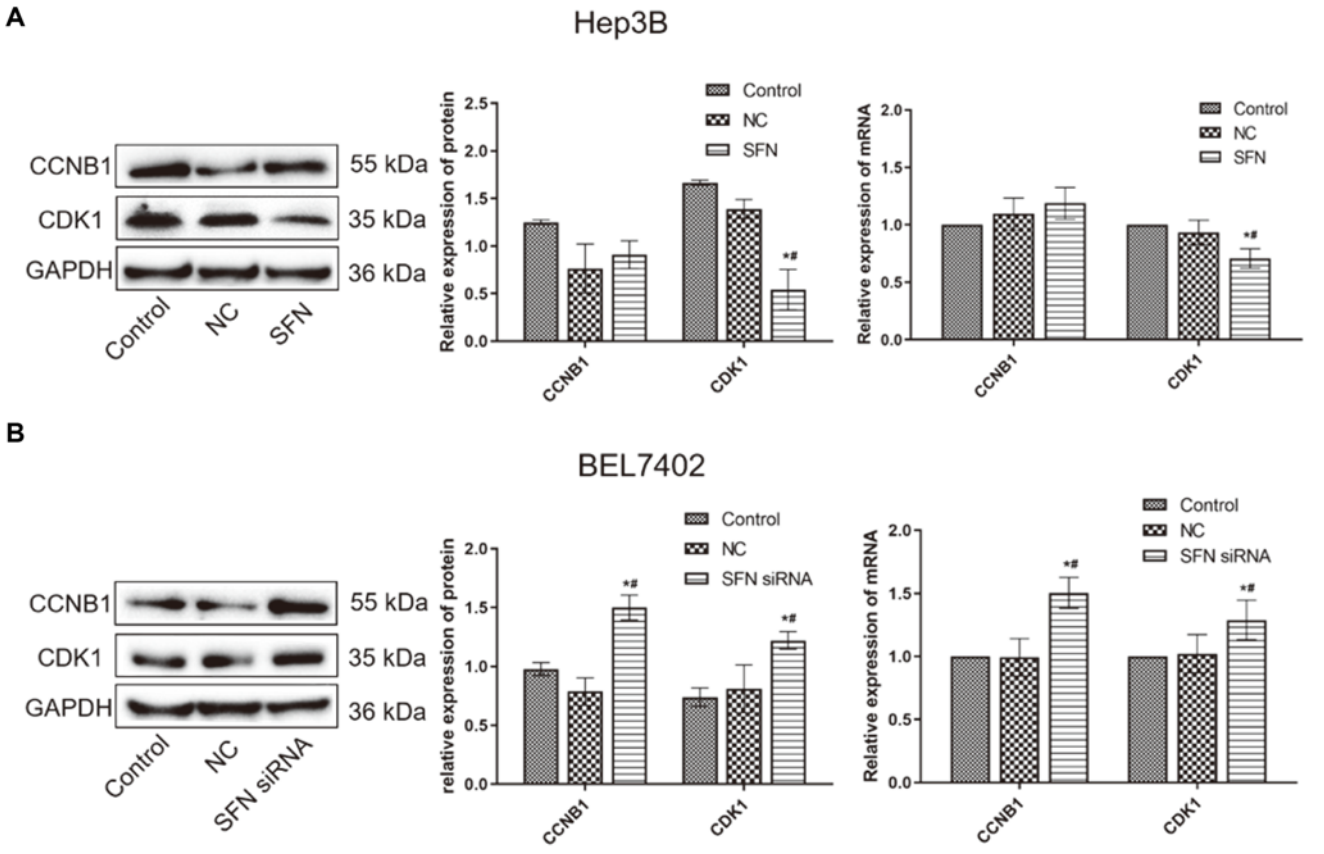

Fig. 4. qRT-PCR and western blotting detection of CCNB1 and CDK1 expression (N = 3, error bars indicate S.D.). (A) CCNB1 and CDK1 expression in Hep3B cells; (B) CCNB1 and CDK1 expression in BEL7402 cells. The expression of CCNB1 mRNA and protein in Hep3B cells transfected with SFN-pcDNA3.1(+) was not different from that of the control group, but the expression of CDK1 was significantly decreased. The mRNA and protein expression of CCNB1 and CDK1 in BEL7402 cells transfected with SFN siRNA were significantly increased. *$p < 0.05$ vs. control; #$p < 0.05$ vs. NC; NC: empty vector.

abdominal pain, body weight reduction, obstructive jaundice, and drowsiness [12]. Its early diagnosis is relatively difficult due to the indistinct pain site and lack of specific clinical symptoms. Most patients have been in the middle and late stage after diagnosis, which is also a major dilemma in the clinical treatment of liver cancer [13]. The etiology of HCC is complex and has not been fully explained. It is generally believed that it is regulated by multiple genes through multiple pathways [14]. The expression of stratifin (SFN) in normal tissues and hepatoma tissues was significantly different. Therefore, Hep3B and BEL7402 hepatoma cells were selected and mRNA and protein expression levels of SFN gene were detected by qRT-PCR and western blotting methods. Compared with the expression differences among the three cells, Hep3B cells with low SFN expression and BEL7402 cells with high SFN expression were selected. Hep3B cells were transfected with SFN-pcDNA3.1(+), and BEL7402 cells were transfected with SFN siRNA. The specific role of SFN was explored in the two cells.

CCNB1 (cyclin B1, cyclin B1) is a kind of cell structural protein. It is a chaperone of maturation promoting factor (MPF) and plays an important role in cell division cycle [15]. CCNB1 protein showed periodic changes in the whole cell cycle. It began to express in S phase, increased in G1 phase and peaked in G2/M. At the end of cell division, the cells began to be ubiquitinated and degraded. When the cell cycle regulation was out of balance, the tumor gene expression was abnormal, which made CCNB1 continue to express, leading to the occurrence of tumor [16,17]. Cyclin-dependent kinase (CDK1) is one of the important members of Ser/Thr protein kinase family and plays an important role in cell cycle [18]. It has kinase activity only when combined with CCNB1. CDK1 is the key factor to promote G2 → M phase transformation [19]. Therefore, the orderly cell cycle is achieved by the co-regulation of CDK1 and CCNB1.

SFN has low expression in many kinds of tumors and promotes the development of tumors. Low expression of SFN and the high expression of Akt in primary breast

cancer tissues were negatively correlated [20]. It was confirmed that the low expression of SFN could cause the high expression of Akt, indicating that the low expression of SFN was closely related to the Akt mediated breast cancer development process [21]. It was also reported that SFN recombinant transfection could inhibit the growth of nasopharyngeal carcinoma CNE1 and CNE2 cells and Akt overexpression, the activity and proliferation of Rat1-Akt cells and its tumorigenicity and growth in nude mice. At the same time, it was confirmed that SFN played an anti-cancer role by regulating Akt-p27 signal transduction pathway, interfering with cell apoptosis and cell cycle [22,23]. SFN may be involved in cell proliferation and apoptosis in gastric cancer. SFN overexpression was associated with an unfavorable prognosis. It may be a promising target for the treatment of gastric cancer [24]. However, there is no report of SFN in HCC.

In this study, we found that up-regulation of SFN gene reduced the activity of Hep3B cells, increased apoptosis, decreased the ratio of cell cycle G0/G1, increased the ratio of S phase, and decreased cell migration. Down-regulation of SFN gene reduced the cell viability of BEL7402, but had no effect on cell apoptosis, migration, and invasion. At the same time, we also found that in Hep3B cells, up-regulation of SFN lead to down-regulation of CDK1. Down-regulation of SFN led to up-regulation of CCNB1 and CDK1 in BEL7402. Up-regulation of SFN inhibited the expression of CDK1 and CCNB1, which resulted in abnormal cell division and inhibition of tumor cell proliferation.

5. Conclusions

In conclusion, SFN had a significant regulatory effect on the cell cycle and apoptosis of liver cancer cells. The high expression of SFN could inhibit the proliferation of liver cancer cells and induce apoptosis, which may be achieved by down regulating CCNB1 and CDK1, but its specific mechanisms need to be further studied.

Author Contributions

Conceived and designed the experiments: Ying Hui and Xue Qin; Execution of experiments: Hao Zeng, Ying Hui and Wenzhou Qin; Data analysis: Peisheng Chen and Ying Hui; Discussion of results: Hao Zeng, Lifang Huang, Wenfu Zhong and Xue Qin; Wrote the manuscript: Liwen Lin, Hui Lv and Hao Zeng, Critical reading of manuscript: Ying Hui, Yi Feng, and Xue Qin.

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Disclosure Statement

The authors declare no conflicts of interest.
Neither ethical approval nor informed consent was required for this study.

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