Int J Clin Exp Med 2019;12(2):1791-1796
www.ijcem.com /ISSN:1940-5901/IJCEM0081175

Original Article

Reduced Sp1 expression inhibits proliferation and migration of HepG2 cells

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Received June 5, 2018; Accepted November 12, 2018; Epub February 15, 2019; Published February 28, 2019

Abstract: Background: Specificity protein 1 (Sp1) is a ubiquitously expressed transcription factor involved in the regulation of a large number of genes including housekeeping genes as well as actively regulated genes. Increasing evidence has indicated that Sp1 is closely correlated with the occurrence and progression of many tumors. However, its function in hepatocellular carcinoma (HCC) is still not well understood. Here, we explored the effects of Sp1 on the proliferation and migration of the HepG2 cell line. The plasmid expression vector of short hairpin RNA (shRNA) targeting Sp1 was constructed and transfected into HepG2 cells by Lipo2000 transfection reagent. Immunocytochemistry, Western blot, and quantitative real-time PCR were applied to determine Sp1 expression, and MTT and transwell assays were used to analyze cell proliferation and migration, respectively. The results show significantly decreased Sp1 expression can inhibit the proliferation and migration of HepG2 cells after transfection with Sp1 shRNA, which then affects biological behavior of HepG2 cells. So Sp1 may be a therapeutic target and potential diagnostic marker in HCC.

Keywords: Transcription factor, hepatocellular carcinoma, proliferation, migration

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed cancers worldwide, and it has been listed as the second leading death cause among cancer-related deaths [1, 2]. Although there are several achievements in early detection, and modern surgical techniques combining with various treatments such as radiotherapy and chemotherapy are applied, the prognosis of HCC patients remains poor [3, 4]. Studies have shown that only 30%-40% of patients are amenable to potentially curative therapies, for which most patients are often found to be at an advanced stage of disease upon diagnosis [5]. Moreover, the overall 5-year survival rate of HCC patients is not satisfactory, as the incidence of recurrence and metastasis remains high after hepatic resection [6].

Specificity protein 1 (Sp1) is a member of the Sp transcription factor family containing C_2H_2-type zinc fingers, and other members include Sp2, Sp3, and Sp4. The family members play important roles in regulation of cell survival, growth, and tumor development and progression through binding to GC-rich sequences of many cellular and viral genes [7, 8]. Different from Sp2 and Sp4, Sp1 and Sp3 have the same consensus-binding sites and are expressed ubiquitously, indicating that they can act as positive or negative regulators of gene expression [9, 10]. Several studies have shown that enhanced expression of Sp1 is correlated with angiogenic potential of cancer tissue and poor prognosis in patients with cancer [11, 12].

Although Sp1 has been associated with the occurrence and development of cancer, it is unclear whether Sp1 expression can affect biology behavior of HCC cell lines. Therefore, in this study Sp1 expression was inhibited and proliferation and migration of HepG2 cells was examined to investigate the roles of Sp1 in HCC.

Materials and methods

Cell line and cell culture

The human HCC cell line HepG2 was obtained from the Chinese Academy of Scien-

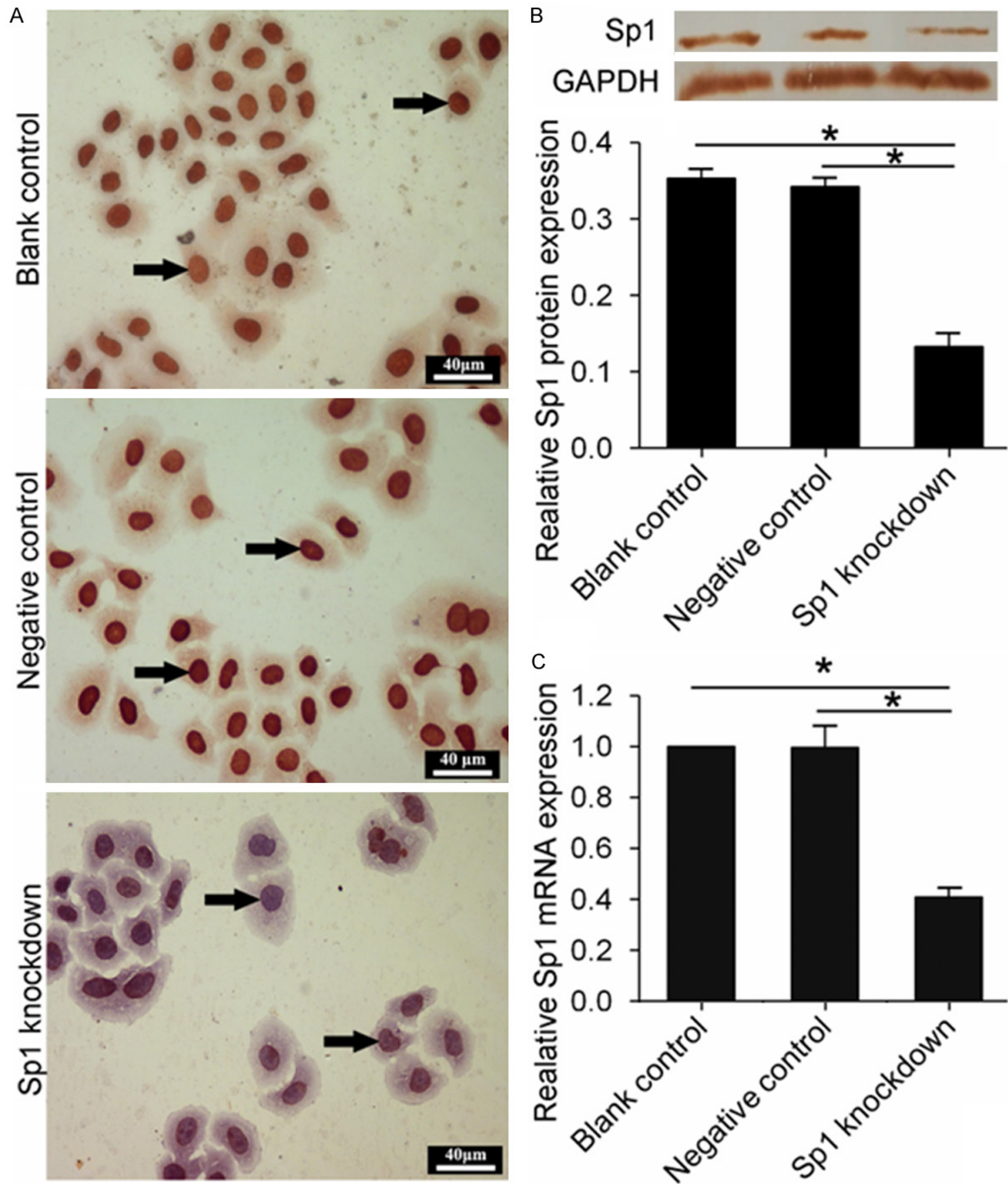

Figure 1. Sp1 expression of HepG2 cells after transfection. A. Representative photograph of Sp1 protein expression in HepG2 cells were detected by Immunocytochemistry (400×), Nuclei of HepG2 cells (Arrow). B. Sp1 protein expression of HepG2 cells by Western blot. C. Sp1 mRNA expression of HepG2 cells by qPCR. $^{*}P < 0.05$.

ces Cell Bank (Shanghai, China) and was maintained in RPMI1640 (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell transfection

A total of 1×10^5 cells/mL HepG2 cells were seeded and grown in six-well plates. When the density of HepG2 cells reached approximately 80% were transfected with 5 μg/mL shRNA tar-

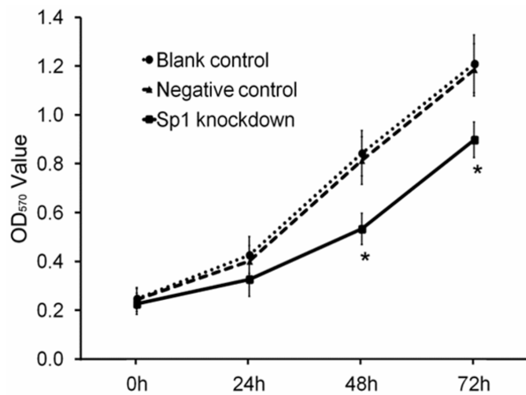

Figure 2. Down-regulation of Sp1 repressed HepG2 cells proliferation. *$P < 0.05$.

geting Sp1 (Genesil, Wuhan, China) using Lipo2000 transfection reagent (Invitrogen, Carlsbad, USA) following manufacturer's instructions.

Immunocytochemistry

A total of 1×10^5 cells/mL HepG2 cells were seeded onto the coverslip and grown overnight in six-well plates after transfection 72 hours. Then, incubated with 4% paraformaldehyde for 10 minutes, 0.5% Txiton X-100 for 20 minutes, 3% H_2O_2 for 10 minutes, and sheep serum for 10 minutes. Rabbit monoclonal antibody to Sp1 (1:200, ab124804, Abcam, Cambridge, UK) was incubated overnight at 4°C, HRP labeled goat anti-rabbit IgG polymer (PV-6001, ZSJB-Bio, Beijing, China) was incubated for 20 minutes, and slides were treated with DAB detection kit (ZSJB-Bio, Beijing, China) to visualize positive staining. Coverslips were observed and photographed under a light microscope (CX41, Olympus, Japan) at 400× magnification.

Western blot analysis

HepG2 cells after transfection 72 hours later were washed twice with cold PBS and total cellular protein was extracted using Epiquik whole cell extraction kit (Epigentek, Farmingdale, USA). Protein extracts (20 μg) were resolved on SDS-PAGE and transferred to PVDF membranes. The membranes were then blocked with 5% nonfat milk powder, and following overnight incubation of membranes with rabbit monoclonal antibody to Sp1 (1:1000, ab124804, Abcam, Cambridge, UK), specific reactive bands were detected using an anti-Rabbit IgG secondary antibody conjugated to HRP (1:1000, Beyotime, Shanghai, China). Immune-reactive bands were visualized with a DAB detection kit (ZSJB-Bio, Beijing, China). Equal loading was verified using a rabbit anti-GAPDH antibody (1:1000, Goodhere, Hangzhou, China).

Quantitative real-time PCR

Expression of Sp1 and reference gene GAPDH was measured by quantitative real-time PCR (qPCR). Primer sequences of Sp1 (Forward: TGGTGGGCAGTATGTTGT, Reward: GCTATTGGCATTGGTGAA) and GAPDH (Forward: GGAGTCAACGGATTTGGT, Reward: GTGATGGGATTTCCATTGAT). Total RNA was isolated from HepG2 cells after transfection 48 hours using TriZol reagent (Invitrogen, Carlsbad, USA), and cDNA was synthesized by reverse transcription (Beyotime, Shanghai, China). qPCR reactions were performed in 20 μL total volumes, and expression of Sp1 and GAPDH was analyzed using the Stratagene Mx3000P software (Agilent Technologies, Inc., Santa Clara, USA) and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ normalization method.

Cell proliferation assay

MTT cell proliferation assays were performed according to manufacturer's instructions (Beyotime, Shanghai, China). A total of 1×10^4 cells/mL was plated in 96-well plates after transfection 24, 48, and 72 hours. After 24 hours, 10 μL MTT (5 mg/mL) was added and incubated for 4 hours, and 100 μL Formanzan dissolved liquid was added and incubated for 4 hours. Absorbance (570 nm) was read using a microplate reader (Bio-Rad, Hercules, USA).

Cell migration assay

HepG2 cells after transfection 48 hours later were seeded on the upper transwell insert with 8-μm pore membrane (Corning, Lowell, USA) containing serum-free medium and the lower chamber was filled with RPMI I640 medium supplemented with 10% FBS. After 24 hour incubation, the cells on the upper surface of the insert were removed, and the cells on the lower surface of the insert were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The numbers of migrated cells were

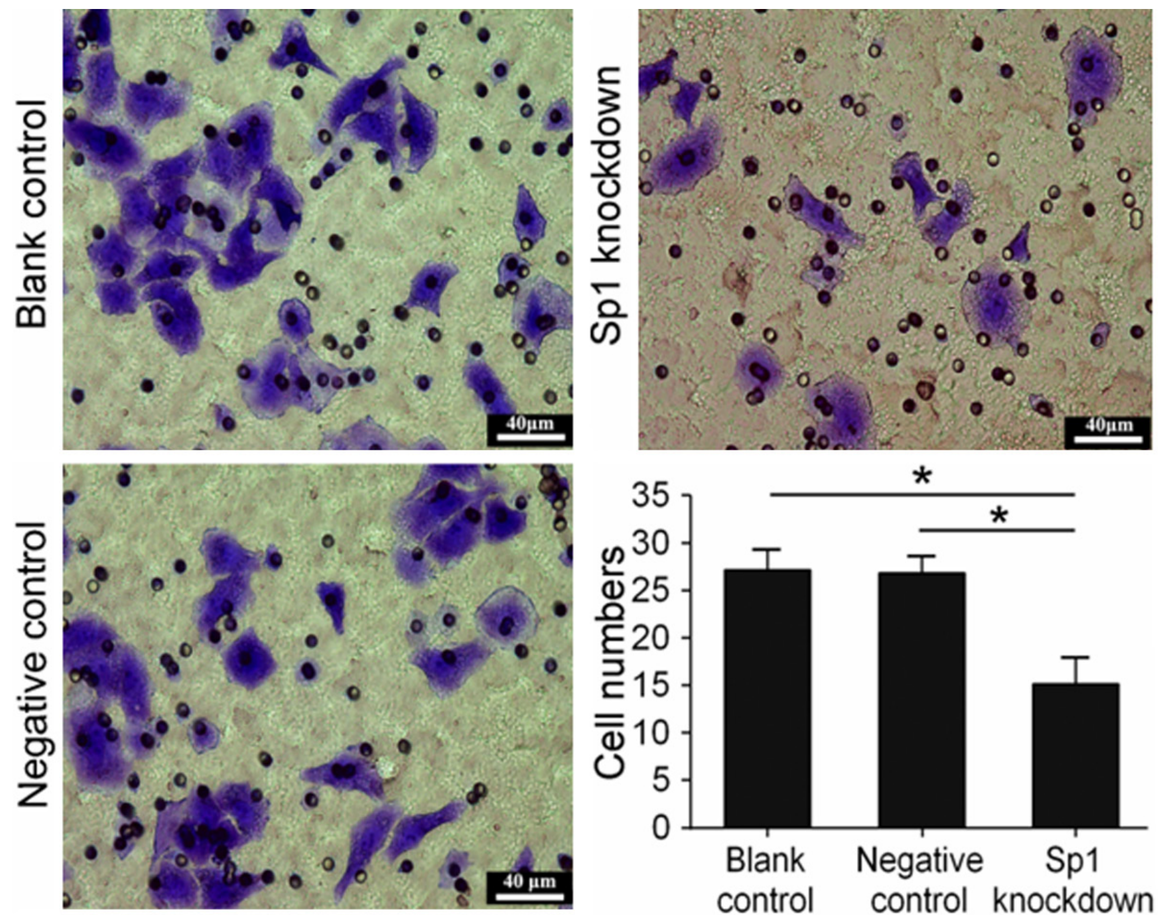

Figure 3. Effect of Sp1 on HepG2 cells migration through a transwell chamber. $^*P < 0.05$.

counted under a light microscope (CX41, Olympus, Japan) at 100× magnification.

Statistical analysis

All data were analyzed by SPSS17.0 (IBM, Armonk, USA) and expressed as mean ± SD. Data were analyzed by one-way ANOVA, and $P < 0.05$ was considered statistically significant.

Results

Sp1 expression of HepG2 cells after transfection

Positive products of Sp1 protein were brown particles mainly distributed in the nuclei of HepG2 cells, and immunocytochemistry results showed Sp1 expression of Sp1 knockdown group obvious weaker than that of negative control and blank control group ($P < 0.05$), and there was no significant difference between the negative control and blank control group ($P > 0.05$, **Figure 1A**). Sp1 protein (**Figure 1B**) and mRNA expression (**Figure 1C**) was also significantly decreased in the Sp1 knockdown group, as compared with the negative control and the blank control group by Western blot and qPCR, respectively ($P < 0.05$). Furthermore, there were no significant difference between negative control and blank control group ($P > 0.05$).

Effects of Sp1 on proliferation of HepG2 cells

To investigate the effects of Sp1 on the proliferation of HepG2 cells, MTT assay was performed following the procedure described in Methods every 24 hours. The results show that Sp1 knockdown significantly reduced HepG2 cells proliferation in comparison to negative control and blank control group after transfection 48 and 72 hours ($P < 0.05$), and had no difference among groups after transfection 24 hours ($P > 0.05$). There were no significant dif-

ference between negative control and blank control group ($P > 0.05$, **Figure 2**). The result suggests that reduced Sp1 expression in HepG2 cells suppressed cell growth.

Effects of Sp1 on migration of HepG2 cells

Transwell migration assay was performed to investigate the effects of Sp1 on the migratory behavior of HepG2 cells. Sp1 knockdown significantly reduced the number of cells that migrated into the lower side of transwell membranes in comparison to cells of negative control and blank control group ($P < 0.05$), and there was no significant difference between the negative control and blank control groups ($P > 0.05$, **Figure 3**). The result suggests that reduced Sp1 expression inhibit the metastatic potential of HepG2 cells effectively.

Discussion

Tumors are often caused by multiple mutations of oncogenes and their products by a number of signal transduction pathways regulating the onset and development of tumors [13, 14]. Sp1 controls number of cellular processes by regulating expression of critical cell cycle, differentiation, and apoptosis-related genes containing proximal GC/GT-rich promoter elements [15]. Current evidence indicates that Sp1 acts as a signal transducers in occurrence, development, and prognosis of multiple tumors. On the one hand, Sp1 as an upstream gene regulated expression of E-cadherin [16] and MALAT1 [17] in lung tumors and SHIP2 [18] in gastric cancer. However, on the other hand, Sp1 as a downstream target gene was medicated by miR-520d-5p [19] in colorectal cancer, miR-205 [20] in esophageal squamous cell carcinoma, and miR-634 [21] in gastric cancer. This study focused on Sp1 because not only previous reports having demonstrated that the expression of Sp was significantly increased in HCC tissues as compared with adjacent normal tissues [22], but also Sp1 over-expression was found in HCC cell lines as compared with normal hepatic cell lines [23]. Both of the previous reports suggested that Sp1 may functionally participate in HCC occurrence and development.

Uncontrolled cell proliferation and aggressive tumor cell metastasis are two essential steps during cancer progression. The former lead to the tumor growth and the latter promote tumor transferring to distant sites. Therefore, in this study the effects of Sp1 on tumor growth and migration of HCC cell line were investigated. Sp1 knockdown in HepG2 cells significantly inhibited cell proliferation and cell migration *in vitro*, indicating that Sp1 expression has a close relationship to biological behavior of HepG2 cells, but the molecular mechanism remain unknown. Future work will explore the molecular mechanism of Sp1 in HCC.

In summary, our studies provide new insights into the molecular mechanism involved in the regulation of HCC growth and provide novel therapeutic targets and strategies for treating HCC.

Acknowledgements

This study was supported by Hainan Provincial Natural Science Foundation of China (Grant No. 814301).

Disclosure of conflict of interest

None.

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