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Original Article

The silence of p66^{Shc} in HCT8 cells inhibits the viability via PI3K/AKT/Mdm-2/p53 signaling pathway

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Abstract: Colon cancer is the second most common cause of cancer-related death, indicating that some of its cancer cells are not eradicated by current therapies. The previous studies demonstrated that p66^{Shc} protein, a member of Shc family, is highly expressed in colon cancer cells, but the role of p66^{Shc} in the progress of colon cancer still unknown. In this study, we found that p66^{Shc} highly expressed in colon cancer tissue and colon cancer cell line SW620 cells, HCT8 cells, HCT116 cells and $CaCO_2$ cells. The silence of p66^{Shc} in HCT8 cells reduced the proliferation and accelerated the apoptosis, in addition, the expression of pro-apoptotic proteins caspase-3, caspase-9, Bax was enhanced and the expression of anti-apoptotic protein Bcl-2 was declined. Moreover, the cell cycle arrest in G0/G1 phase after HCT8 cells treated with p66^{Shc} siRNA. Furthermore, after HCT8 cells treated with p66^{Shc} siRNA, the phosphorylation of PI3K and AKT was significantly suppressed, and the expression of Mdm-2, a downstream of AKT, was obviously prohibited, while the expression of p53 was enhanced. These results indicate that the silence of p66^{Shc} in HCT8 cells inhibits the viability via PI3K/AKT/Mdm-2/p53 signaling pathway, it may provide a promising approach to prevent the progress of colon cancer cell.

Keywords: Colon cancer, p66^{Shc}, HCT8 cells, apoptosis, PI3K/AKT/Mdm-2, p53

Introduction

Colon cancer is the fourth most common cause of cancer diagnosed in males and the third in females. Cancer statistics in the globe discovered that approximately 96,830 new cases and 39,590 deaths from colon cancer will occur in the United States in 2014 [1]. And in China, colon cancer has become the fifth malignant tumor and its incidence has shown an significant increasing trend over the past decade [2]. Although colon cancer patients with early stage can be treated successfully with surgical resection, the patients with terminal stage are refractory. Therefore the effective therapeutic approaches for advanced colon cancer patients are still needed.

In mammalian cells, the Shc A family of adaptor proteins contains three members, p46^{shc}, p52^{shc}, p66^{shc} [3, 4]. The previous studies revealed that Shc proteins can exert mitogenic to promote cell proliferation [5, 6] and anti-apoptotic effects [7]. Recent advances indicate that p66^{Shc} protein is dramatically expressed in epithelial cells and its aberrant expression is identified to be associated with several types of human cancer [8-10]. Therefore, p66^{Shc} proteins may serve as a target in regulating apoptosis and cell proliferation.

A mass of previous studies demonstrated that PI3K/AKT signaling was activated and excessive expressed in multi cancer tissue, such as, gastroenteric tumor, breast cancer and pancreatic cancer [11]. This pathway serves to inhibit many tumor suppressor proteins such as the Bad, FOXO transcription factors, the tuberin/hamartin complex and GSK3 which negatively regulate cell survival, proliferation, and growth [12]. Mayo et al. discovered that PI3K can activate the cyclin-dependent kinase-2 (CDK2) and cyclin-dependent kinase-4 (CDK4), promoting the cells to enter S phase and inducing DNA synthesis [13]. In addition, the activation of AKT which may phosphorylate and inhibit BAD, the

phosphorylated BAD depolymerized with BCL-2, then the unbonded BCL-2 plays anti-apoptotic role [14]. Thus blocking this pathway could therefore simultaneously inhibit the proliferation of tumor cells and sensitize them toward apoptosis.

According to the aforementioned, we hypothesis that the viability of colon cancer cells is able to be suppressed when inhibited the expression of p66^{Shc}.

In this study, we explored the expression of p66^{Shc} in colon cancer tissue and colon cancer cell line cells, we also detected the effects of silenced p66^{Shc} in HCT8 cells on the proliferation, apoptosis, pro-apoptotic and anti-apoptotic proteins expression, cell cycle distribution. Finally the possible mechanism which involved in this process was explored.

Materials and methods

Materials

Cell Counting Kit-8 was obtained from Dojindo (Japan). Cell culture plates were ordered from Corning (NY, USA). RNeasy mini kit was purchased from Qiagen (Valencia, CA). RIPA lysis buffer and PVDF membrane were obtained from Bio-Rad (Hercules, CA, USA). Annexin V/fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Beyotime biotech company (China). RPMI 1640 medium, fetal bovine serum, glutamine, and gentamicin were purchased from Invitrogen (Carlsbad, CA, USA). The primary antibodies including caspase-3, caspase-9, Bax, Bcl-2, β-actin were acquired Cell Signaling Technology (Beverly, MA), and the p66^{Shc} antibody was obtained from (Abcam). The scramble siRNA and p66^{Shc} siRNA were commercial synthesized from Funeng company (Shanghai, China).

Cell lines

The human colon cancer cell lines NCM460, HCT8, HCT116, SW620 and $CaCO_2$ cells were obtained from Funeng biotechnology company (Shanghai, China). Cells were maintained in RPMI 1640 media plus 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin at 37°C and 5% CO_2.

RT quantitative-PCR analysis

HCT8 cells treated with control, scramble siRNA and p66^{Shc} siRNA were used to isolate total RNA respectively by using RNeasy kit according to the manufacturer's protocol. Briefly, first-strand cDNA was reverse-transcribed from 1 μg total RNA using the Super-Script First-Strand cDNA System (Invitrogen), and was amplified by Platinum SYBR Green qPCR SuperMix-UDG. A master mix was prepared for each PCR reaction, which included Platinum SYBR Green qPCR SuperMix-UDG, forward primer, reverse primer, and 10 ng of template cDNA. PCR conditions were 5 min at 95°C, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The forward and backward primer sequences for p66^{Shc} were AATTTGGGCCTCTTGTACAGTT and TACCTCACAGGCCTAGGCGAGG.

CCK-8 assay

HCT8 cells were seeded in a 96-well plate at a concentration of 5×10^3 cells/well for 24 hours. Then scramble siRNA and p66^{Shc} siRNA were added into the medium respectively, the group without any treatment as control. At each time point, HCT8 cells were continually incubated with 10 μL CCK-8 for 4 h. Then the optical density (OD) was determined using an enzyme-linked immunosorbent assay plate reader (Bioreader) with a reference wave length of 450 nm. The culture medium was replaced every two days.

Apoptosis assay

The apoptosis of HCT8 cells treated with control, scramble siRNA and p66^{Shc} siRNA was analyzed using the Apoptosis Detection Kit according to the manufacturer's instructions. Cells were seeded in 6-wells plate at a density of 1×10^5 cells per well with RPMI 1640 medium for 24 hours. Then cells were digested and resuspended in 300 μL binding buffer containing 5 μL Annexin V-FITC and 5 μL PI solution and incubated at room temperature in the dark for 20 min. Stained cells were analyzed by flow cytometry (FACScan; BD Biosciences).

Cell cycle analysis

Cell cycle analysis was carried out by flow cytometry according to a standard protocol. HCT8 cells with the treatment of control, scramble siRNA and p66^{Shc} siRNA were gained by centrifugation respectively, then washed with cold PBS, and fixed with cold 70% ethanol for 12 hours. After this the fixed cells were stained with PI solution consisting of 50 μg/mL PI, 20 μg/mL RNase A, and 0.1% Triton X-100. After

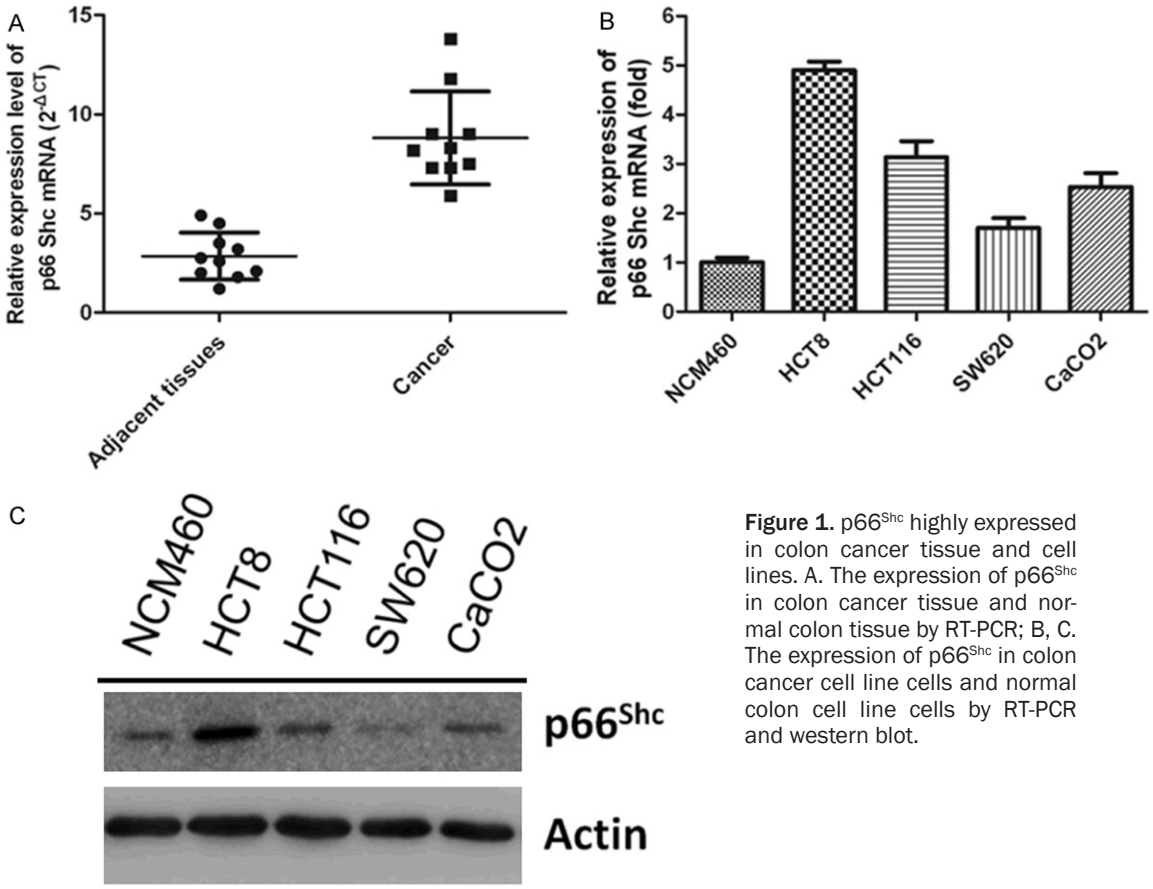

Figure 1. p66Shc highly expressed in colon cancer tissue and cell lines. A. The expression of p66Shc in colon cancer tissue and normal colon tissue by RT-PCR; B, C. The expression of p66Shc in colon cancer cell line cells and normal colon cell line cells by RT-PCR and western blot.

0.5 h incubation in the dark, the stained cells were detected in a FACScan flow cytometer. The distribution of cells in the different cell cycle phases was analyzed using Multicycle software (Phoenix Flow Systems, San Diego, CA).

Western blot

After HCT8 cells treated with control, scramble siRNA and p66Shc siRNA for 24 hours respectively, total cell lysates were prepared using RIPA lysis buffer. For western blot analysis, denaturated protein lysates (50 mg) were pipetted to SDS-PAGE gel. Separated proteins were transferred to PVDF membrane and blocked with 5% skim milk in TBST (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) for 1 hr at room temperature. The membranes were then probed with specific primary antibodies followed by peroxidase-conjugated secondary antibody, and visualized using an ECL detection system.

Statistical analysis

The data were expressed as mean ± standard deviation (SD) and analyzed using IBM SPSS 19.0 statistic software. Statistical differences between the treated and control groups were determined by One Way ANOVA. Differences between means were considered significant if $P<0.05$.

Results

p66Shc highly expressed in colon cancer tissue and cell lines

Firstly we explored the expression of p66Shc in colon cancer tissue by RT-PCR. As **Figure 1A** showed, the expression of p66Shc was significantly higher in cancer tissue than adjacent normal colon tissue. In addition, there was also a statistic difference in the expression of p66Shc between colon cancer cell line SW620 cells ($P<0.05$), HCT8 cells ($P<0.01$), HCT116 cells ($P<0.01$), $CaCO_2$ cells ($P<0.01$) and NCM460 cells (normal colon cell line) (**Figure 1B**). Moreover, the western blot results further demonstrated the expression of p66Shc in colon cancer cell line cells was dramatically higher than normal colon cells (**Figure 1C**).

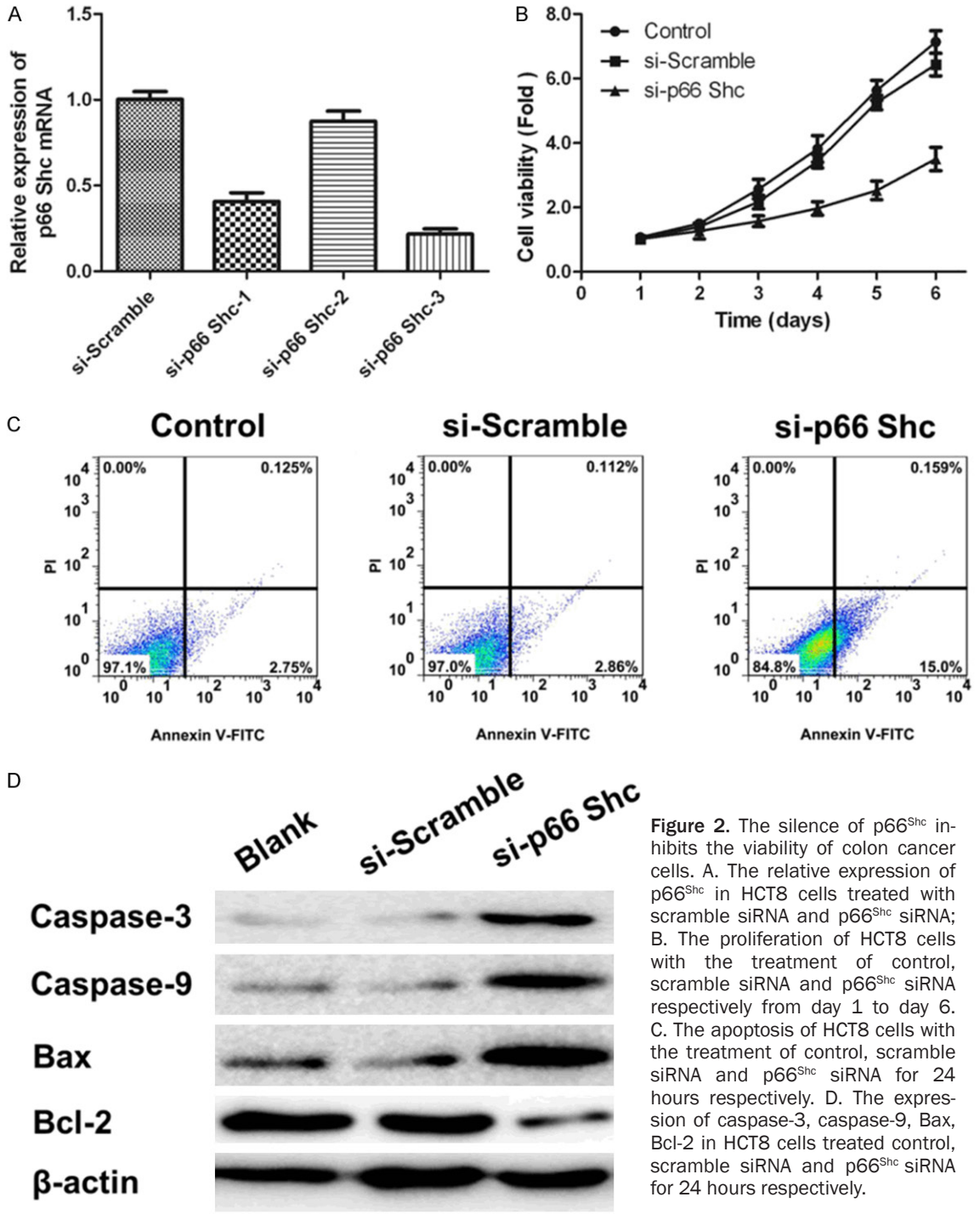

Figure 2. The silence of p66Shc inhibits the viability of colon cancer cells. A. The relative expression of p66Shc in HCT8 cells treated with scramble siRNA and p66Shc siRNA; B. The proliferation of HCT8 cells with the treatment of control, scramble siRNA and p66Shc siRNA respectively from day 1 to day 6. C. The apoptosis of HCT8 cells with the treatment of control, scramble siRNA and p66Shc siRNA for 24 hours respectively. D. The expression of caspase-3, caspase-9, Bax, Bcl-2 in HCT8 cells treated control, scramble siRNA and p66Shc siRNA for 24 hours respectively.

The silence of p66Shc inhibits the viability of colon cancer cells

We have demonstrated that p66Shc highly expressed in colon cancer cells, so what will happen after knockdown p66Shc in colon cancer cells. As shown in **Figure 2A**, the expression of p66Shc was obviously reduced in HCT8 cells treated with p66Shc siRNA, suggesting the expression of p66Shc was successfully suppressed in HCT8 cells. After HCT8 cells treated with p66Shc siRNA, we found that the proliferation of HCT8 cells was significantly prohibited from day 3 to day 6 when compared to the control and scramble siRNA groups (**Figure 2B**). Additionally, the apoptosis of HCT8 cells was dramatically

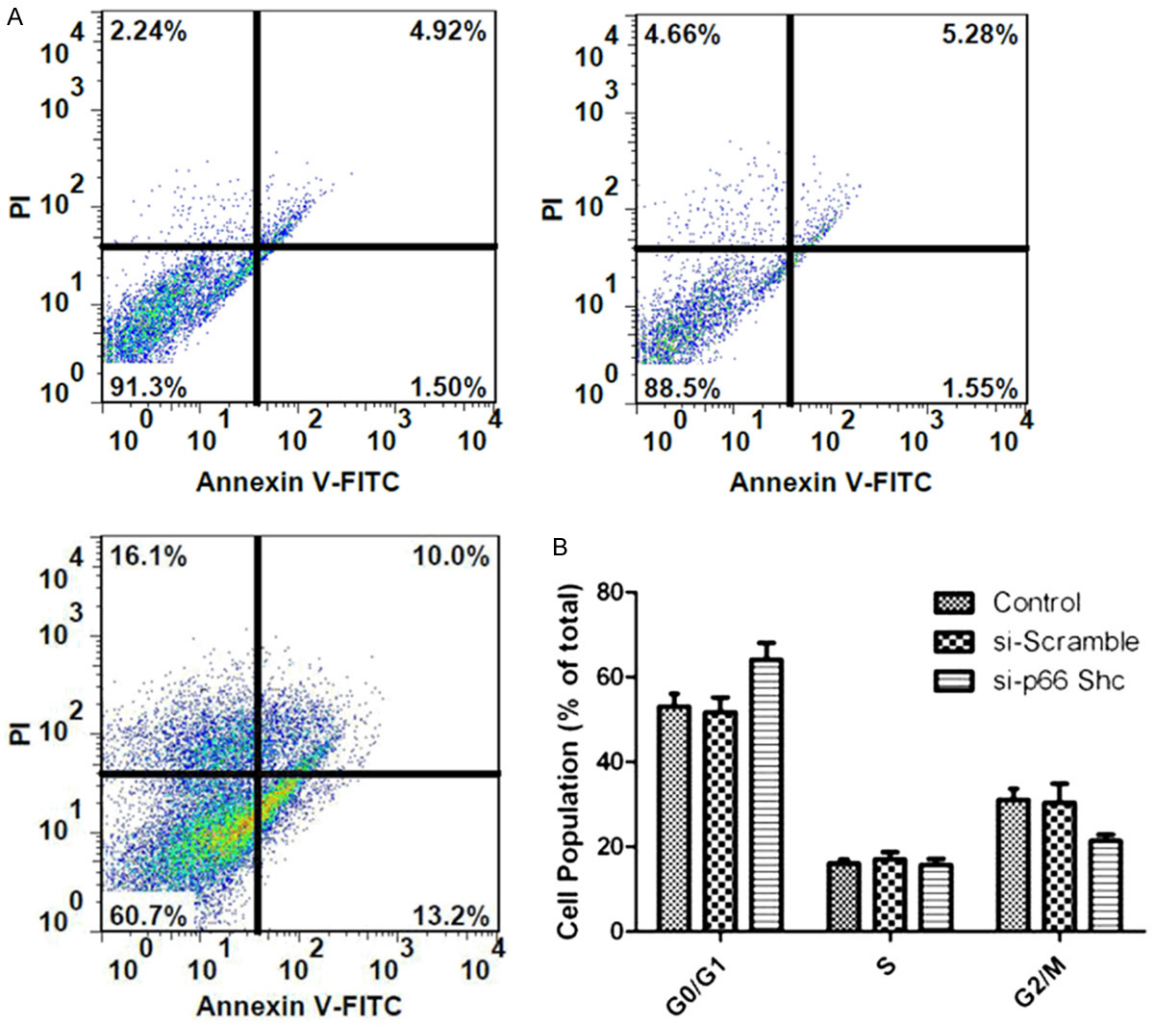

Figure 3. The silence of p66Shc changes the cell cycle distribution of HCT8 cells. A. The effect of p66Shc siRNA on HCT8 cell cycle was explored by flow cytometry; B. The quantitative analysis of HCT8 cell cycle treated with control, scramble siRNA and p66Shc siRNA for 24 hours respectively.

increased in p66Shc siRNA treated group (**Figure 2C**). Furthermore, the expression of caspase-3, caspase-9 and Bax (pro-apoptotic proteins) was significantly enhanced in p66Shc siRNA treated group than control and scramble siRNA groups. On the contrary, the expression of Bcl-2 (anti-apoptotic protein) was declined in p66Shc siRNA treated group (**Figure 2D**). These results revealed that the viability of HCT8 cells can be inhibited after silence p66Shc in colon cancer cells.

The silence of p66Shc induces cell cycle arrest in G0/G1 phase

We also explored whether the silence of p66Shc affected cell cycle distribution. As **Figure 3** showed, in compared to the control and scramble siRNA treated group, the proportion of cells in G0/G1 phase was increased and the proportion of cells in G2/M phase was declined in p66Shc siRNA treated group, which suggests the silence of p66Shc induces cell cycle arrest in G0/G1 phase.

The silence of p66Shc inhibits the viability of HCT8 cells via PI3K/AKT/Mdm-2/p53 signaling pathway

Previously we have proved that the silence of p66Shc in HCT8 cells was able to inhibit HCT8 cells' viability, we also explored the mechanism which involved in this process. As shown in **Figure 4A**, the phosphorylation of PI3K and AKT was significantly suppressed in p66Shc siRNA treated group when compared to the control and scramble siRNA treated groups. Also the expression of Mdm-2, a downstream of

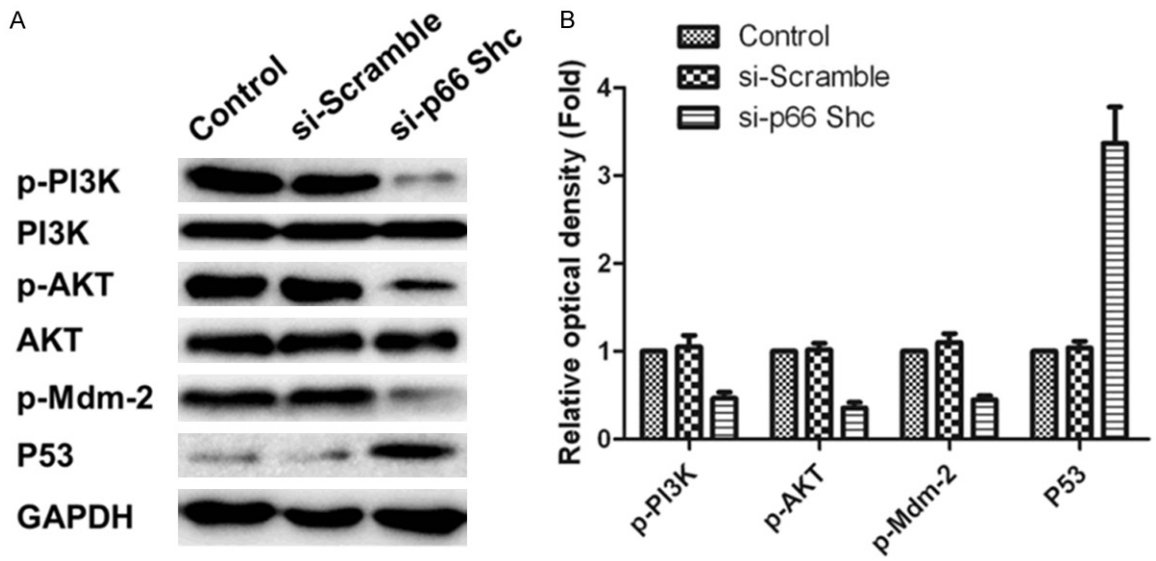

Figure 4. The silence of p66Shc inhibits the viability of HCT8 cells via PI3K/AKT/Mdm-2/p53 signaling pathway. A. The expression of PI3K, AKT, Mdm-2, p53 were detected in HCT8 cells treated with control, scramble siRNA, p66Shc siRNA for 24 hours respectively by western blot; B. The gray scale value analysis of PI3K, AKT, Mdm-2, p53 expression.

AKT, was obviously prohibited when compared to the other groups. In contrast, the expression of p53 was enhanced in p66Shc siRNA treated group than the control and scramble siRNA groups. The gray scale value analysis of PI3K, AKT, Mdm-2, p53 expression further confirmed this result (**Figure 4B**). The results revealed that the silence of p66Shc played an important role in the viability inhibition in HCT8 cells via PI3K/AKT/Mdm-2/p53 signaling pathway.

Discussion

To explore an effective approach to treat the terminal colon cancer is beneficial for the patients. In this study, we found that p66Shc highly expressed in colon cancer tissue and colon cancer cell line cells. The aim of this study was thus to identify the effects of silence of p66Shc on the viability of colon cancer cells which may ultimately initiate the apoptotic cascade leading to cancer cell death.

Recent advances indicate that p66Shc protein is able to promote cell proliferation [5, 6] and reduce cell apoptosis [7]. And p66Shc protein is dramatically expressed in several types of human cancer [8-10]. It is consistent with our findings, we found that p66Shc protein highly expressed in colon cancer tissue and colon cancer cell line cells. Moreover, after silenced p66Shc in HCT8 cells, the expression of pro-apoptotic proteins caspase-3, caspase-9, Bax was increased and the expression of anti-apoptotic protein Bcl-2 decreased. Further studies revealed that the expression of PI3K, AKT, Mdm-2 was suppressed and p53 expression was enhanced. So we speculated that the highly expressed p66Shc activated the PI3K/AKT signaling and inhibited the expression of p53 which lead to progression of colon cancer.

The PI3K-Akt signaling pathway is identified to play an important role in the genesis of some human cancers [15]. The activation of PI3K/Akt signaling can promote the proliferation of cancer cells and prohibit the apoptosis of cancer cells [16, 17]. Mdm-2 is a downstream molecule of Akt, which is associated with the initiation and progression of multi human cancer [18, 19]. The previous studies demonstrated that the highly expression of Mdm-2 was able to enhance the cells' viability, prolong the survival length, promote cell proliferation, boost the growth of tumors [20]. Recent advance revealed that the abnormal expression of Mdm-2 was closely related to the tumor invasion, metastasis, and poor prognosis [21], especially in esophagus cancer, gastric cancer, colon cancer and liver cancer. The activated AKT1 can phosphorylate Mdm-2, then dissociates the AKT1-Mdm-2 compound, the dissociative Mdm-2 enters the nucleus and combines with p53 to form a Mdm-2-p53 compound, thus inhibited the transcriptional activity of p53 and induce p53 degradation [22, 23].

The tumor suppressor gene p53 is important in the etiology of cancer [24]. Agarwal et al. reported that the increased expression of wild-type p53 in response to DNA damage arrests cells late in the G1 stage of the cell cycle by stimulating the synthesis of inhibitors of cyclin-dependent kinases, such as p21/WAF1 [25]. This tumor suppressor protein not only arrests cells late in the G1 stage of the cell cycle, but it is also capable of inducing cell apoptosis [26]. It has been shown that p53 up-regulated the expression of Bax (pro-apoptotic protein) and down-regulated the expression of Bcl-2 (anti-apoptotic protein) [27, 28]. Recent observations found that p53 also plays an important role in the alteration of the Bcl-xL (pro-proliferative protein)/Bax ratio [29], resulting in accelerating the apoptosis of cancer cells.

In summary, in this study we found that the silence of p66^{Shc} in HCT8 cells inhibits its viability via suppressing the expression of PI3K, AKT, Mdm-2 and promoting p53 expression, resulting in cell cycle arrests in the G1 stage, accelerates the expression of pro-apoptotic proteins caspase-3, caspase-9, Bax and declines the expression of anti-apoptotic protein Bcl-2. It may provide a promising approach to prevent the progress of colon cancer cell.

Disclosure of conflict of interest

None.

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