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Full Length Research Paper

Reversion of malignant phenotype of human hepatocellular carcinoma cells by saikosaponin D *in vitro*

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The present study aimed to investigate the effect of Saikosaponin D (SSD) on the malignant phenotype of HepG2 cells and its potential mechanism in order to provide evidence for prevention and treatment of hepatocellular carcinoma (HCC) with SSD. HepG2 cells in the logarithmic phase were randomly divided into two groups: SSD group and control group. Cells in the SSD group were treated with 10 mg/L SSD for 48 h and those in the control group were untreated. Cell inhibition rate was measured by methlthiazolyl tetrazolium (MTT) assay, cell morphology detected by Giemsa staining followed by observation under a contrast phase microscope, the contents of alpha-fetoprotein (AFP) and albumin (ALB) in the supernatant measured by chemiluminescence and radioimmunoassay, respectively. Cell migration was determined by transwel chamber assay and flow cytometry done to measure the cell cycle. Reverse transcriptase- Polymerase chain reaction (RT-PCR) and immunohistochemistry were performed to measure the messenger ribonucleic acid (mRNA) and protein expression of p27, respectively. The growth of HepG2 cells was significantly inhibited after SSD treatment for 48 h (P<0.05). Cells in the SSD group had normal differentiation, became small and round and had small, round nucleus and reduced nuclear-cytoplasmic ratio. When compared with control group, the ALB level was significantly increased (P<0.05) and AFP level markedly reduced (P<0.05). In addition, the number of migrating cells in the SSD group was lower than that in the control group (P<0.01). The SSD treated cells had dramatically higher proportion of cells in G1 phase (P<0.05) and markedly lower proportion of cells in S phase (P<0.05). Furthermore, the mRNA and protein expressions of p27 in the SSD treated HepG2 cells were higher than in those without SSD treatment. SSD can reverse the malignant phenotype of HepG2 cells, which may be associated with up-regulation of p27 expression, arrest of cell cycle in G1 phase and promoted differentiation of HepG2 cells.

Key words: Saikosaponins D, HepG2, malignant phenotype, p27.

INTRODUCTION

Bupleurum is one of Traditional Chinese Medicine and is found to have the anti-tumor effect. SSD is a pharmacologically potent triterpenoid saponin in the Bupleurum. To reverse the malignant phenotype of HCC cells has been an important strategy for the prevention and treatment of HCC. Studies (He et al., 2006; Han et al., 2006) have demonstrated that SSD can induce the apoptosis of HCC cells and reverse the multidrug resistance of HCC. To date, the effect of SSD on the malignant phenotype of HCC is seldom reported. In the present study, we observed the effects of SSD on the proliferation, synthesis and invasion of HCC cells (HepG2) and detected the cell cycle and expression of differentiation related gene in HepG2 cells following SSD treatment, which aimed to explore the mechanism underlying the reverse of malignant phenotype of HCC

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cells by SSD.

MATERIALS AND METHODS

SSD (Jiangxi Herbfine Hi-tech Company Limited) (HPLC>98), RPMI-1640, trypsin (Gibco, USA), newborn calf serum (Hangzhou Sijiqing Biological Engineering Materials Company Limited), methlthiazolyl tetrazolium (MTT) (Beijing Huamei Biotech Company Limited), Trizol (15596-026, Invitrogen, USA), one step RT-PCR kit (Cat: 210212) (QIAGEN, Germany), PCR primers (Shanghai Sangon Biological Engineering Technology and Services Company Limited 100 bp deoxyribonucleic acid (DNA) Ladder (Cat: MD 109), 6×loading buffer (Beijing Tiangen Biotech), SYBR GREEN 1 (AMERSCO), and HepG2 cells (Institute for Viral Hepatitis of Chongqing Medical University) were used in the present study.

Detection of cell proliferation by MTT assay

HepG2 cells in logarithmic phase were seeded into a 96-well plate (4000 cells per 100 µl per well) followed by incubation overnight. Then, these cells were divided into 5 groups and treated with SSD in RPMI-1640 at five different concentrations (0, 2.5, 5, 10 and 20 mg/L) (200 µl/well) and 5 wells were included in each group. Incubation was performed for 24, 48 and 72 h and then 0.5% MTT solution was added to each well (20 µl/well) followed by incubation for another 4 h. The supernatant was removed and dimethyl sulfoxide (DMSO) was added (150 µl/well) followed by gentle shaking for 10 min. Absorbance (A) was measured at 570 nm in a microplate reader and inhibition rate (IR) was calculated as follow:

$$IR=(A_{control} - A_{experiment}) / A_{control} \times 100\%.$$

Giemsa staining for morphological examination

Cells were seeded into a 6-well plate (10000 cells/ml/well) followed by incubation over night. Then, cells were divided into SSD group and control group and three wells in each group. In the SSD group, cells were treated with SSD at 10 mg/L in RPMI-1640 (3 ml/well). In the control group, cells were maintained in RPMI-1640 without SSD. Incubation was done for 48 h and cells were rinsed three times. Subsequently, these cells were fixed in methanol-glacial acetic acid for 10 min and stained with Giemsa for 20 min. After washing with phosphate buffer saline (PBS) three times, cells were air-dried and observed under an inverted phase contrast microscope.

Transwell chamber assay

The Transwell chamber was hydrated with serum-free RPMI-1640 for 1 h. Then, 600 µl of RPMI-1640 containing 10% serum was added to the lower chamber and cell suspension was added to the upper chamber. The cell density was 2×10^6/ml and 200 µl of cell suspension was added to each well. Three wells were included in each group. In the SSD group, cells were treated with 10 mg/L SSD and those in the control had no treatment. Incubation was done at 37°C for 18 h and cells were then washed in PBS twice. Subsequently, cells were fixed in 4% paraformaldehyde and stained with crystal violet. Following washing in PBS twice, cells were observed under a microscope and five fields were randomly selected followed by counting the number of migrating cells. The mean number of migrating cells represents the ability of cancer cells to migrate.

Detection of AFP and ALB

HepG2 cells were traditionally maintained and cell suspension was prepared at 4×10^5/ml. Then, cells were seeded into a 6-well plate (40000 cells/ml/well). Incubation was performed overnight and cells were randomly assigned into two groups each of which included 6 wells. In the SSD group, cells were treated with 10 mg/L SSD in RPMI-1640 (3 ml/well). In the control group, cells were maintained in RPMI-1640 without SSD. Incubation was done for 48 h and supernatant collected. Chemiluminescence and radioimmunoassay were performed to measure the contents of AFP (Yuande Biotech, China) and ALB (Ruiqi Biotech, China), respectively.

Detection of cell cycle

Cells were digested in 0.25% trypsin and then collected followed by fixation in 70% pre-cold alcohol overnight. Before detection, the alcohol was removed and cells were rinsed in PBS twice. Following treatment with 1 ml of PI at 4°C in dark for 30 min, cells were filtered in 400-mm mesh followed by detection of cell cycle by flow cytometry (BECKMAN-COULTER, USA).

Detection of p27 mRNA expression

The complementary deoxyribonucleic acid (cDNA) sequence of p27 was obtained from the national center for biotechnology information (NCBI) and primer 5.0 was employed to design the primers: forward:5'-GGATAAGTGAAATGGATACTACATC -3'; reverse: 5'-AAAAAGAGGGGAAAACCTATTCTAC -3', the anticipated size was 228 bp. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal reference and the primers were as follows: forward: 5'-AGGTCGGAGTCAACGGATTTG -3', reverse: GTGATGGCATGGACTGTGGT -3', and the anticipated size was 532 bp. Cells in different groups were collected and total ribonucleic acid (RNA) was extracted using Trizol. Amplification of target gene and internal reference was performed according to the manufacturer's instructions. Then, products were subjected to electrophoresis and Band Scan 5.0 was employed to measure the optical density (OD). The relative expression of p27 was calculated as follow:

$$OD_{target\ gene}/OD_{internal\ reference}.$$

Detection of p27 protein expression

Cells were grown on the slide and immunohistochemistry was performed with SB method according to the manufacturer's instructions. In the negative control, primary antibody was replaced with PBS. Slides were observed under a microscope and 5 fields were randomly selected and cells were counted at a mediate magnification. A total of 50 cells were counted in each field and p27 positive cells were characterized by yellowish brown granules in the nucleus. The proportion of p27 positive cells was calculated.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and

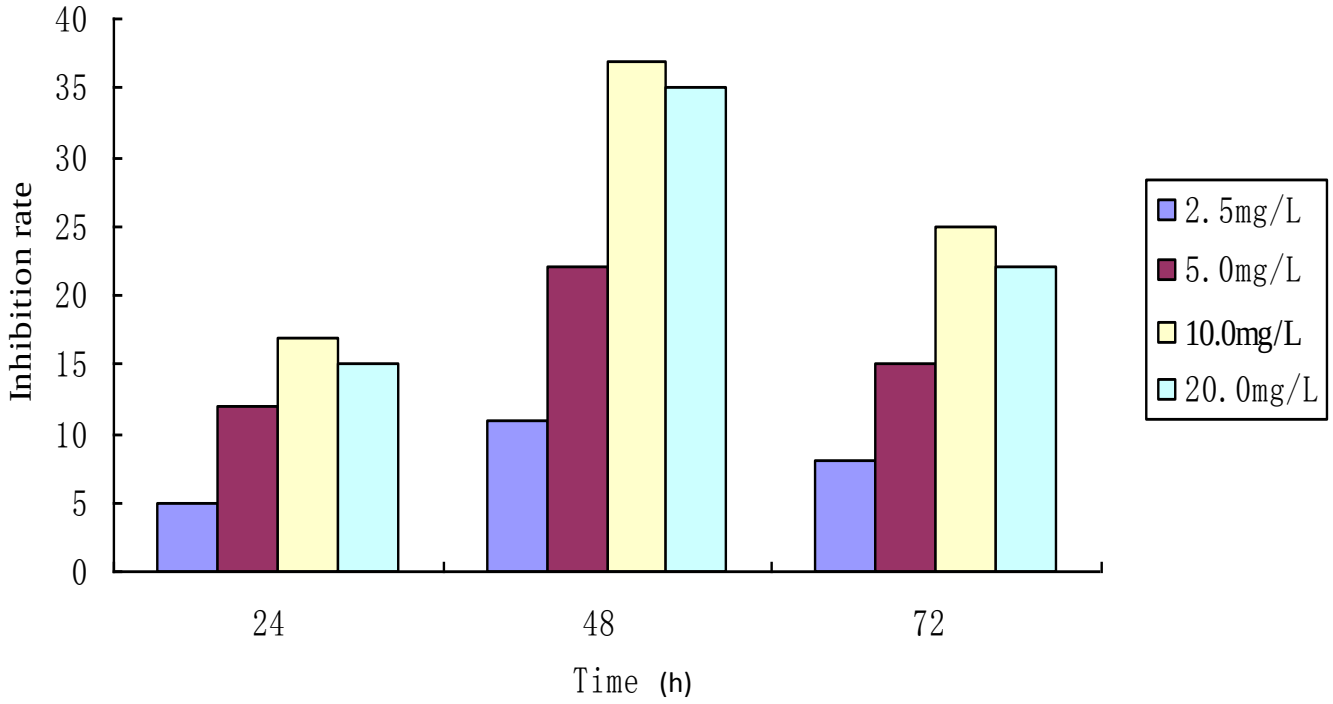

Figure 1. Inhibition rate of HepG2 cell growth following treatment with SSD at different concentrations and for varied durations.

statistical analysis was performed with SPSS version 13.0. Comparisons among different groups were done with one way analysis of variance and those between two groups with t test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Effect of SSD on HepG2 cell proliferation

HepG2 cells were treated with SSD at different concentrations for different durations and cell proliferation was determined. When compared with control group, treatment with > 5 mg/L SSD for 24 h or longer significantly inhibited the cell proliferation ($P<0.05$). The inhibition was more obvious with the increase of SSD concentration and/or prolongation of treatment. The inhibition of cell proliferation was the most obvious after treatment with 10 mg/L SSD for 48 h ($P<0.01$) (Figure 1). Thus, treatment with 10 mg/L SSD for 48 h was used in the following experiments

Effect of SSD on the morphology of HepG2 cells

After treatment with 10 mg/L SSD for 48 h, the adherent HepG2 cells were reduced as compared to control group. The intercellular space was also increased and number of suspended cells elevated. Giemsa staining showed the differentiation of HepG2 cells in the SSD group had a tendency to become normal, the cells became smaller and round, the nucleus appeared to be smaller, round or oval, the nuclear-cytoplasmic ratio reduced, the number of nucleolus decreased, and nucleolus became light or obscure (Figure 2).

Effect of SSD on the migration of HepG2 cells

Following treatment with 10 mg/L SSD for 48 h, the number of migrating cells was 41.00±4.64, which was significantly lower than that in the control group (50.60±4.04) ($P<0.01$). This finding suggests the ability of HepG2 was significantly compromised after SSD treatment (Figure 3).

Effect of SSD on the secretion of AFP and ALB

Following 10 mg/L SSD treatment for 48 h, the AFP level in the supernatant of HepG2 cells was 70.3±5.7 mg/L which was markedly lower than that in the control group (118.2±15.6 mg/L) ($P<0.01$). After SSD treatment, the ALB concentration was 31.1±4.9 mg/L which was significantly higher than that in the control group (24.7±2.8 mg/L) ($P<0.05$). These findings suggest that SSD treatment can decrease the AFP secretion but

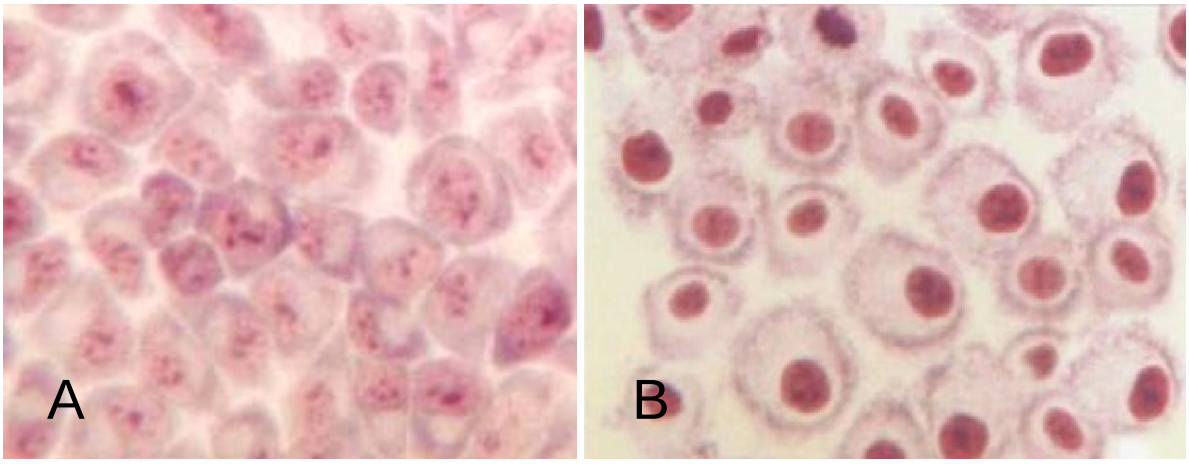

Figure 2. Morphology of HepG2 cells following SSD treatment for 48 h (Giemsa staining, ×400) A: control group; B: SSD group.

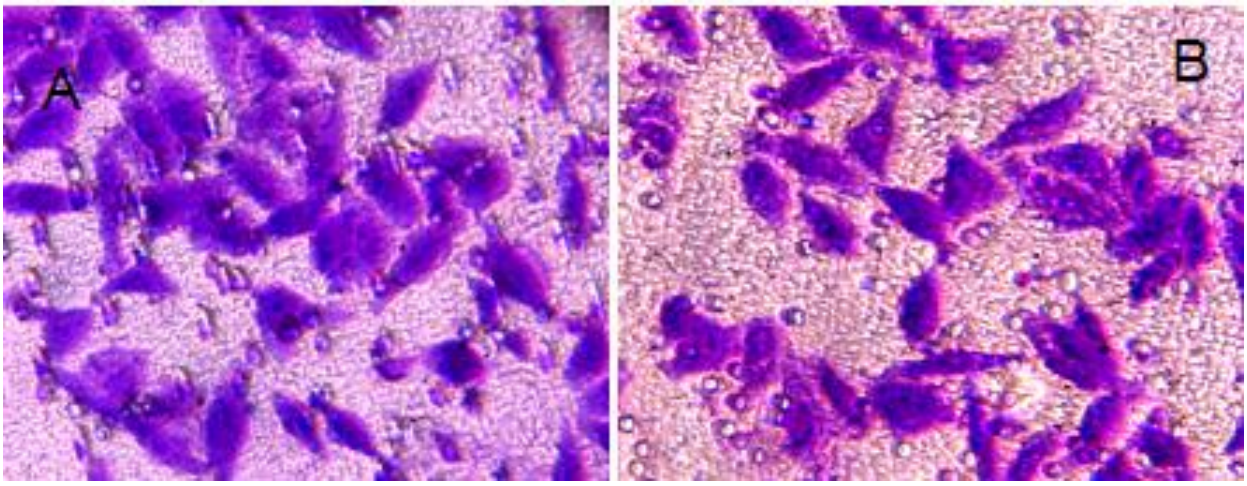

Figure 3. Effect of SSD on the migration of HepG2 cells (×400) A: control group; B: SSD group.

increase the synthesis of ALB.

Effect of SSD on the cell cycle of HepG2 cells

After 10 mg/L SSD treatment for 48 h, the cell cycle of HepG2 cells was significantly changed. Results showed cells in the S phase were markedly reduced but those in the G1 dramatically increased when compared with control group ($P<0.05$).

Effect of SSD on the mRNA expression of p27 in HepG2 cells

Two bands were identified at 216 bp (p27) and 532 bp GAPDH in both groups. The expression of GAPDH was similar between two groups. Results showed the mRNA expression of p27 in the SSD group (0.566±0.001) was significantly higher than that in the control group (0.335±0.000) ($P<0.05$) (Figure 5).

Effect of SSD on the protein expression of p27 in HepG2 cells

The p27 positive cells had yellowish brown granules in the nucleus of HepG2 cells. In the SSD group, the proportion of positive cells was 33.9±3.1, which was dramatically higher than that in the control group (21.9±1.7) ($P<0.01$). This finding indicates that SSD can

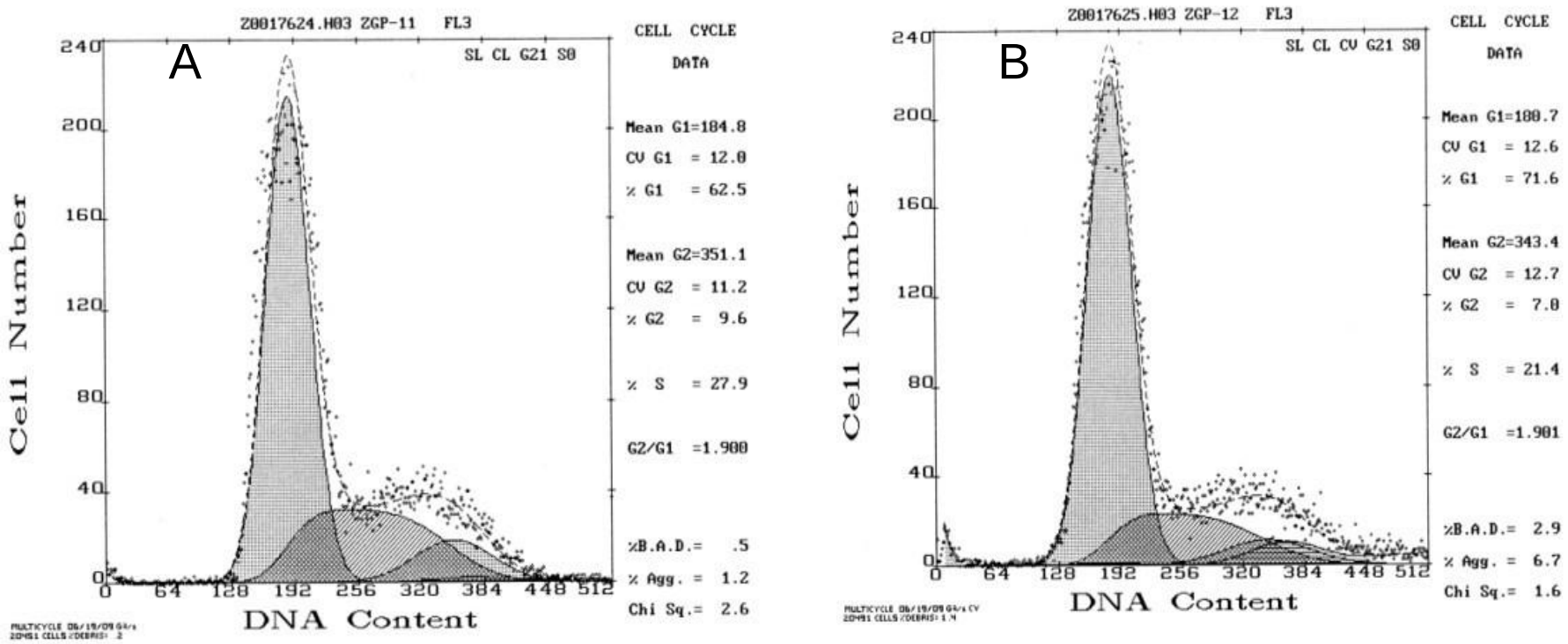

Figure 4. Cell cycle of HepG2 cells following SSD treatment; A: control group; B SSD group.

increase the protein expression of p27.

DISCUSSION

Traditional Chinese medicine and their active ingredient might have advantages in reverse of malignant phenotype of cancer cells. SSD is the most potent Triterpenoid saponin of bupleurum and has been found to affect the malignant phenotype of cancer cells including cell proliferation, cell cycle, morphology, protein synthesis and secretion and infiltration and invasion. Especially, it has favorable promise in reversing the malignant phenotype of HCC cells. The HCC cells have extremely active proliferation, large shape, increased nuclear-cytoplasmic ratio and significantly elevated migration, which are the characteristics of malignant phenotype of HCC cells. The increased secretion of AFP and the compromised synthesis of ALB also reflect the degree of malignancy of HCC. AFP has been accepted as a marker of malignant phenotype of human HCC cells. When the hepatocytes or the cells of fetal gonads become malignant, some genes may be re-activated and AFP which is no long synthesized in adults then is synthesized gain resulting in increased serum AFP. The ALB is synthesized in the liver and reflects the maturation of hepatocytes. Both AFP and ALB can be used to determine the malignant phenotype of HCC cells. In the present study, the cell morphology of HepG2 became nearly normal following SSD treatment, the AFP secretion was reduced but the ALB synthesis increased accompanied by attenuation of migration. These

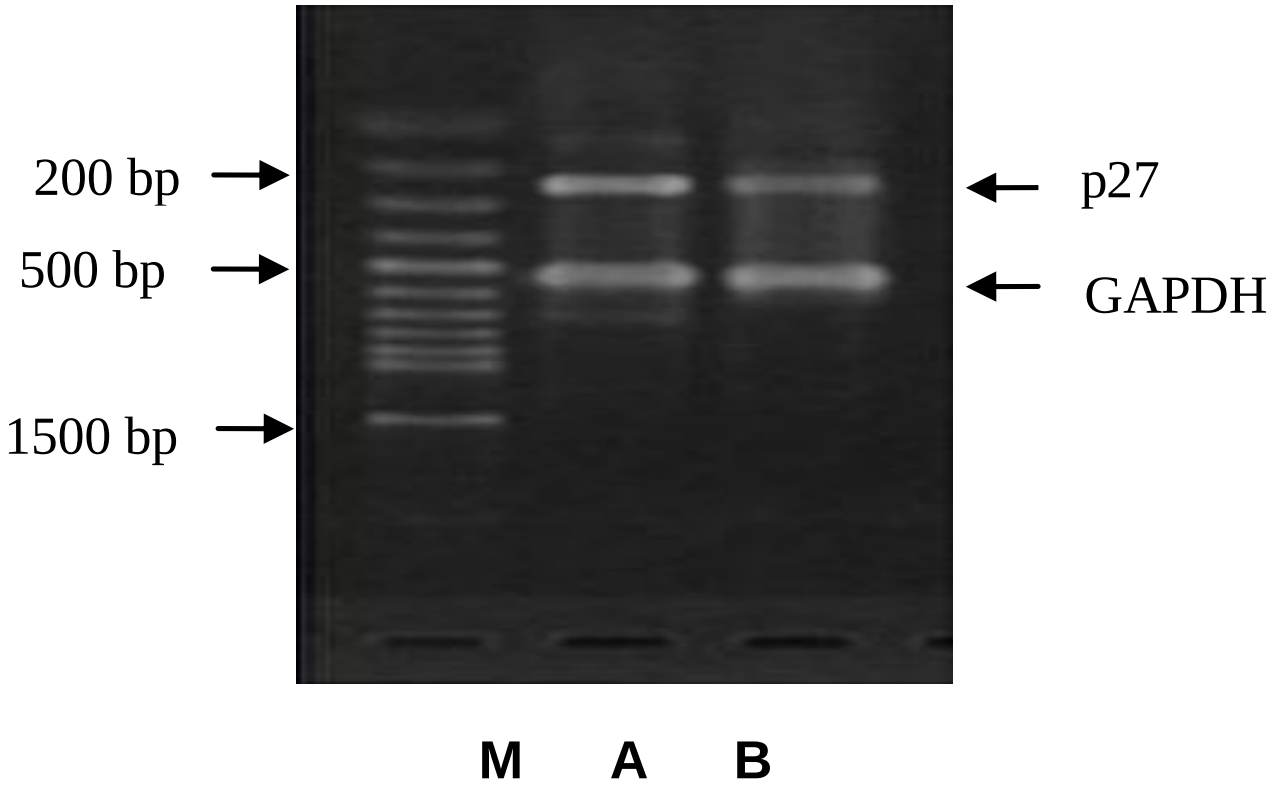

Figure 5. mRNA expression of p27 in HepG2 cells following SSD treatment M: DNA marker; A: SSD group; B: control group.

findings suggest SSD can induce the normal differentiation of HepG2 cells and SSD may reverse the malignant phenotype of HepG2 cells. The normal cell growth depends on the coordinated regulation of cell cycle by different regulators and the imbalance between positive and negative regulatory factors of cell proliferation may result in hyperplasia and disrupted differentiation leading to the tumorigenesis (Boonstra, 2003). The checkpoint dysregulation has been found to be closely related to the tumorigenesis. In the cell cycle, checkpoint functions at three stages: G1/S transition, supervision of S phase and G2/M transition (Golubnitschaja, 2007; Durand et al., 1997). Once the genes regulating cell cycle are abnormally expressed, the cancer cells may not progress into apoptosis under the control of check point which is different from normal cells. Instead, these cells can proliferate indefinitely, which is characterized by increased proportion of cells in the S phase and high proliferative activity.

In the present study, our results showed the cell cycle of HepG2 cells was markedly changed following SSD treatment. The cells in the S phase were significantly reduced but those in the G1 phase dramatically increased. This finding suggests SSD can act at the G1/S transition and arrest the HepG2 cells in G1 phase. It has been confirmed that the induction differentiation of cancer cells may be realized by suppression of telomerase activity, influencing cell signaling pathways, affecting cyclic adenosine monophosphate (cAMP) and tumor genes, etc. Of the tumor related genes, p27 has been demonstrated to be one related to the cell differentiation (Durand et al., 1997; Durand et al., 1998). P27 is one of cyclin-dependent kinase inhibitors (CDKI) and belongs to the CDKI family. It mainly acts as an inhibitor of CDK to negatively regulate the cell cycle. p27 can form a complex with cyclinE/cdk2 in late G1 phase and then inhibit the cdk2 activity. Then, cells cannot transit from G1 phase to S phase where a critical checkpoint of cell cycle, and they may subsequently withdraw from G1/S and progress to differentiate (Dash and El-Deiry, 2004).

Clinical trials showed the p27 expression in the HCC was lower than that in the adjacent normal tissues and the p27 expression was related to the AFP level, pathologic grade, necrosis and metastasis (Wang et al., 2007). Moreover, the p27 protein expression was reduced in the HCC which was associated with the malignancy and invasion of HCC (Wang and Wang, 2002). Fiorentino et al (2001) found that high p27 expression predicted the prolonged survival time of HCC patients and low p27 expression was associated with liver

cirrhosis. In their study, they speculated that elevated p27 expression was an independent factor predicting the prognosis of HCC. In the present study, our findings showed the mRNA expression of p27 was significantly increased following SSD treatment, which indicates SSD can up-regulate p27 expression. The negative regulation of cell cycle by p27 inhibits the proliferation of HCC cells and reverses the malignant phenotype. Our results demonstrate that SSD can revere the malignant phenotype of human HCC cells (HepG2 cells) *in vitro*, which may be related to the down-regulation of p27 expression and subsequent changes in cell cycle. Thus, we speculate that SSD may be used as an inducer of differentiation of HCC cells. The exact mechanism should be further investigated in future studies.

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