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# The expression of SIRT3 in primary hepatocellular carcinoma and the mechanism of its tumor suppressing effects

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**Abstract. – OBJECTIVE: To observe the SIRT 3 expression in primary hepatocellular carcinoma (HCC), and then establish the eukaryotic expression vector of SIRT3 to observe the proliferation and apoptosis of pZsGreen-c1-SIRT3 HepG2 cells. Furthermore, we explored the mechanism of SIRT3 in inhibiting HCC.**

**PATIENTS AND METHODS: Immunohistochemistry was used to detect the expression of SIRT3 in the tumor tissue and para-tumor tissue in 32 patients with HCC and the normal liver tissue in 10 patients. The mRNA of SIRT3 from the normal liver tissue was used as a template, reverse transcription-polymerase chain reaction (RT-PCR) was used to obtain the total sequence of SIRT3 gene, and then the gene was cloned and combined with vector pZsGreen-c1, liposome transfection technology was used to transfect the recombined plasmid into HepG2. The cells were divided into three groups: group A (HepG2 cells as a blank control group), group B (pZsGreen-c1 HepG2 cells as an experimental control group) and group C (pZsGreen-c1-SIRT3 HepG-2 cells as an experimental group). MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) assay was used to detect the growth and proliferation of cells in 3 groups; annexinV/PI double staining was used to detect the apoptotic rates of cells in 3 groups. Western blot was used to detect the protein expression of SIRT3, Fas, Bax and P53, and water-soluble tetrazolium salt (WST-1) was used to detect MnSOD content in 3 groups.**

**RESULTS: Immunohistochemistry results showed that SIRT3 in the tumor tissue sample was positive in 19 patients out of 32 HCC patients; however, there was no strong positive case, the positive rate of SIRT3 expression was 59.38% (19/32). SIRT3 in the para-tumor tissue was positive in 31 HCC patients, the positive rate was 96.88% (31/32), and 18 cases were strongly positive; SIRT3 in normal liver tissue was positive in all 10 cases, the positive rate was 100.0% (10/10), and 7 cases were strongly positive. The differences of SIRT3 positive rate and positive score in tumor tissue from para-tumor tissue and normal liver tissue were statistically significant ($p<0.05$). However, the differences between para-tumor tissue and normal liver tissue were not statistically significant ($p>0.05$). After pZsGreen-c1-SIRT3 transfection, MTT results showed that the OD values in 3 groups were increased with the time, showing time-dependent manner. At 48 h after culture, the OD values in-group C were significantly different from group A and B, and the inhibitory rates were statistically different ($p<0.05$). After 48 h, the OD values and inhibitory rates in-group C showed that the cells were obviously inhibited, and the inhibitory rates were increased with the time, showing time-dependent manner. Flow cytometry results showed that the cell numbers of early stage apoptosis and late stage apoptosis were significantly increased in group C, which was significantly higher than group A and B, the differences were statistically significant ($p<0.01$). Western blot results showed that expression levels of SIRT3, p53, Bax and Fas were not different between group A and B ($p>0.05$); SIRT3, p53, Bax and Fas in group C were significantly increased, which were statistically higher than group A and B ($p<0.01$). WST-1 results showed that MnSOD contents were not statistically different between group A and B ($p>0.01$). MnSOD content in-group C was significantly higher than the other groups, which were statistically significant ($p<0.01$).**

**CONCLUSIONS: SIRT3 shows low expression or deficiency in HCC tissue, indicating that SIRT3 expression can affect the occurrence and development of HCC. SIRT3 can inhibit the growth and proliferation of HepG2 cells and induce HepG2 cell apoptosis. The mechanism may be related to the up-regulation of MnSOD and p53, the up-regulation of Bax and Fas by MnSOD.**

Key Words:
Primary hepatocellular carcinoma, SIRT3, Immunohistochemistry, Western blot, SIRT3 pZsGreen-c1 transfection, Mechanism, MnSOD, p53, Apoptosis.



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## Introduction

Primary hepatocellular carcinoma (HCC) is the most common malignant tumor worldwide, the mortality of which is third among all the cancers, just next to gastric cancer and esophageal cancer[1-3]. China is the highest prevalence area of HCC, the proportion of patients died from HCC in China is about 50% of that in the whole world. In the recent 20 years, although the treatment of HCC has been significantly improved, the morbidity and mortality remain the second among all the cancers[4,5]. SIRT3 is a member of silent information regulator family, which is a major newly discovered histone deacetylase in mitochondria. Mitochondria cannot only produce reactive oxygen species (ROS), but it is also the target organelle of the antioxidant enzyme, and mitochondrion is the place of eliminating ROS[6,7]. ROS is closely related to the development of HCC, thus dysfunction of mitochondria is critical in the development of HCC. Increased SIRT3 expression can eliminate ROS and down-regulate HIF1a expression by up-regulating MnSOD to alleviate the oxidative stress caused by ROS[8,9]. Thus, SIRT3 is closely related to the regulation of ROS. However, there is no report that focuses on the role of SIRT3 in the pathogenesis of HCC. Thus, to further reveal the function of SIRT3 in HCC is significant for providing a reference in the diagnosis and treatment of HCC.

## Patients and Methods

### Experimental Instruments and Reagents

#### *The experimental Instruments*

RM2135 microtome was purchased from Leica Biosystems (Wetzlar, Germany); high speed centrifuge was purchased from Eppendorf AG (Hamburg, Germany); BIO-RAD Trans-Blot equipment was purchased from Bio-Rad Laboratories (Inc., Hercules, CA, USA); electrophoresis system was purchased from Dingguo (Biotechnology Development Center, Beijing, China); super clean bench was purchased from Suzhou Purification Engineering Company, (Suzhou, China); BD FACS Aria flow cytometer was purchased from Becton (Dickinson and Company, Franklin Lakes, NJ, USA); GeneAmp® PCR System 9600 was purchased from Applied Biosystems (Inc., Carlsbad, CA, USA); UV-7502C ultraviolet spectrophotometer was purchased from Jiangyi Instrument (Co., Ltd, Shanghai, China).

#### *Reagents*

Immunohistochemistry Kit (KIT-5020 goat anti-mouse) was purchased from Maixin (Biotechnology Company, Fuzhou, China); mouse anti-human SIRT3 monoclonal antibody was purchased from Abcam (Cambridge, UK); HRP labeled goat anti-mouse IgG was purchased from Zymed Laboratories (Waltham, MA, USA); HRP labeled goat anti-mouse IgG+A+M was purchased from Zymed Laboratories (Waltham, MA, USA); HRP labeled rabbit anti-goat IgG was purchased from Zymed Laboratories, (Waltham, MA, USA); HRP labeled goat anti-rabbit IgG was purchased Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA); goat anti-human SIRT3 antibody was purchased from Abcam plc., (Cambridge, UK); mouse anti-human GAPDH antibody was purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA); rabbit anti-human Fas antibody was purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA); rabbit anti-human Bax antibody was purchased from Cell Signaling Technology Inc., (Boston, MA, USA); mouse anti-human p53 was purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA); RNA extraction kit was purchased from Tiangen Biotechnology Co. Ltd., (Beijing, China); PCR kit was purchased from QIAGEN Inc., (Hilden, Germany); HepG2 cell line was purchased from Yaji Biological Technology Co., Ltd; (Shanghai, China). Lipofectamine™ 2000 was purchased from Thermo Fisher Scientific Inc., (Waltham, MA, USA); MTT kit was purchased from Sigma-Aldrich Company (St. Louis, MO, USA); Annexin V-FITC & PI apoptosis kit was purchased from KeyGen Biotechnology Co., Ltd., (Nanjing, China); BCA protein concentration detection kit was purchased from Thermo Fisher Scientific Inc., (Waltham, MA, USA).

## Methods

#### *The Collection and Grouping of Tumor Tissue Sample, Para-Tumor Tissue Sample and Normal Liver Tissue Sample*

Tumor tissue group: the primary HCC samples and clinical characteristics from 32 patients admitted in Department of Hepatobiliary Surgery in the Second Xiangya Hospital between March, 2011 and November, 2011 were collected (the clinical characteristics are shown in Table I). All the HCC patients did not receive any treatment before surgery, and were all confirmed as prima-

**Table I.** The clinical and pathological characteristics of liver cancer patients.

| Case number | | 32 |
|---|---|---|
| Gender | Male | 23 |
| | Female | 9 |
| Age | >60 | 13 |
| | ≤60 | 19 |
| | Average age | 46.3±8.5 |
| | Age range | 28-71 |
| Hepatitis history | Hepatitis B | 28 |
| | Hepatitis C | 2 |
| | No hepatitis | 2 |
| AFP value | ≥20 μg/L | 22 |
| | <20 μg/L | 10 |
| Tumor size | ≥5 cm | 15 |
| | <5 cm | 17 |
| Liver carcinoma envelop | Yes | 18 |
| | No | 14 |
| Differentiation degree of HCC | High differentiation | 8 |
| | Moderate differentiation | 17 |
| | Low differentiation | 7 |
| Portal vein tumor thrombus | Yes | 9 |
| | No | 23 |

ry HCC by pathological examination. Para-tumor liver cirrhosis group: the non-necrotic para-tumor liver cirrhosis tissue which were more than 2 cm from the tumor edge from the above 32 HCC patients were collected, and were confirmed as liver cirrhosis tissue without carcinogenesis or invasion. Normal liver tissue group: the normal liver tissues were collected from the 10 patients with hepatic hemangioma or traumatic liver rupture, all the patients had no hepatitis history and the tissues were confirmed as normal liver tissue by pathological examination. The collection of all cases and the pathological tissue samples were approved by the Ethics Committee of the Second Xiangya Hospital.

### Immunohistochemistry

The tissue samples were fixed in 10% neutral formalin, dehydrated, embedded in paraffin, dewaxed and hydrated; then, antigen retrieval was completed; the primary antibody and secondary antibody were added respectively, and 3,3'-diaminobenzidine (DAB) was used to develop, hematoxylin was used to dye the samples and then the samples were observed under the microscope. The assessment of immunohistochemistry results was completed by 2 experienced pathologists. 5 fields of each slide were randomly selected to calculate the positive rate of the slides: the positive rate (%) = number of positive cases (weak positive + positive + strong positive)/total case number×100%.

### Synthesis and Transfection of SIRT3 Eukaryotic Vector

Eukaryotic vector pZsGreen-c1 was purchased from Clontech Laboratories Inc., (Mountain View, CA, USA). The total RNA was extracted from the para-hemangioma tissue during surgery, and reversely transcripted to cDNA, and then the gene was amplified by PCR. The PCR product was digested with restriction enzyme, and then the gene was connected to pZsGreen-c1. The recombined plasmid was imported into E.coli to extract more plasmids. The transfection was completed according to the protocol for Lipofectamine™ 2000. pZsGreen-c1 and pZsGreen-c1-SIRT3 were transfected into HepG2 respectively.

### The Cell Viability Detected by MTT

There were three groups: group A (HepG2 cells as blank control group), group B (pZsGreen-c1 HepG2 cells as an experimental control group) and group C (pZsGreen-c1-SIRT3 HepG-2 cells as an experimental group). 100 μl cell suspension was seeded in 96-well plate ($5\times10^3$/well), then 20 μl 5 g/L MTT solution and 150 μl DMSO (dimethyl sulfoxide) solution were added respectively. The OD values at 0 h, 24 h, 48 h, 72 h and 96 h were detected to draw a growth curve, and the inhibitory rates were calculated to draw an inhibitory rate curve.

### The Apoptotic Rates Detected by AnnexinV/PI Staining

1-$5\times10^5$ cells were collected in the test tube, 500 μl binding buffer was added to re-suspend the cells. 5 μl Annexin V-FITC (fluorescein isothiocyanate) was added, and then 5 μl propidium iodide was added and mixed. The mixture was kept at room temperature in darkness for 15 min, and then it was detected by flow cytometry within 1 h.

### Expression Levels of SIRT3, Fas, Bax and p53 Detected by Western Blot in 3 groups

The total proteins were extracted from 3 groups, BCA protein assay was used to detect the concentration of the proteins. The proteins were loaded and received sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto the membrane and blocked with 5% skimmed milk overnight; the membrane was washed with phosphate buffered saline and tween

**Table II.** SIRT3 expression in tumor tissue, para-tumor cirrhosis tissue and normal liver tissue.

| Group | Case number | SIRT3 expression | | | | Positive rate (%) |
|---|---|---|---|---|---|---|
| | | - | + | ++ | +++ | |
| Tumor tissue group | 32 | 13 | 14 | 5 | 0 | 59.38* |
| Para-tumor cirrhosis group | 32 | 1 | 0 | 13 | 18 | 96.88 |
| Normal liver tissue group | 10 | 0 | 1 | 2 | 7 | 100.00 |

Note: *$p<0.05$ the expression in tumor tissue group compared with para-tumor cirrhosis group and normal liver tissue group.

(PBST) for 3 times; the corresponding primary antibody was added and incubated for 2 h at room temperature, the membrane was washed with PBST for 5 min for 3 times. Then, the secondary antibody was added and incubated at room temperature on a shaker for 1 h, and washed with PBST for 5 min for 3 times. ECL substrate was added and the membrane was developed. The bends were analyzed by Gel pro4.0, and the relative value of each bend was calculated (the gray value of target protein/the gray value of GAPDH).

### MnSOD Content Detected by WST-1

The cells were collected and homogenized in 500 μL homogenization buffer, and then centrifuged for 1000 rpm/min at 4°C for 5 min. The supernatant was transferred into another 1 ml tube to detect the SOD activity in cytoplasm and mitochondria. The reagents were added according to the protocol, and SOD enzyme solution was added and incubated at 37°C for 20 min. The OD values of each well were detected at 450 nm wave-length. MnSOD inhibitory rate=[(control 1-control 2)-(sample-control 3)]/(control 1-control 2), MnSOD active unit = inhibitory rate/(1-inhibitary rate)U. The protein concentrations of 3 groups were detected and the MnSOD active unit was converted to U/mg protein.

### Statistical Analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for analysis. The data were analyzed by ANOVA or independent-samples *t*-test. The data were presented as mean± standard deviation (SD). The numeration data were compared by using non-parametric rank sum test. $p<0.05$ represents that there is a statistical difference, and $p<0.01$ represents there is significant statistical difference.

## Results

### The Expression of SIRT3 in Tumor Tissue, Para-Tumor Cirrhosis Tissue and Normal Liver tissue

Immunohistochemistry results showed that SIRT3 in the tissue sample was positive in 19 patients out of 32 HCC patients; however, there was no strong positive case and 13 cases were negative, the positive rate of SIRT3 expression was 59.38% (19/32); SIRT3 in para-tumor tissue was positive in 31 HCC patients, the positive rate was 96.88% (31/32), and 18 cases were strongly positive, only 1 case was negative. SIRT3 in normal liver tissue was positive in all 10 cases, the positive rate was 100.0% (10/10), and 7 were strongly positive (as shown in Table II). The positive scores in tumor tissue, para-tumor tissue and normal liver tissue were 2.31±3.20, 8.72±3.35 and 9.20±4.62 (as shown in Table III). The differences of SIRT3 positive rate and positive score in tumor tissue from para-tumor tissue and normal liver tissue were statistically significant ($p<0.05$).

**Table III.** The positive scores of SIRT3 in HCC group, paratumor cirrhosis tissue and normal liver tissue.

| Group | Case number | Positive score | Mean±SD |
|---|---|---|---|
| Tumor tissue group | 32 | 74 | 2.31±3.20* |
| Para-tumor cirrhosis group | 32 | 279 | 8.72±3.35 |
| Normal liver tissue group | 10 | 92 | 9.20±4.62 |

Note: *$p<0.05$ the expression in tumor tissue group compared with para-tumor cirrhosis group and normal liver tissue group.

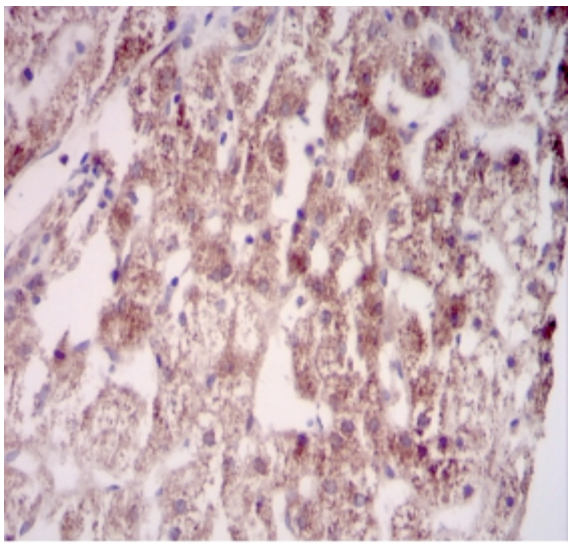

**Figure 1.** SIRT3 expression in the normal liver tissue (×200).

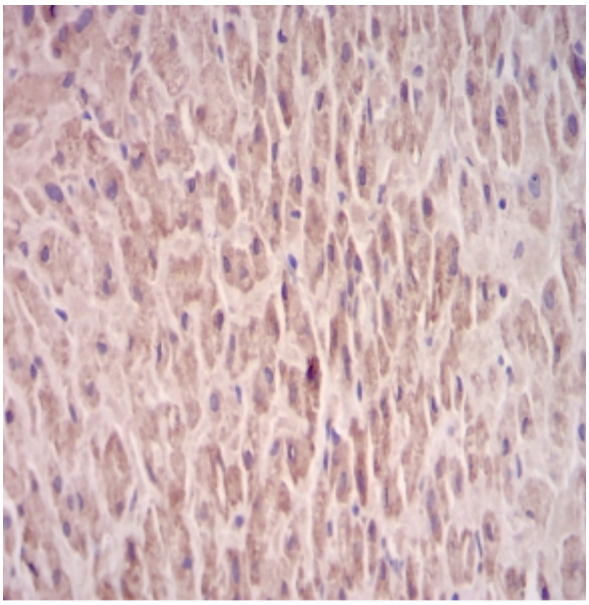

**Figure 2.** SIRT3 expression in para-tumor cirrhosis tissue (×200).

However, the differences between para-tumor tissue and normal liver tissue were not statistically significant ($p>0.05$).

As shown in Figure 1, SIRT3 is highly expressed in normal liver tissue, mainly distributing in the cytoplasm. As shown in Figure 2, SIRT3 is also highly expressed in para-tumor cirrhosis tissue, mainly distributing in the cytoplasm. As shown in Figure 3 and Figure 4, SIRT3 expression is obviously decreased in tumor tissue, mainly distributing in the cytoplasm.

### *Cell Viability Detected by MTT*

MTT results showed that the OD values in 3 groups were increased with the time, showing time-dependent manner. At 48 h after culture, the OD values in-group C were significantly different from group A and B, and the inhibitory rates were statistically different ($p<0.05$) as shown in Table IV and Figure 5. After 48 h, the OD values and inhibitory rates in groups C showed that the cells were

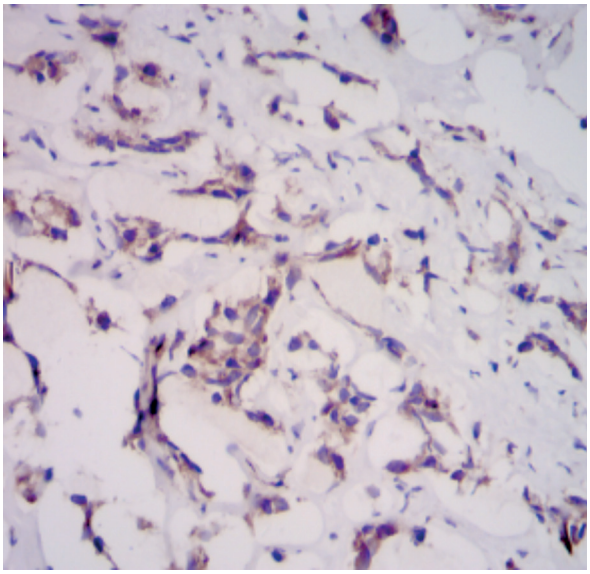

**Figure 3.** SIRT3 expression in tumor tissue (×200).

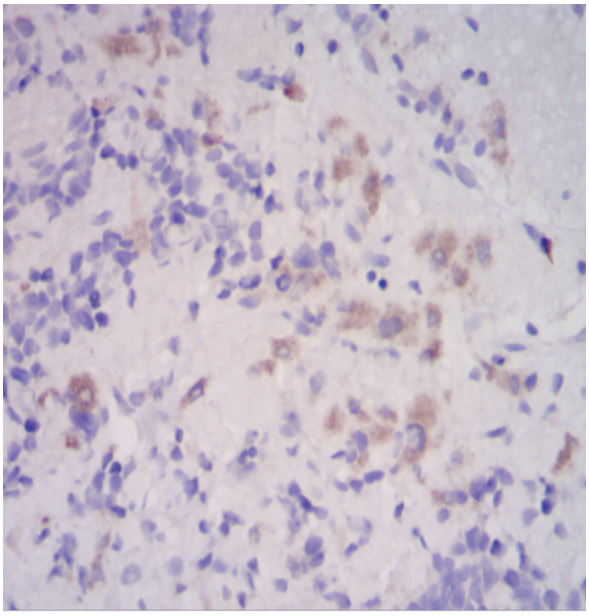

**Figure 4.** SIRT3 expression in tumor tissue (×200).

**Table IV.** MTT OD values of 3 groups at different time points.

| Group | 0 h | 24 h | 48 h | 72 h | 96 h |
|---|---|---|---|---|---|
| Group A | 0.506±0.008 | 0.604±0.017$^{\Delta}$ | 0.715±0.016$^{\Delta}$ | 0.845±0.012$^{\Delta}$ | 0.914±0.016$^{\Delta}$ |
| Group B | 0.505±0.017 | 0.598±0.016$^{\Delta}$ | 0.696±0.007$^{\Delta}$ | 0.806±0.010$^{\Delta}$ | 0.872±0.018$^{\Delta}$ |
| Group C | 0.498±0.015 | 0.554±0.009$^{\Delta}$ | 0.620±0.015$^{*\S}$ | 0.701±0.16$^{*\S\&}$ | 0.738±0.017$^{*\S\&}$ |

Note: $^{*}p<0.05$ compared among A, B and C; $^{\Delta}p<0.05$ compared within A, B and C; $^{\S}p<0.05$ when 0 h, 24 h compared with 48 h, 74 h, 96 h in group C; $^{\S}p<0.05$ when 48 h compared with 72 h, 96 h in group C.

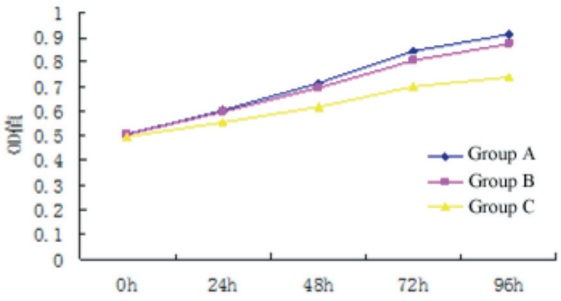


**Figure 5.** Growth curve of 3 groups.

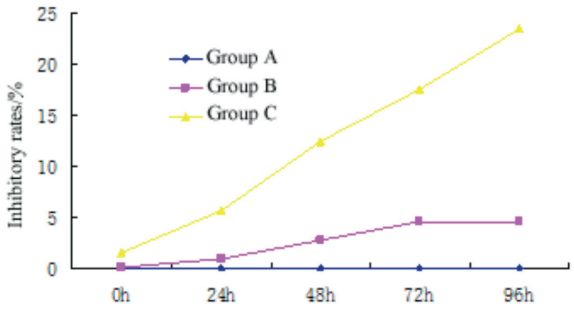


**Figure 6.** Inhibitory rates of 3 groups at different time points.

**Table V.** MTT inhibitory rates of 3 groups at different time points (%).

| Group | 0 h Inhibitory rate (%) | 24 h Inhibitory rate (%) | 48 h Inhibitory rate (%) | 72 h Inhibitory rate (%) | 96 h Inhibitory rate (%) |
|---|---|---|---|---|---|
| Group A | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Group B | 0.13±0.01 | 1.01±0.03 | 2.74±0.13 | 4.64±0.23 | 4.54±0.22 |
| Group C | 1.62±0.05 | 5.64±0.72 | 12.43±1.09$^{*\Delta}$ | 17.52±2.87$^{*\Delta}$ | 23.44±3.30$^{*\Delta}$ |

Note: $^{*}p<0.05$ compared among A, B and C; $^{\Delta}p<0.05$ compared within A, B and C.

obviously inhibited, and the inhibitory rates were increased with the time, showing time-dependent manner (as shown in Table V and Figure 6).

### *Apoptosis Detected by flow Cytometry*

After culture for 72 h, the early stage apoptotic rate and late stage apoptotic rate in group A were 2.35±0.98% and 2.09±1.02%; the early stage apoptotic rate and late stage apoptotic rate in group B were 2.69±1.12% and 2.86±1.21%; the early stage apoptotic rate and late stage apoptotic rate in group C were 9.26±2.35% and 9.11±2.55%, which were obviously higher than group A and B, there were significant differences ($p<0.01$) as shown in Figure 7 and 8, Table VI, the apoptotic rates were not statistically different between group A and B ($p>0.05$). It suggests that PZsGreen-c1-SIRT3 can increase the apoptotic rate of HepG2 cells.

### *Expression levels of SIRT3, Fas, Bax and p53 Detected by Western Blot*

After 72 h, Western blot results showed that expression levels of SIRT3, p53, Bax and Fas were not different between group A and B ($p>0.05$); SIRT3, p53, Bax and Fas in group C were significantly increased (0.812±0.259, 0.476±0.205,

**Table VI.** The apoptotic rates of 3 groups.

| Group | Early stage apoptotic rate (%) | Late stage apoptotic rate (%) |
|---|---|---|
| Group A | 2.35±0.98 | 2.09±1.02 |
| Group B | 2.69±1.12 | 2.86±1.21 |
| Group C | 9.26±2.35$^{**}$ | 9.11±2.55$^{**}$ |

Note: $^{**}p<0.01$ group C compared with group A or B.

**Table VII.** The apoptotic rates of 3 groups.

| Group | SIRT3 | Fas | Bax | P53 |
|---|---|---|---|---|
| Gropu A | 0.250±0.143 | 0.179±0.014 | 0.188±0.012 | 0.121±0.013 |
| Group B | 0.248±0.141 | 0.198±0.013 | 0.211±0.035 | 0.117±0.012 |
| Group C | 0.812±0.259** | 0.476±0.205** | 0.550±0.121** | 0.490±0.216** |

Note: **$p$<0.01 group C compared with group A or B.

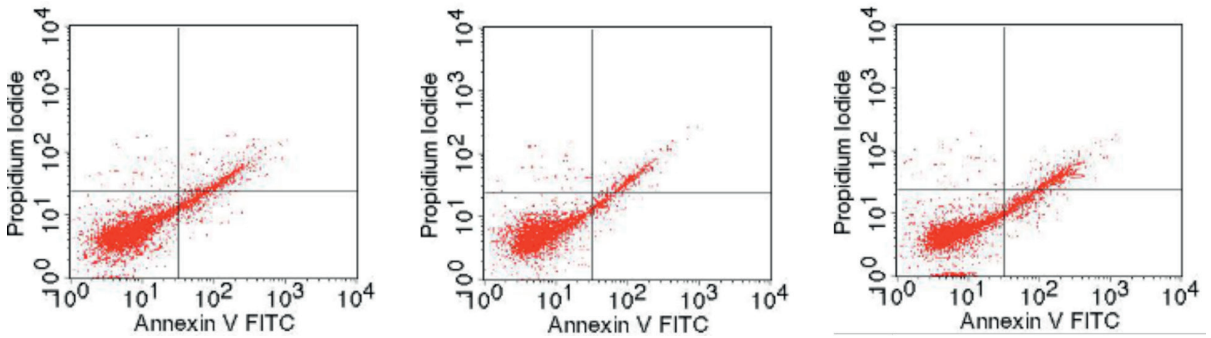


**Figure 7.** ***A***, Flow cytometry results of group A. ***B,*** Flow cytometry results of group B. ***C,*** Flow cytometry results of group C.

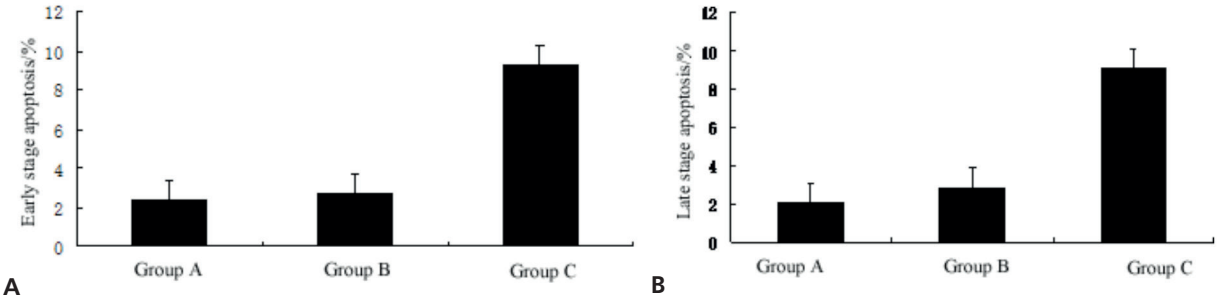


**Figure 8.** ***A,*** Early stage apoptosis of 3 groups. ***B,*** Late stage apoptosis of 3 groups.

0.550±0.121 and 0.490±0.216), which were statistically higher than group A and B ($p$<0.01) (as shown in Figure 9 and 10, Table VII).

### *MnSOD detected by WST-1*

After 72 h, the MnSOD enzyme active units in groups A and B were 0.588±0.264 U and 0.575±0.211 U; the MnSOD contents in group A and B were 0.408±0.115 U/mg and 0.408±0.119 U/mg, there was no statistical difference. MnSOD active unit and content in group C were 2.612±0.334 and 2.271±0.452 U/mg, which were significantly higher than group A and B, there were statistically significances ($p$<0.01) as shown in Table VIII and Figure 11.

## Discussion

Liver cancer is a common malignant tumor of high malignancy worldwide, and HCC is the most common type. Although the diagnosis and treat-

**Table VIII.** MnSOD contents in 3 groups.

| Group | MnSOD enzyme active unit (U) | MnSOD content (U/mg) |
|---|---|---|
| Group A | 0.588±0.264 | 0.408±0.115 |
| Group B | 0.575±0.211 | 0.408±0.119 |
| Group C | 2.612±0.334** | 2.271±0.452** |

Note: **$p$<0.01 group C compared with group A or B.

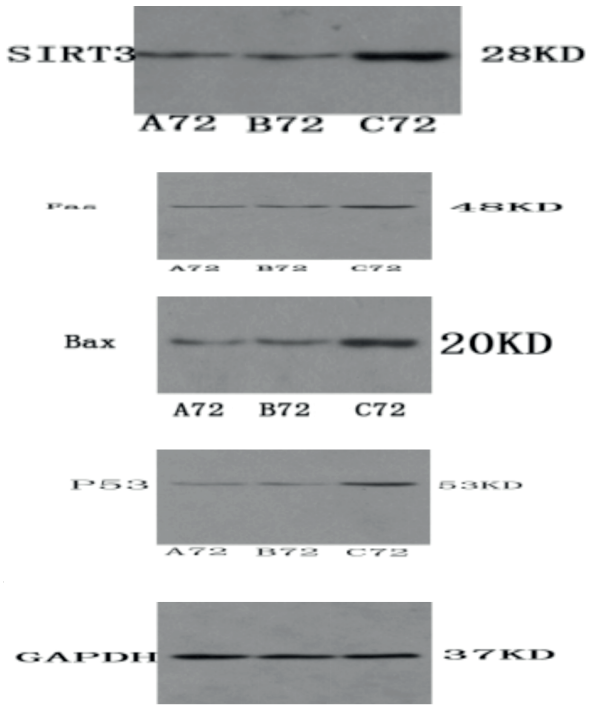


**Figure 9.** Expression levels of SIRT3, Fas, Bax and p53 detected by Western blot.

ment of HCC have been improved in the recent years, the median survival time is still less than 3 months. Thus, exploring a novel therapeutic target or effective drug remains significant.

SIRT3 is a sirtuin located in mitochondria in mammals, which is the only confirmed gene related to longevity. It can regulate energetic metabolism. In the study by Kim et al[13], SIRT3 expression in human breast cancer and some other cancers is decreased, and the rate of cancer in SIRT3 knockout mice is significantly increased. Increased SIRT3 expression has an antitumor effect by up-regulating MnSOD in mitochondria. Low SIRT3 expression may increase ROS in mitochondria and further promote the growth of tumor cells. It has been shown that SIRT3 is decreased in colorectal cancer, osteosarcoma and leukemia[14-16]. Oxidative stress plays a critical role in the development of liver cancer, and the increased ROS level is a characteristic in all HCC animal models[16]. Thus, ROS plays an important role in the development of liver cancer, and we further hypothesize the SIRT3 gene is also significant in the development of liver cancer.

In immunohistochemistry results, SIRT3 expression was high in the para-tumor tissue and normal liver tissue, which was in accordance with the other studies. Meanwhile, it was shown that SIRT3 expression was low or deficient in tumor tissue. Although in this study the positive rate of SIRT3 was up to 59.38% (19/32), 14 cases were weakly positive

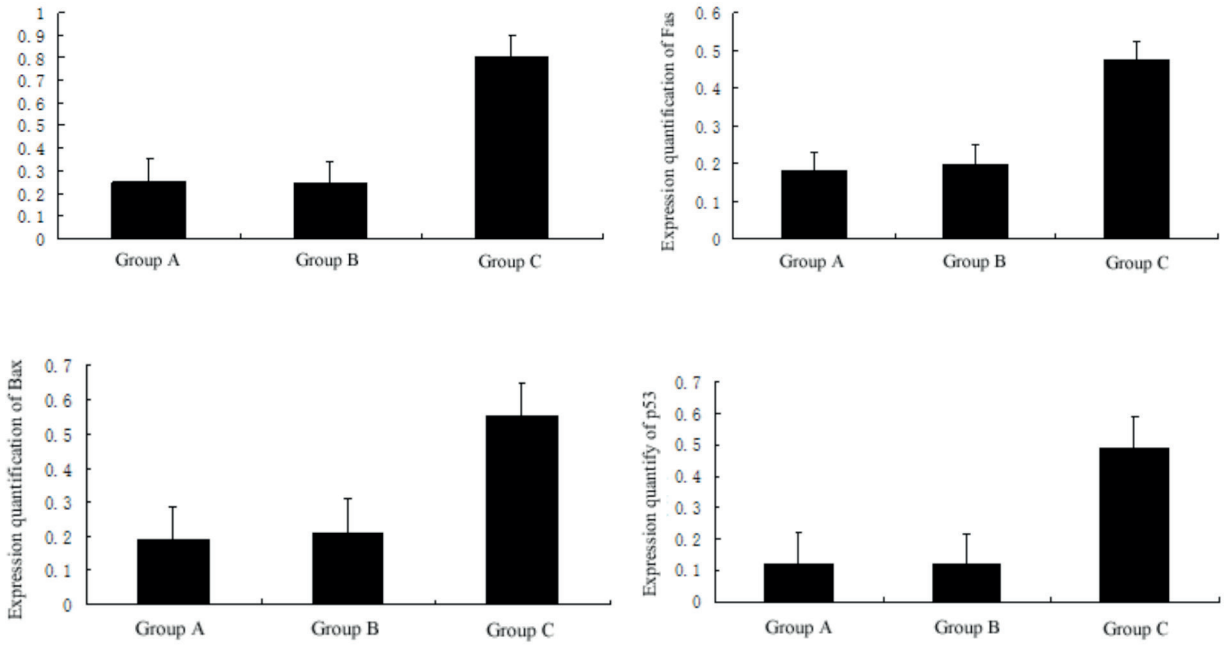


**Figure 10.** ***A,*** Expression quantification of SIRT3 by Western blot. ***B,*** Expression quantification of Fas by Western blot. ***C,*** Expression quantification of Bax by Western blot. ***D,*** Expression quantify of p53 by Western blot.

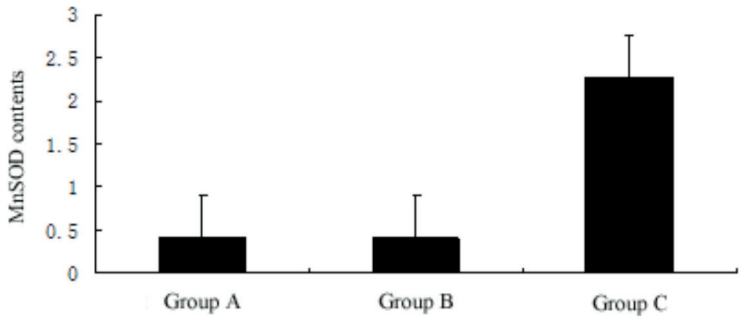


**Figure 11.** MnSOD contents in 3 groups.

and no one was strongly positive, and there were 13 negative cases. It indicates that SIRT3 in liver tumor tissue is lower or deficient compared with normal liver tissue or para-tumor tissue.

After transfection, MTT results showed that the inhibitory rate of HepG2 cells was increased with the time, the growth and proliferation abilities were gradually decreased. It suggests that high SIRT3 expression can inhibit the growth and proliferation of HepG2 cells. Then, whether high SIRT3 expression inhibits HepG2 cells by inducing the apoptosis was further explored.

It is reported that the decrease or inhibition of apoptosis is the pathogenesis of many tumors. Tumor cells can malignantly proliferate due to down-regulation of apoptotic genes[17]. Some antitumor drugs kill the tumor cells by inducing apoptosis. During apoptosis, there are many extracellular activating apoptotic enzymes, Fas/Fasl, Bcl-2/Bax and P53 that are studied most thoroughly[18].

In our work, Annexin V/PI staining was used to detect the apoptosis. The results showed that at 72 h after transfection, the early apoptotic rate and late apoptosis rate in-group C were higher than the other 2 groups; there were statistical significance ($p$<0.01). It suggests that PZsGreen-c1-SIRT3 can increase the apoptosis of HepG2 cells, and SIRT3 can inhibit growth and proliferation of HepG2 cells by inducing apoptosis.

We also detected expression levels of Fas, Bax and P53 by Western blot after culture for 72 h, and found that Fas, Bax and P53 in group C were significantly higher than group A and B, which further verified the significance of apoptosis.

The main free radical scavenger in mitochondria is MnSOD, which can convert superoxide anion into $H_2O_2$, and then into $H_2O$ to maintain the oxidation-reduction balance[19]. Thus, MnSOD expression is very important to oxidation-reduction in the cells. Oberley et al[20] have reported that MnSOD activity is significantly decreased in malignant tumor, which is also verified in variable tumor cells. We hypothesized that SIRT3 could inhibit the growth and proliferation of tumor cells by up-regulating MnSOD expression. In our study, we used WST-1 assay to detect the MnSOD active unit and content in different groups after culture for 72 h. The results showed that MnSOD enzyme active unit and content in-group C were significantly higher than group A and B, which were statistically different ($p$<0.01). It suggests that pZsGreen-c1-SIRT3 can decrease ROS level to inhibit HCC by up-regulating MnSOD.

It is reported that MnSOD can also down-regulate Bcl-2 and up-regulated Bax, Bax/Bcl-2 ratio and caspase-3 activity to induce apoptosis. Bcl-2 family is important in regulating cell survival or apoptosis, and Bcl-2/Bax ratio is especially important. pZsGreen-c1-SIRT3 may also up-regulate MnSOD to increase Bax, and further inhibit Bcl-2 and decrease Bcl-2/Bax ratio, and further induce cell apoptosis to inhibit the growth and proliferation of HepG2 cells[21].

In some studies, it is also shown that MnSOD can also induce apoptosis through death receptor, (Fas). After combining with Fas ligand, it can combine with Fas-associated protein with death domain (FADD), FADD can further convert caspase-8 zymogen to active caspase-8, and finally activate caspase-3 to induce apoptosis[22]. In this study, Fas expression was significantly increased in group C, indicating that pZsGreen-c1-SIRT3 can also up-regulate Fas expression by up-regulating MnSOD to inhibit the growth and proliferation of HepG2 cells.

The well-known tumor inhibitor gene p53 can stop the progress of cell cycle, repair the DNA and induce apoptosis. P53 is also a tar-

get to inhibit tumor growth and proliferation[23]. In our study, p53 expression in-group C after 72 h was significantly higher than group A and B, which were statistically different ($p<0.01$). Thus, high SIRT3 expression may also inhibit tumor by up-regulating p53.

## Conclusions

SIRT3 shows a low expression or deficiency in HCC tissue, indicating that SIRT3 expression can affect the occurrence and development of HCC. SIRT3 can inhibit the growth and proliferation of HepG2 cells and induce HepG2 cell apoptosis. The mechanism may be related to the up-regulation of MnSOD and p53, and the up-regulation of Bax and Fas by MnSOD.

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### Conflict of interest

The authors declare no conflicts of interest.

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