



## Effects of *AFP* gene silencing on Survivin mRNA expression inhibition in HepG2 cells

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**ABSTRACT.** We investigated the effects of alpha-fetoprotein (*AFP*) gene silencing on Survivin expression in HepG2 cells. Small interfering RNA technology was used to downregulate AFP expression in HepG2 cells. An enzyme-linked immunosorbent assay was used to measure AFP concentration in the supernatant before and after transfection. An MTT assay was used to detect cell proliferation activity before and after transfection. We performed flow cytometric analysis to detect the cell apoptosis rate, and reverse transcription-polymerase chain reaction to detect Survivin mRNA levels before and after transfection. Forty-eight hours after transfection, AFP concentration in the supernatant of the experimental group significantly decreased, hepatocellular carcinoma cell growth was inhibited by 43.1%, and the apoptosis rate increased by 24.3%. Survivin mRNA expression was reduced by 78.0% in HepG2 cells. These indicators in the control group and in the

blank group did not change significantly. Silencing of AFP expression in HepG2 cells can effectively inhibit the growth of hepatoma cells and promote apoptosis, which may be useful for reducing intracellular Survivin mRNA levels.

**Key words:** Alpha-fetoprotein; Hepatocellular carcinoma; Survivin; RNA interference

## INTRODUCTION

Alpha-fetoprotein (AFP) has been widely used as a serum marker of hepatocellular carcinoma (HCC) in clinical diagnosis, treatment, and prognosis (Debruyne and Delanghe, 2005). Previous studies showed that AFP plays an important role in cell proliferation and apoptosis of HCC (Yang et al., 2008; Li et al., 2009). Survivin is a recently discovered member of the apoptosis inhibitor protein family that is expressed in a variety of tumors but remains silent in normally differentiated tissues. This protein has become a hotspot in recent studies (Chau et al., 2007). Studies have shown that Survivin in HCC tissues is highly expressed and is an independent factor of poor prognosis (Yang et al., 2011). Few studies have examined the relationship between liver cell AFP and Survivin in China and abroad. In this study, HepG2 in liver cells was examined using RNA interference technology, which was used to silence *AFP*. Apoptosis and Survivin gene expression changes were detected to explore the feasibility of *AFP* gene targeting RNA interference therapy for treating hepatoma.

## MATERIAL AND METHODS

### Materials

RPMI-1640 medium was obtained from GIBCO BRL (Grand Island, NY, USA). Fetal bovine serum was provided by Hangzhou Evergreen Biological Company (Hangzhou, China). Lipofectamine 2000 transfection reagent was purchased from Lvyang (Jiangsu, China). The AFP quantitative enzyme-linked immunosorbent assay (ELISA) kit was provided by the Zhengzhou Antulvke Biological Engineering Co., Ltd. (Henan, China). RNA interference series and related primers of the *AFP* gene were designed and synthesized by Shanghai Sangon Biological Engineering Company (Shanghai, China).

### Methods

#### *Cell culture*

The hepatoma cell line HepG2 was donated by the Digestive Institute of Nanchang University. The cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, inoculated at 37°C and 5% CO<sub>2</sub>, and cultured in a saturated humidity incubator.

#### *Experimental grouping*

In this study, the experimental cells were divided into 3 groups, including the experi-

mental group, the liposome + empty plasmid control group, and the blank control group.

### ***AFP determination in HepG2 cell supernatant using ELISA***

HepG2 cells were inoculated into the medium and cultured, and the cultured cell supernatant was collected before transfection and 12, 24, and 48 h after transfection. Centrifugation at 3000 rpm and 4°C for 10 min was used to collect the supernatant. According to the kit instructions, ELISA methods were used to determine AFP levels.

### ***siRNA silencing of the AFP gene in HepG2 cells***

This study targeted the following *AFP* siRNA gene fragment: 5'-AACTCAGTGAGG ACAAACTAT-3'. Primer design and synthesis for this sequence were conducted by Shanghai Sangon (Shanghai, China). The upstream primer was 5'-AAA TAC ATC CAG GAG AGC CA3', while the downstream primer was 5'-CTG AGC TTG GCA CAG ATC CT-3'.

The vector was constructed according to method reported by Yuan et al. (2004). siRNA expression plasmids for the *AFP* gene were prepared as described by Yang et al. (2008). For plasmid transfection, HepG2 cells in the logarithmic growth phase were digested with 0.25% trypsin, and serum and antibiotic-free RPMI-1640 medium was used to resuspend the cells. In 3 wells for each group, plasmids and diluted Lipofectamine 2000 were added for transfection and the cells were incubated at room temperature for 4 h. The medium was changed at 12 and 24 h, and the cells were harvested at 48 h.

### ***Detection of cell proliferation and apoptosis***

HepG2 cell proliferation was detected using the MTT assay for HepG2 cells collected before transfection and 12, 24, 36, and 48 h after transfection. Nine wells were examined in each group, with a final volume of 200 µL in each well; next, 20 µL MTT solution was added to each well (3 g/L). The cells were incubated for 6 h. The medium was aspirated, and 150 µL dimethyl sulfoxide was added to each well and mixed. The absorbance was measured at 490 nm using a microplate reader. Apoptosis of HepG2 cells was detected using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining.

Flow cytometry analysis of HepG2 cells was conducted before transfection and 12, 24, and 48 h after transfection. EDTA-free trypsin was used for digestion, total cells were collected, washed twice with phosphate-buffered saline, and the supernatant was discarded. Next, 400 µL binding buffer was added to resuspend the cells, and 6 µL Annexin V-FITC and 4 µL PI were added. The cells were incubated at room temperature in the dark for 15 min. An excitation wavelength of 488 nm was used for apoptosis detection with red and green fluorescence channels.

### ***Reverse transcription-polymerase chain reaction detection of the Survivin gene***

A semi-quantitative reverse transcription-polymerase chain reaction method was performed to detect Survivin gene expression before and after transfection in each group. Gene-specific primers for Survivin were used for amplification. The forward primer sequence was 5'-CCG CAT ACA GTG GTC GAG AGA-3', while the reverse primer sequence was 5'-GTG TAG GGT GTA GAA TCC TGT TCA-3'.

## RESULTS

### AFP levels in supernatant of transfected cells in each group

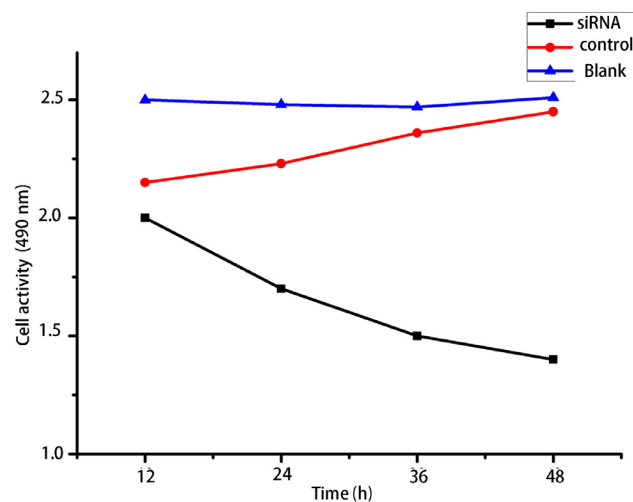
ELISA was used to detect the AFP concentration in the supernatant of HepG2 cells. The results are shown in Table 1.

**Table 1.** AFP levels in the supernatant of HCC HepG2 cells (ng/mL).

Groups	Experimental	Negative	Blank
Before siRNA	988.45 ± 12.58	925.27 ± 5.25	939.56 ± 7.56
After 12 h	549.81 ± 6.54	895.02 ± 7.53	921.00 ± 6.55
After 24 h	275.76 ± 3.27	904.65 ± 4.38	959.83 ± 10.74
After 48 h	154.23 ± 7.78	876.21 ± 14.62	979.71 ± 8.25

### Decreased HepG2 cell growth and viability after *AFP* siRNA transfection

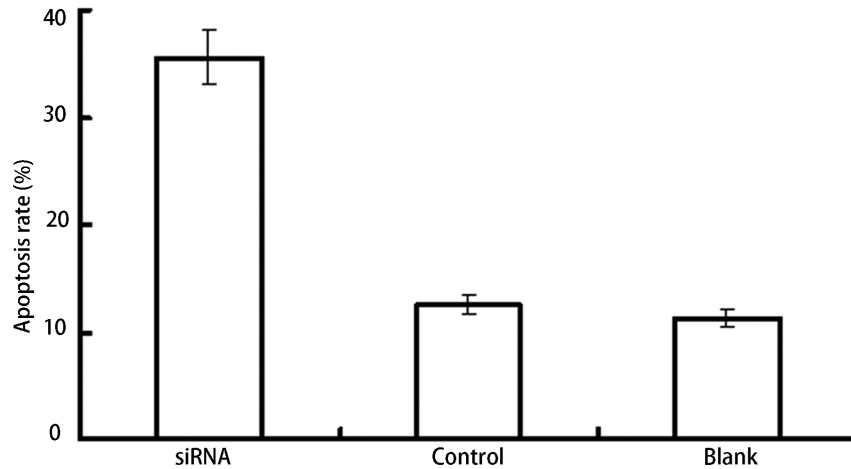
HepG2 cells were collected before transfection and 12, 24, 36, and 48 h after transfection, and an MTT assay was conducted to detect cell viability. The results revealed significant differences between the 24 and 48 h after transfection groups and the control group ( $P < 0.01$ ).



**Figure 1.** Effects of *AFP* siRNA on HepG2 cell proliferation detected by MTT assay.

### Increased HepG2 cell apoptosis after *AFP* siRNA transfection

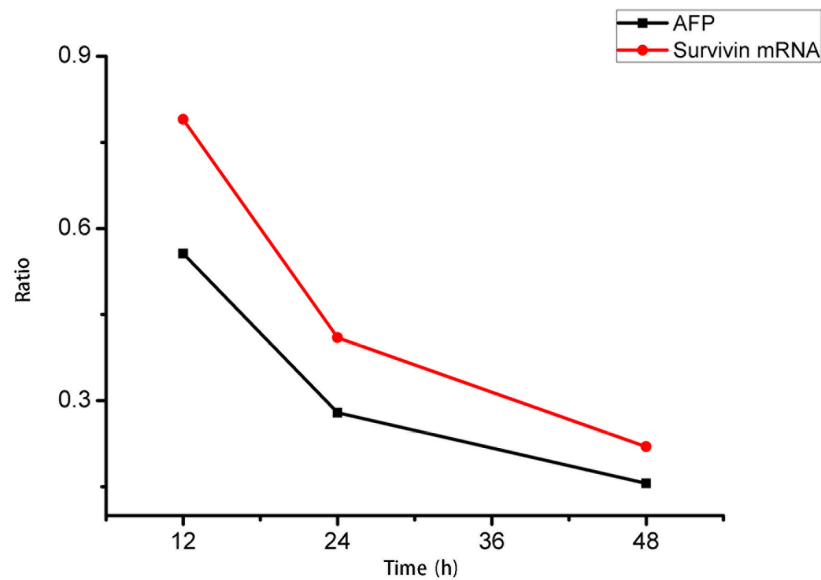
We determined HepG2 cell apoptosis rates 48 h after *AFP* siRNA transfection in each group. The results showed that the *AFP* siRNA-transfected cell apoptosis rate was 35.6%, which increased to 24.3% compared with the value before transfection. The apoptosis rates were 12.5 and 11.3% in the negative control and blank groups, respectively. The apoptosis rate in the experimental group was significantly higher than that in the positive and negative control groups; the difference was statistically significant ( $P < 0.05$ ).



**Figure 2.** HepG2 cell apoptosis 48 h after *AFP* siRNA transfection.

### Effect of HepG2 cell *AFP* siRNA on Survivin gene expression

The cells in the transfection and control groups were collected at 12, 24, and 48 h after transfection. The *AFP* gene was amplified, subjected to electrophoresis, and analyzed using an ultraviolet gel image analyzer. At 24 and 48 h after *AFP* siRNA transfection, Survivin mRNA levels were decreased by 59 and 78%, respectively, compared with that before transfection. *AFP* levels in HepG2 cell supernatants and Survivin mRNA expression in HepG2 cells were very consistent.



**Figure 3.** *AFP* and Survivin mRNA expression changes after *AFP* siRNA transfection.

## DISCUSSION

Because a large number of people have chronic hepatitis B infection, primary liver cancer remains a prevalent disease with high incidence (Zheng et al., 2012). Recently, the treatment of liver cancer has improved, particularly serological detection technology based on AFP and combined modern imaging technology, improving the early diagnosis of liver cancer and increasing early treatment in patients with liver cancer (Wu, 2008). AFP is widely used as a biomarker for HCC diagnosis, monitoring, and follow-up, but studies of its biological function have only recently increased. Numerous studies confirmed that AFP has complicated biological functions, such as promoting liver cancer cell proliferation, inhibition of apoptosis, and immune evasion (Li et al., 2009; Coumar et al., 2013).

In this study, siRNA was used to silence *AFP* gene expression in HepG2 cells. The results showed that 48 h after transfection, AFP concentration in the supernatant of the hepatoma cell culture solution significantly decreased (from  $988.45 \pm 12.58$  to  $154.23 \pm 7.78$ ). The difference was significant compared with the control group, indicating that the transfection was successful. Forty-eight hours after transfection, the growth of liver cancer cells in the experimental group was inhibited by 43.04% and the apoptosis rate increased by 24.3%. After silencing of *AFP* gene expression in HCC cells, cell proliferation decreased and apoptosis increased; the results were similar to those of Yang et al. (2008).

Survivin is an inhibitor of apoptosis protein that shows decreased expression in well-differentiated adult tissues besides the thyroid, thymus, and gonads. It shows positive expression in the vast majority of tumor tissues. These unique expression characteristics make it a target for cancer diagnosis and therapy (Rödel et al., 2012; Coumar et al., 2013). Studies have shown that Survivin was highly expressed in hepatoma cells and that it can regulate cell division, proliferation, and apoptosis (Zhu et al., 2005; Zhao et al., 2011; Liu et al., 2013).

We used RNA interference to downregulate *AFP* gene expression in hepatoma HepG2 cells and found that the apoptosis rate of liver cancer cells increased. We also found that during transfection, as AFP concentration in the culture supernatant continuously decreased, Survivin mRNA levels also decreased, and both were consistent.

Therefore, apoptosis rate changes may be associated with decreased expression of Survivin, and silencing of the *AFP* gene can inhibit Survivin expression in HepG2 cells, promoting apoptosis. Consistent with our results, Li et al. (2007) found that in Bel7402 cells, inhibition of AFP expression using an AFP antibody significantly lowered Survivin levels and increased hepatocellular apoptosis. Thus, AFP inhibited the activity of tumor necrosis factor-related apoptosis-inducing ligand by upregulating Survivin expression.

Wu (2008) compared 6646 liver cancer surgery patients from 1999-2003 and 5524 liver cancer surgery patients before 1998 in Shanghai Eastern Hepatobiliary Hospital and found that tumor-free survival time was not significantly different. This indicated that surgery, which is currently the most effective treatment available for liver cancer, did not improve the efficacy in small HCC patients. Thus, developing other treatments for HCC is very important. AFP plays an important role in the occurrence and development of HCC. We found that blocking AFP expression using siRNA may be useful for controlling AFP-positive HCC.

## CONCLUSIONS

*AFP* gene silenced by RNAi inhibited cell proliferation and induced apoptosis through suppression of Survivin mRNA expression. After *AFP* siRNA transfection at 48 h, *AFP* expression was almost completely inhibited, cell proliferation activity was decreased by 43.1%, cell apoptosis rate was increased by 24.3%, and the Survivin mRNA expression was reduced to 22.0% in the experimental group.

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