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Silencing alpha-fetoprotein expression induces growth arrest and apoptosis in human hepatocellular cancer cell

Xiaojun Yang [a], Youcheng Zhang [a,*], Lingyi Zhang [b], Lin Zhang [a], Jie Mao [a]

[a] *Department of General Surgery, Lanzhou University Second Hospital, Lanzhou, 730030, Gansu, China*
[b] *Department of Digestion Medicine, Lanzhou University Second Hospital, Lanzhou, 730030, Gansu, China*

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Abstract

The expression of alpha-fetoprotein (AFP), a tumor-associated antigen, is silenced in normal adult hepatocyte but reactivated in human hepatocellular carcinoma (HCC). To investigate the roles of AFP in the regulation of cell growth, we silenced AFP expression in the HCC cell line Huh7 by transfection of specific Stealth™ RNAi. After the transfection for 48 h, the expression of AFP gene was almost abolished, the cell proliferation was inhibited by 46.15%, and the number of cells undergoing early apoptosis was significantly increased to 63.93%. Inhibition of AFP expression also resulted in an increased in Bax/Bcl-2 ratio, the release of cytochrome *c* from mitochondria and activation of caspase-3. The results suggest that AFP may positively regulate cell proliferation by enhancing the apoptosis resistance via dysfunction of the p53/Bax/cytochrome *c*/caspase-3 signaling pathway in AFP-producing HCC cell line. As such, the knockdown of AFP gene should be further investigated in vivo as a novel approach to HCC treatment.

Keyword: AFP; RNA interference; Hepatocellular carcinoma; Cytochrome *c*; Apoptosis

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies rated fifth in incidence and the third in mortality worldwide [1]. The mean survival time from establishment of the diagnosis is only about 4 months, it causes approximately 600,000 deaths annually [2]. Despite advances in surgical and nonsurgical therapies for hepatocellular cancer, there is still no satisfactory method for improving the overall survival rate of affected patients. Although the major etiological agents have been identified, the molecular pathogenesis of HCC remains unclear [3]. Further study on the molecular mechanisms underlying HCC as well as identification of new targets of molecular therapy is critically needed.

Tumor-associated antigens such as alpha-fetoprotein (AFP) may be used as a target for cancer selective gene therapy. AFP gene is highly expressed in the fetal liver but transcriptionally silent in normal adult hepatocytes. However, reactivation of this protein is always demonstrated in more than 70% of hepatocellular carcinomas (HCC) [4]. The physicochemical and structural properties of this 70-kDa glycoprotein have been extensively described [5] since its relationship with HCC was reported in

* Corresponding author. Tel.: +86 931 8942240.
E-mail address: zhangychmd@yahoo.com.cn (Y. Zhang).

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the mid-1960s. In the last 2 decades, the growth regulatory properties of AFP have aroused interest as a result of studies involving ontogenetic and oncogenic growth in both cell culture and animal models. A myriad of studies have documented that AFP is capable of regulating growth in ovarian, placental, uterine, hepatic, phagocytic, bone marrow, and lymphatic cells [6,7], in addition to various neoplastic cells [8,9]. Subsequent studies revealed that AFP could induce apoptosis in various tumor cell culture lines (heptoma and lymphoblastomas) in just a few hours. Induction of tumor cell death was shown at high levels of AFP (>100 μg/ml) [10], whereas lower amounts of AFP (<100 μg/ml) failed to inhibit growth in any of the cell lines but rather showed a slight stimulating effect in many cell lines, including Bel7402, HepG2, SMMC-7721, and NIH3T3 cells [9,11,12]. These in vitro data demonstrated that AFP could induce either stimulatory or inhibitory growth activity, depending on the relative concentration administered [13]. Although there have been considerable research indicating that AFP can modulate apoptotic signals, the precise mechanism of AFP-mediated cell growth regulation and apoptosis resistance remains obscure.

Recently, small interfering RNA (siRNA) have been shown to inhibit the expression of a corresponding target gene in mammals [14], whereby siRNA molecules are separated into single strands and incorporated into the RNA induced silencing complex (RISC), which then cleaves the corresponding cellular mRNA. RNAi serve as a powerful technology to specifically block the expression of target genes [15,16]. To characterize the roles of AFP in modulating cell growth and survival, we silenced AFP expression by Stealth RNAi and evaluated the effects on cell proliferation and apoptosis in the HCC cell line Huh7, which expresses high levels of AFP. We also explored the possible molecular mechanisms that underlie the apoptosis induction by silencing AFP in Huh7 cells.

2. Materials and methods

2.1. Design and synthesizing of Stealth RNAi targeting AFP gene sequence

According to the siRNA design guidelines [17,18], three different RNAi target sequences were selected corresponding to nucleotides 152–176 (HSS303-Stealth RNAi), 245–269 (HSS304-Stealth RNAi), 546–570 (HSS305-Stealth RNAi) of the human AFP mRNA (GenBank Accession No. NM00 1134). The three 5-nucleotide modified synthetic stealth RNAi targeting AFP were customarily synthesized by Invitrogen Inc without overhanging at the 3′end, and Stealth RNAi duplexes whose GC content is similar to that of each duplex siRNA (low GC content) from Invitrogen Inc were used as negative control. Sequences of the three synthesized oligonucleotides are: HSS303: sense 5′-UAAACUUAUCU CUGCAGUACAUUGG-3′, anti-sense 5′-CCAAU GUACUGGAGAGAUAAGUUUA-3′; HSS304: sense 5′-AAUUGCAGUCAAUGCAUCUUUCA CC-3′, anti-sense 5′-GGUGAAAGAUGCAUUG ACUGCAAUU-3′; HSS305: sense 5′-CAUACAG GAAGGGAUGCCUUCUUGC-3′, anti-sense 5′-G CAAGAAGGCAUCCCUUCCUGUAUG-3′. These target sequences were submitted to a BLAST search to ensure that only the AFP gene was targeted.

2.2. Cell culture and transfection

The HCC cell line Huh7, a generous gift from Dr. Wenzhe Ho, Children's Hospital of Philadelphia (USA), was maintained and grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Inc., USA) supplemented with 10% fetal bovine serum and 100 U/ml penicillin G/streptomycin. The cells at about 70% confluence were used for all the experiments in the present study.

Synthesized Stealth RNAi against AFP were transfected into Huh7 cells using the Lipofectamine™ RNAi Max transfection Agent (Invitrogen Inc., USA) according to the manufacturer's protocol. Meanwhile, the negative control was used to prevent induction of nonspecific cellular events caused by introduction of the oligonucleotide into cells. The transfection efficiency of each duplex siRNA was confirmed by using BLOCK-iT™ Alexa Fluor® Red Fluorescent (Invitrogen Inc., USA) according to the manufacturer's instructions. The duplex siRNA is not homologous to any known genes. Uptake of the Fluorescent Oligo, which correlates strongly with uptake of Stealth™ RNAi was used to measure transfection efficiency during optimization. Reverse transfection was applied to deliver Stealth™ RNAi, fluorescent oligo or negative control duplexes into Huh7 cells. Briefly, the complexes were prepared inside the wells, then cells and medium were added and incubated at 37 °C in a CO^2 incubator until assayed for gene knockdown. Cell number added per well was optimized such that 24 h after plating, cell confluence is 30–50%.

2.3. Reverse transcription polymerase chain reaction-assess Stealth RNAi effects

Huh7 cells (3×10^5) were seeded onto 6-well plates. Total RNA was extracted using Trizol reagent (Invitrogen Inc., USA) following the manufacturer's instruction at 48 or 72 h after transfection. First strand cDNA synthesis and amplification were performed using two step real time RT-PCR kit (TakaRa, Japan). Quantitative PCR amplifications were performed with a 7000 Sequence Detection System (Applied Biosystems, USA). Reactions were carried out in a 25 μl reaction volume containing 12.5 μl of 2×SYBR® *Premix Ex Taq*™ (TakaRa, Japan). β-Actin was used as an internal standard. The sequences of primers are: AFP sense 5′-GGAAGTCT GCTTTGCTGAAGA-3′ and anti-sense 5′-CACAC CGAATGAAAGACTCGT-3′ (GenBank Accession No. NM001134.1), β-actin sense 5′-GCAAGCAGG AGTATGACGAGT-3′ and anti-sense 5′-GCAAGC AGGAGTATGACGAGT-3′ (GenBank Accession No. NM031144) (Shanghai Sangon, China). Thermal cycle conditions: 95 °C for 10 s, followed by 40 cycles of 95 °C 5 s, 60 °C 30 s. The ΔC_t of each group was calculated by formula: $\Delta C_t = Ct_{AFP} - Ct_{\beta\text{-actin}}$. $\Delta\Delta C_t$ was calculated by $\Delta Ct_{treated} - \Delta Ct_{control}$. The fold-change for AFP expression levels of the treated groups were calculated using $2^{-\Delta\Delta Ct}$.

2.4. AFP proteins detection by enzyme-linked immunosorbent assay (ELISA)

The supernatant of Huh7 cells were collected after transfection with Stealth RNAi or negative control for 48 or 72 h. AFP proteins in supernatant of Huh7 cell was measured using a commercially available human AFP ELISA kit (Autobio Co. Ltd., China) according to the manufacturer's instructions. The optical absorbance of the samples was measured at 450 nm using a microplate reader from Bio-Rad, USA. Standards of defined concentrations were run in each assay allowing the construction of a calibration curve by plotting absorbance versus concentration. The AFP protein concentrations in the supernatant were then calculated from this calibration curve and expressed in ng/ml.

2.5. Dimethylthiazolyl-2,5-diphenyl-tetrazolium bromide (MTT) assay

After reverse transfection of Huh7 cells with Stealth RNAi or negative control duplexes in 96-well plates, MTT was added (24 μl/well of 5 g/L solution in PBS) at either 24, 48 or 72 h. The plate was then incubated at 37 °C for 4 h, the reaction was stopped by addition of 180 μl DMSO. The crystallized MTT was dissolved and the absorbance was measured using an ELISA reader (Victor Co., Finland) at 490 nm wavelength. All samples were assayed repeatedly in 6 wells. The percentage of cell proliferation for each group with different treatments were calculated using the formula: cell proliferation ratio = (A_{490}Treatment/A_{490}Control) × 100%.

2.6. Apoptosis assessment with Hoechst 33258, transmission electron microscopic (TEM) and flow cytometry (double staining with FITC/PI)

The cells undergoing apoptosis were visualized with Hoechst 33258 staining [19]. Briefly, 48 or 72 h after transfection, Huh7 cells plated on glass microscope slides in 6-well plates were fixed with 70% ethanol for 10 min followed by staining with Hoechst 33258 (Beyotime, China, 500 μl/well) at room temperature in dark for 5 min. The cells were then washed twice with PBS, examined and immediately photographed under a fluorescence microscope (Nikon Corporation, Chiyoda-ku, Tokyo, Japan) with an excitation wavelength of 350 nm. Apoptotic cells were defined on the basis of nuclear morphology changes, such as chromatin condensation and fragmentation.

We also detected the apoptosis cells by transmission electron microscopic (TEM). The Huh7 cells were digested with 0.25% trypsinase and collected at 72 h after transfection. Rinsed twice with PBS, the cells were prefixed with 0.3% glutaraldehyde overnight, post-fixed with 10 mL/L osmic acid, dehydrated in graded ethanol, embedded in Epon 812 mixture, and cut into 0.05 μm thick sections on an ultramicrotome. The cells were observed under JEM-1230 (JEOL Ltd., Japan) electronmicroscopy.

To quantitatively analyze the effects of Stealth RNAi on cell apoptosis, Huh7 cells were transfected with Stealth RNAi or negative control in culture flasks. After 48 or 72 h, cells were harvested by trypsinization and rinsed with cold PBS twice. After centrifugation (4 °C, 1000*g*) for 10 min, cells were resuspended in 200 μl binding buffer and then treated with 10 μl Annexin V-FITC and 5 μl propidium iodide (Sigma, USA) for 15 min at room temperature. Flow cytometric analysis of cells was performed with an EpicsXL Coulter flow cytometer (BECKMAN-COULTER, USA). FITC−/PI− cells

were counted as normal control, The quantity of FITC+/PI− cells corresponded to early apoptosis, while that of FITC+/PI+ cells corresponded to late apoptosis or secondary necrosis [20].

2.7. Protein extraction and Western immunoblot analysis

Huh7 cells (5×10^5) were seeded into culture flasks. After 48 or 72 h transfection, cells were collected and washed twice by cold PBS. Mitochondrial and cytosolic fractions were prepared by resuspending the cell pellets with 500 μl fractionation buffer, containing 20 mM/L Hepes PH 7.5, 10 mM/L KCl, 1.5 mM/L $MgCl_2$, 1 mM/L EDTA, 1 mM/L dithiothreitol, 250 mM/L sucrose, and protease inhibitor cocktail, and incubated for 20 min on ice. Cells were then disrupted by 25 strokes with Dounce homogenizer. After homogenization, unbroken cells and large debris were removed by centrifugation at 800*g* for 10 min at 4 °C .The supernatants containing mitochondria were further centrifuged at 10,000*g* for 25 min at 4 °C. The resulting supernatants were saved as cytosolic extracts at −70 °C for further analysis. The mitochondrial pellets were lysed with 100 μL of fractionation buffer, saved as mitochondrial fractions, aliquoted and frozen at −70 °C.

Protein concentrations of mitochondria and cytoplasm were determined using the bicinchoninic acid protein assay reagent (Applygen Technologies Inc., China). Equal amounts of protein (20 μg) were separated on a 12–15% SDS polyacrylamide gel and transferred to PVDF membrane. Membranes were blocked in 5% nonfat dry milk in TBST (Tris-buffered saline containing 0.1% Tween-20) for 1 h at room temperature and incubated with the primary antibody (dilution 1:400–2000) in TBST containing 1% milk overnight at 4 °C. The primary antibodies used were anti-AFP, cytochrome *c* (Beijing Biosynthesis Biotechnology Co. Ltd., China), P53, Bcl-2, Bax, Procaspase-3 (Santa Cruz Biotech, USA). After washing 4 times with TBST, the blots were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:2500, Santa Cruz Biotech, USA) at room temperature for 2 h. Immunocomplexes were visualized by incubation of the filters with Enhanced Chemiluminescence reagent (Chemicon International, USA) and exposed on X-ray film. To confirm equal protein loading, membranes were reprobed with anti-β-actin antibody (Santa Cruz Biotech) in 1:2000 dilution. Densitometric analyses were performed using Scion Image software.

2.8. Statistical analysis

Data were expressed as mean ± SD. One-way ANOVA followed by Bonferroni correction was used to compare the data among three or more groups, a Student's *t*-test was also used. All statistical analyses were performed using the SPSS 13.0 software package for Windows (SPSS Inc., Chicago, IL) and a value of $p < 0.05$ was considered significant.

3. Results

3.1. Transfection efficiency and conditions

In the present study, stealth RNAi was used to determine the effect of AFP gene silencing on cell growth and survival. To optimize conditions for siRNA knockdown, we first determined transfection efficiency using BLOCK-iT™ Alexa Fluor® Red Fluorescent control. Twenty-four hours after reverse transfection, Huh7 cells were observed using a fluorescent microscope. The transfection efficiency was evaluated by counting the red fluorescent cells, which was over 95% when using 10 nM/L final concentration of oligo duplex and 1×10^5/ml Huh7 cells (Fig. 1). Therefore, we used 10 nM/L siRNA and 1×10^5/ml Huh7 cells to perform subsequent experiments. In the cocktail group, the final concentration for each of the three stealth RNAi was 5 nM/L.

3.2. Stealth RNAi specifically reduces AFP expression in Huh7 cells

Following transfection of Huh7 cells with the stealth siRNA targeting AFP, we measured steady state mRNA levels by real time RT-PCR. Results showed an obvious decrease of AFP mRNA levels with the AFP RNAi while the levels of the housekeeping gene β-actin remained relatively unchanged. Of the three stealth RNAi against AFP, HSS304 showed a more potent suppression of AFP mRNA expression than HSS303 or HSS305 did. Comparatively, the cocktail of the three stealth RNAi showed the strongest suppression while transfection of negative control had no effect on AFP mRNA expression. Quantification analysis revealed that HSS304 reduced AFP mRNA by 96.9% and 97.4% of the blank control at 48 and 72 h after transfection, respectively. The reduction rates of the cocktail RNAi were 97.1% (48 h) and 98.5% (72 h) of the blank control, whereas HSS303, HSS305, and negative control transfected group were 95.6%, 89.5%, 15% at 48 h and 97.2%, 87.3%, 18% at 72 h after transfection,

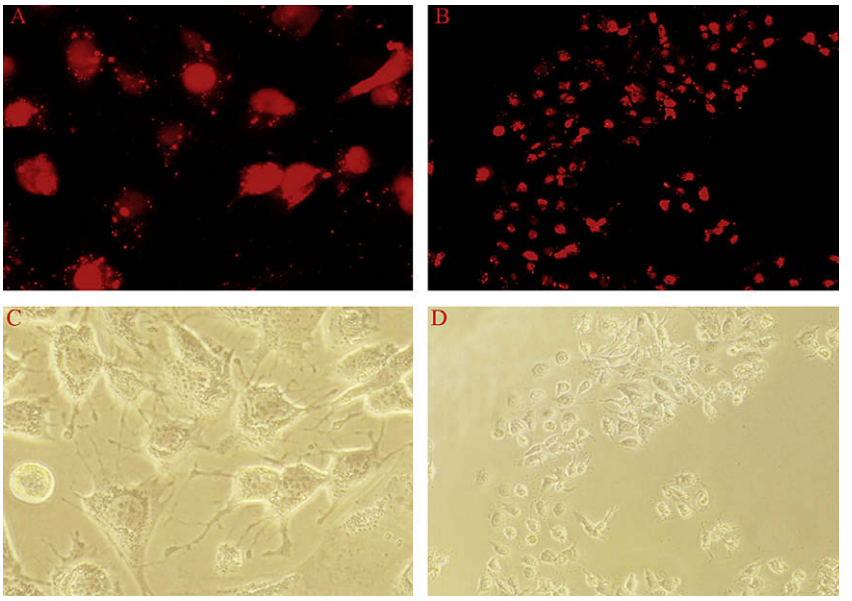


Fig. 1. BLOCK-iT™ Alexa Fluor® Red Fluorescent Control. The BLOCK-iT™ Alexa Fluor Red Fluorescent Control (10 nM/L) was transfected into Huh7 cells using Lipofectamine™ RNAi MAX Transfection Reagent. Twenty-four hours after transfection, growth medium was removed and replaced with PBS. Cell localization of the Alexa Fluor Red Fluorescent control oligo is seen with fluorescent microscope (A, 400× and B, 100×). Nearly 100% of the cells took up the control oligo and retained a normal morphology, as seen in bright-field (C, 400× and D, 100×).

respectively. There was a significant difference between blank control and the stealth RNAi groups (Fig. 2A, $p < 0.01$).

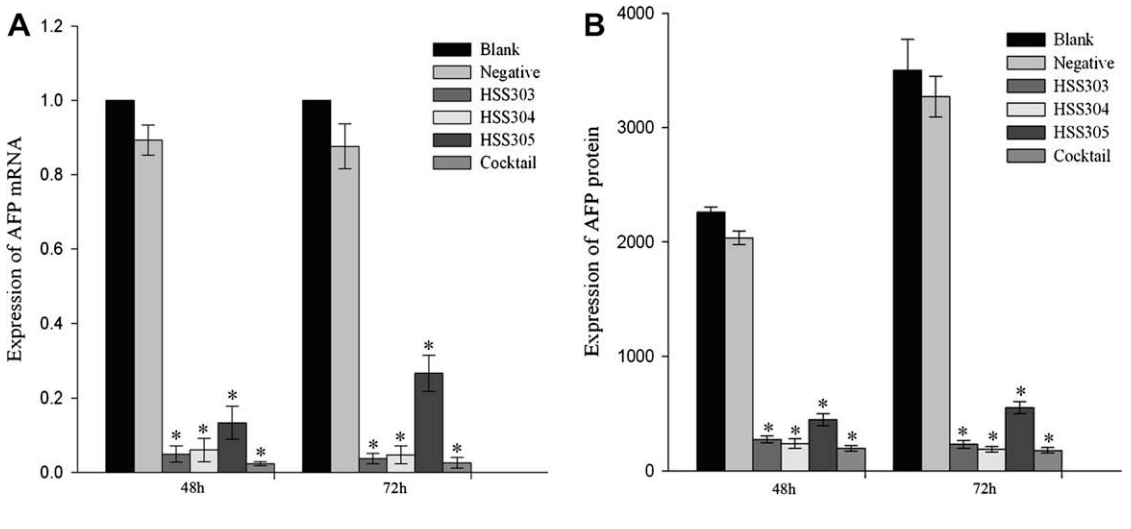
Compared to the blank control, the results obtained from ELISA ($n = 3$) of supernatant also demonstrated that AFP protein secretion was also significantly reduced after transfection of the stealth RNAi (HSS303, HSS304, and HSS305) and the RNAi cocktail for 48 and 72 h; the cocktail had the most potent effect. In contrast, transfection of negative control had no obvious silencing effect on secretion of AFP protein. The corresponding values of AFP protein reduction for blank control, negative control, HSS303, HSS304, HSS305, and RNAi cocktail groups at 48 and72 h after transfection are shown in Fig. 2C. Distinct differences were seen between the blank control and RNAi groups. The cocktail of the three stealth RNAi showed the most effective inhibition at 72 h after transfection (Fig. 2B, $p < 0.01$).

3.3. Silencing of AFP gene decreases viability in Huh7 cells

To determine whether silencing AFP gene affects cell proliferation in Huh7 cells, metabolic activity at 24, 48, and 72 h after transfection was determined by MTT assay. The cell viability was reduced significantly after treatment with the stealth RNAi against AFP (HSS303, HSS304, and HSS305) and the RNAi cocktail at 24, 48, and 72 h as compared with the negative control or blank control (Fig. 3A, $p < 0.05$). The cocktail of the three stealth RNAi had the most potent suppression ($p < 0.01$). The inhibition rates of cell proliferation were 34.06%, 47.61%, and 65.36% after 24, 48, and 72 h of transfection, respectively. In contrast, transfection of the negative control had no obvious effect on cell proliferation. The corresponding values for blank control, negative control, HSS303, HSS304, HSS305, and the cocktail groups after 24, 48, and 72 h of transfection are shown in Fig. 3B.

3.4. Silencing of AFP gene induces apoptosis in Huh7 cells

To assess whether silencing AFP gene could also affect cell survival, we performed Hoechst 33258 staining. Results showed that the cells transfected with HSS304 and the cocktail displayed obviously chromatin condensation, nuclear staining was much brighter than it was in the blank control cells (Fig. 4A). Transmission electron microscopy revealed that in Huh7cells transfected with HSS304 or cocktail groups, dense chromatin appeared near the nucleus membranes, nuclear fragmentation and apoptotic bodies also occured, which presented some typical manifestations of cell apoptosis; while no typical manifestation of cell apoptosis was observed in the control groups and the nuclei appeared to undergo karyokinesis, a symbol of a vigorous cell proliferation (Fig. 4B). In order to further investigate the apoptotic inducing effect of silencing AFP expression in Huh7 cells, we classified and counted apoptotic cells at the early stage, terminal stage and necrotic stage, respectively, by flow cytometry



C

The value of AFP protein in supernant after transfection(Mean ± SD,ng/ml)

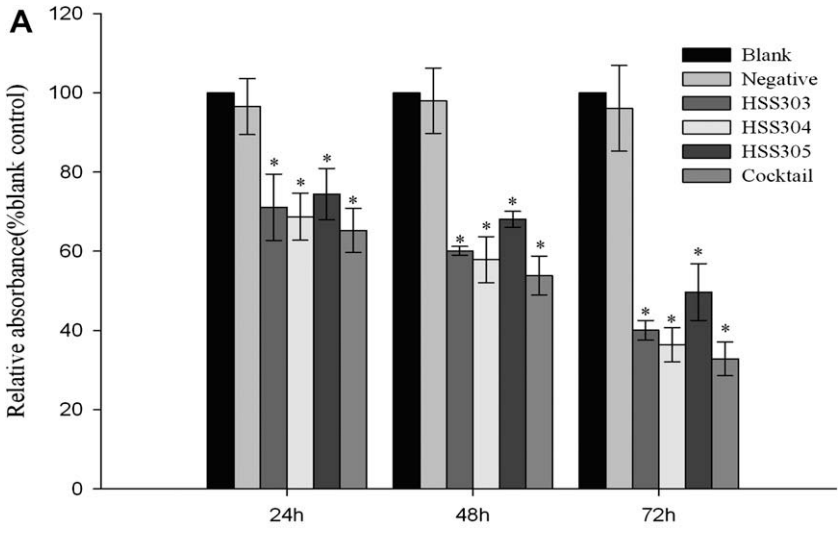
Time(h)	Blank	Negative	HSS303	HSS304	HSS305	Cocktail
48	2258.36±47.02	2034.53±59.75	277.00±29.59 *	239.53±42.40 *	450.40±52.99 *	198.10±29.70 *
72	3498.86±272.80	3271.06±178.39	233.93±34.30 *	190.23±22.02 *	555.40±52.64 *	180.60±24.70 *

Fig. 2. Expression of AFP mRNA and protein at 48, 72 h after transfection with HSS303. HSS304, HSS305, cocktail of the three stealth RNAi against AFP, negative control RNAi or blank control. (A) Relative expression of AFP mRNA level (% blank control) analyzed by quantitative RT-PCR ($^{*}p < 0.01$, vs. blank group); (B) expression of AFP proteins in supernatant of Huh7 cells were detected by ELISA after transfection ($^{*}p < 0.01$, vs. blank group); (C) the corresponding values of AFP proteins in the six groups at 48, 72 h after transfection ($^{*}p < 0.01$, vs. blank group). All experiments were repeated three times and data were expressed as mean ± SD. One-way ANOVA followed by Bonferroni correction was used to compare the data among six groups.

using double staining with FITC/PI. After transfection for 48 and 72 h, the apoptotic cells were markedly increased in HSS304 and cocktail groups compared to those in the negative and blank control groups. Their values are shown in Fig. 4D ($p < 0.01$). The results showed that early stage apoptotic cells were markedly increased compared to terminal stage cells after 48 h transfection, and the apoptotic cells were mainly at the early apoptotic stage after 72 h transfection (Fig. 4C and D), which was more significant in the cocktail treated cells.

3.5. Effect of silencing AFP gene on apoptosis-related molecules in Huh7 cells

Although AFP mainly distributed in supernatant as a secreting protein, we still detected its expression in cytoplasm in western blot when analyzing the effect of silencing AFP on the expression of other genes we were interested in. The results showed that AFP protein was absent in HSS304 and cocktail groups compared with the protein in negative and blank control groups at 48 and 72 h after transfection (Fig. 5). To explore the possible mechanism underlying the apoptosis induction after silencing AFP in Huh7 cells, we performed Western blot analysis for P53, Bax/Bcl-2, procaspase-3, and cytochrome *c* expression. It has been assumed that accumulation of P53 protein represents P53 dysfunction, because missense mutations of P53 stabilize the protein by increasing its half-life considerably compared with the wild-type protein [21], thus the accumulated P53 protein that was detected in this study was therefore assumed to be stabilized by mutation. We found that mutant P53 protein decreased significantly in HSS304 and cocktail groups compared to the negative and blank control groups at 48 and 72 h after transfection ($p < 0.05$). However, Bax/Bcl-2 ratio was increased in HSS304 and cocktail groups, it was 5.67-, 6.64-fold (at 48 h) and 7.45-, 8.97-fold (at 72 h) higher than that of the blank control group after transfection (Fig. 5, $p < 0.01$). We also analyzed the procaspase-3 levels in Huh7 cells of all four groups and studied the location of cytochrome *c* in mitochondrial intermembrane space and cytoplasm. The results showed that both the levels of procaspase-3 and cytochrome *c* in mitochondrial intermembrane space were reduced distinctly in HSS304 and cocktail groups compared to the negative and blank control groups at 48 and 72 h after



B

The value of relative absorbance(%blank control,Mean±SD)

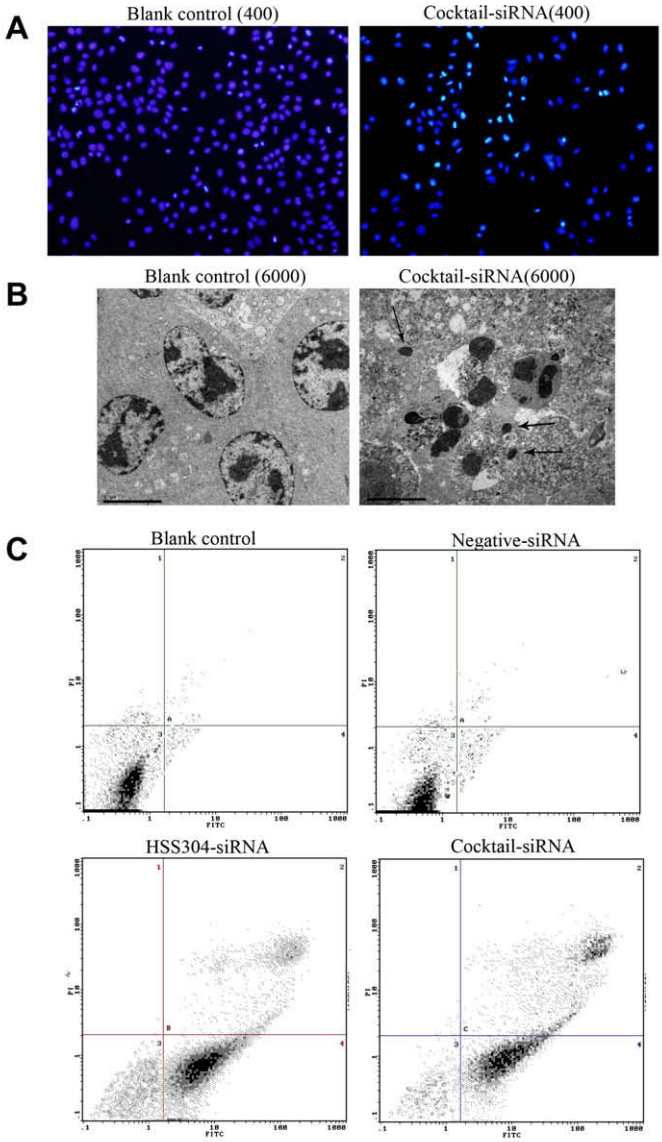
Time(h)	Blank	Negative	HSS303	HSS304	HSS305	Cocktail
24	100.00	96.52±7.07	71.10±8.39 *	68.69±5.95 *	74.42±6.46 *	65.25±5.57 *
48	100.00	98.00±8.29	60.10±1.10 *	57.87±5.82 *	68.07±2.01 *	53.85±4.94 *
72	100.00	96.08±10.80	40.00±2.50 *	36.37±4.34 *	49.68±7.16 *	32.28±7.28 *

Fig. 3. Silencing AFP with stealth RNAi against AFP (HSS303, HSS304, and HSS305) or cocktails affects cell proliferation in Huh7 cells. (A) The metabolic activity was determined by the MTT assay at 24, 48, and 72 h after transfection. (B) The values of their relative absorbance were calculated in terms of the percentage of the blank control. Assays were performed in triplicates and are representative for six independent experiments, the relative OD values of 490 nm are presented as mean ± SD ($n = 6$) ($^{*}p < 0.05$ compare with blank group).

transfection. Besides, cytochrome *c* levels in cytoplasm were elevated markedly (Fig. 5, $p < 0.05$). It indicated that cytosolic translocation of mitochondrial cytochrome *c* occurred in AFP expression-silenced Huh7 cells. Therefore, silencing AFP gene expression inhibits cell proliferation and induces apoptosis in Huh7 cells. The apoptosis effect may be mediated by the dysfunction of P53/Bax/cytochrome *c*/caspase-3 signaling pathway.

4. Discussion

AFP is a major serum protein of embryonic plasma that is involved in the regulation of differentiation, proliferation, and apoptosis in developing cells [5,13,22]. The biological roles of AFP in mice have been established that AFP is required for brain sexual differentiation. AFP null females are infertile. The brain and behavior of female AFP null mice were masculinized and defeminized by exposure to prenatal estrogen, which is protected by AFP protein in wild-type female mice [23,24]. It has also been well characterized as a physiological carrier/transport protein for the delivery of various ligands, including fatty acids, drugs, hormones, and heavy metals into developing and malignant cells [5,13]. The specific expression and internalization of AFP is restricted to developing cells, such as embryonic cells, activated immune cells and tumor cells, which suggests its important regulatory roles in cell growth and differentiation [5,13,22]. In mid 1990s, it was reported that AFP and MAB 167H.1 acted against AFP receptor blocked programmed cell death in HL60 cells and that inhibition of AFP receptor expression was accompanied by induction of apoptosis [25]. Subsequent studies revealed that AFP positive forms of stomach cancer are characterized by significantly lower apoptotic index than AFP negative forms [26]. In 1997, it was demonstrated that AFP may be responsible for resistance of hepatoma HepG2 cells to cytotoxic effect of tumor necrosis factor [27], which is consistent with data supporting a role for AFP in apoptosis inhibition. However, subsequent studies showed that AFP induced apoptosis in various human tumor cell cultures in concentrations exceeding (100–200 μg/ml) [28,29]. During the last decade, it has been confirmed from a multitude of studies that AFP is a growth regulator and modulates the ontogenic



D

The value of apoptotic ratio(Mean±SD%)

Time(h)	apoptosis	Blank	Negative	HSS304	Cocktail
48	early stage	7.97±1.30	8.47±2.95	48.27±13.21 *	63.93±11.38 *
	terminal stage	2.27±0.91	2.77±1.39	10.60±1.95 *	17.33±2.24 *
72	early stage	7.07±1.35	8.10±2.07	60.73±12.64 *	69.23±9.71 *
	terminal stage	1.80±0.44	2.87±1.12	12.47±2.87 *	18.57±6.37 *

Fig. 4. Silencing AFP gene can induces apoptosis in Huh7 cell. (A) The cells undergoing apoptosis were visualized with Hoechst 33258 staining; (B) the apoptosis cells were visualized with TEM, the arrows indicate the apoptotic bodies in the cocktail–siRNA group; (C) representative analysis by two-parameter Annexin V-FITC/PI flow cytometry of Huh7 cells at 72 h after transfection. The proportion of FITC+/PI− cells corresponds to early apoptosis, and that of FITC+/PI+ cells corresponds to late apoptosis or secondary necrosis; (D) apoptotic ratio were determined by flow cytometry, assays were performed in triplicates and are representative for three independent experiments, the results are presented as mean ± SD ($^{*}p < 0.01$ vs. blank group).

growth and tumor progression, even though the overall findings remain controversial and their interpretations are still being debated [9,12,30,31]. Previous studies also implicated that the higher levels of AFP in the serum of liver cancer subjects is the reason for tumor development rather than merely a

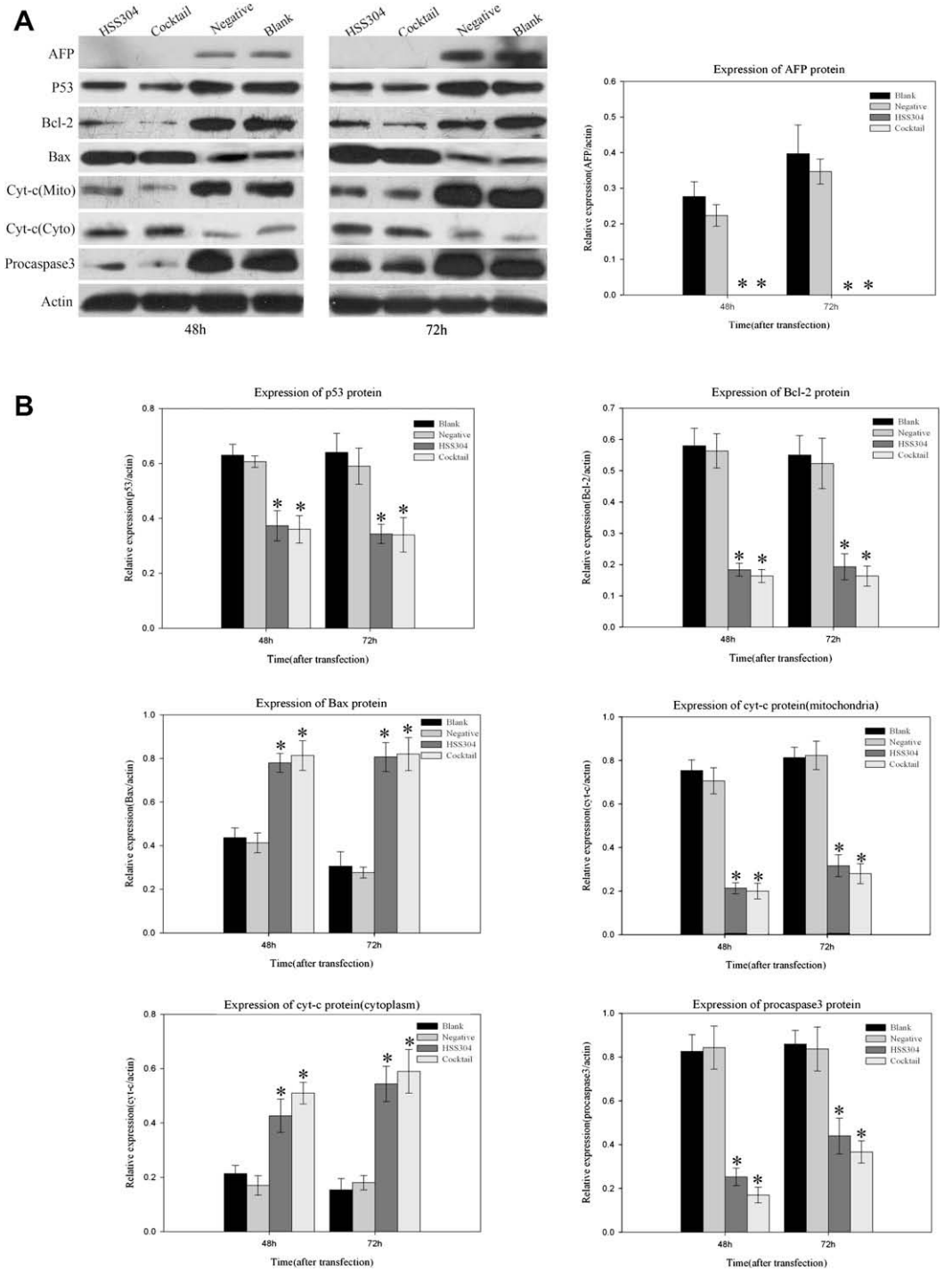


Fig. 5. Western blot detects the expression of AFP, P53, Bcl-2, Bax, cytochrome *c* (mitochondria/cytoplasm), and procaspase-3 in Huh7 cells at 48, 72 h after transfection with stealth RNAi (HSS304, the cocktail of three stealth RNAi against AFP, negative control RNAi) or blank control. (A) The bands shown here are from a representative experiment repeated three times with similar results; (B) the bands were quantified by densitometry using software Glyko Bandscan 5.0 (Glyko Inc., USA), and protein levels were normalized against those of β-actin respectively, data were expressed as mean ± SD. One-way ANOVA followed by Bonferroni correction was used to compare the data among four groups ($^{*}p < 0.05$ vs. blank group).

concomitant oncofetal protein. Although fewer studies indicate that AFP could causes apoptosis in tumor cells [28,32], the data from most current research demonstrates AFP as an enhancer of tumor growth. The down-regulation of alpha-fetoprotein is able to suppress the growth of malignant hepatocyte cells [11,33,34]. On the other hand, high levels of AFP in the fully developed HCC, or in the serum of the host, are associated with more aggressive behavior, and increased anaplasia [35]. Therefore, these studies suggest that AFP functions to constitute one of the fundamental steps in the progression of hepatoma. However, the precise mechanism of AFP-mediated cell growth regulation and apoptosis inhibition remains to be elucidated. Thus revealing the intracellular mechanisms underlying the growth regulation and apoptosis inhibition will provide further insights into the understanding of the biological role of AFP, particularly in the case of hepatocellular carcinoma.

In recent years, RNAi is more capable to specifically silence particular genes, it can be used as a powerful tool for investigating the functions of genes and genetic therapy for carcinoma [36,37]. To explore the role of AFP in tumorigenesis and tumor progression, we silenced the expression of AFP in the HCC cell line Huh7 by using RNAi in the present study. In order to validate Stealth RNAi, we measured each oligomer's effects on both AFP mRNA and protein levels. The results demonstrated that the stealth RNAi against AFP (HSS303, HSS304, and HSS305) and the cocktail of the three stealth RNAi can silence AFP expression effectively at both mRNA and protein levels in Huh7 cell after transfection. In addition, MTT assay confirmed that the proliferation of Huh7 cells was reduced significantly after AFP gene was silenced by RNAi. Furthermore, using FACS methods (double staining with FITC/PI), we observed that in comparison with non-silencing siRNA group and blank control, the stealth RNAi against AFP (HSS303, HSS304, and HSS305) and the cocktail caused accumulation of early stage apoptotic cells at 48 and 72 h after transfection (AFP was silenced). The presence of nuclear chromatin condensation and apoptotic bodies in these cells was also confirmed by Hoechst 33258 staining and TEM observation.

Based on the above results, it is clear that AFP may play an important role in the proliferation and apoptosis resistance of hepatoma cells which produce high levels of AFP. Although high concentrations of AFP or AFP-derived peptides have displayed some growth inhibitory properties [22], AFP concentration as low as below 100 μg/ml in Huh7 cells and most HCC patients could still exhibit apoptosis inhibition. Wang et al. [11] have studied *in vitro* effects of a hairpin AFP-siRNA expressing plasmid on AFP expression in SMMC-7721 cells, and they reported that the AFP-siRNA expressing plasmid down-regulated the expression of AFP about 34%, and inhibited SMMC-7721 cell proliferation without inducing apoptosis. As they did not knockdown AFP expression efficiently, we believe that the remaining low concentration of AFP may still have some apoptosis inhibition effect. In our study, we silenced expression of AFP almost completely (98.5%) with transfection of the cocktail of the three stealth RNAi at 72 h, and we did detect the overt apoptosis in AFP silenced cells, even in HSS305 treated cells with 85% reduction of AFP protein.

The wild-type p53 is a very important tumor suppressor gene for the inhibition of tumor cell proliferation. Some studies have shown that hepatocellular carcinoma is associated with abnormal expression of p53 gene [38,39]. Functional inactivation of p53 is often a critical step in the pathway to tumorigenesis. When p53 gene is mutated, it can not suppress the growth of tumor cells; on the contrary, it works as an oncogene. Furthermore, mutant p53 can bind with the wild-type p53 resulting in loss of its activity. Previous investigation demonstrates that interaction between p53 and transforming growth factor beta pathways targets chromatin modification and transcription repression of the alpha-fetoprotein gene [40]. Aberrant expression of AFP during tumorigenesis may require functional inactivation of the p53 protein [41]. In order to explore the possible mechanism underlying the apoptosis induction through silencing AFP in Huh7 cells and to verify whether silencing AFP expression effects on mutant P53 expression, Western blotting for mutant P53 was performed. The results revealed that silencing AFP expression may reduce the expression of mutant P53 protein in Huh7 cells. The down-regulation of mutant P53 should not be the off-target effect of the RNAi because the three stealth RNAi are not homologous to any known genes, and the negative control siRNA does not affect the expression of mutant P53. We suggested that silencing AFP may inhibit the expression of mutant p53 proteins in Huh7 cells.

P53 may induce cell apoptosis by transcriptionally down-regulating Bcl-2 and up-regulating Bax expression [42,43], which has been suggested to determine the survival or death of cells following an apoptotic stimulus [44]. Bcl-2 is an upstream effector molecule in the apoptotic pathway and a potent suppressor of apoptosis. It has been shown to form a heterodimer complex with the proapoptotic member Bax, thereby neutralizing its proapoptotic effects. Low expression of the apoptosis inducer Bax correlates with poor response to therapy and shorter overall survival in solid tumors. Since the ratio of Bax/Bcl-2 is important in determining whether the cells will undergo apoptosis or survival [45], we then determined whether Bax and Bcl-2 are regulated by mutant P53 proteins in Huh7 cells after AFP is silenced by the stealth RNAi. By immunoblotting, we demonstrated that silencing AFP may reduced the expression of mutant P53 (in a direct or indirect way), leading to a decrease in the expression of Bcl-2 but an increase in the expression of Bax, the Bax/Bcl-2 ratio in Huh7 cells was therefore changed which is similar to tocotrienol-rich fraction (TRF) of palm oil mediated induction of apoptosis [46].

During apoptosis, Bcl-2 family proteins regulate the release of mitochondrial apoptotic molecules such as apoptosis inducing factor (AIF), and also it was found that Bax triggers cytochrome *c* release from mitochondrial whereas Bcl-2 inhibits it [47]. To verify whether the elevation of Bax/Bcl-2 ratio in Huh7 cells could trigger the release of cytochrome *c* from mitochondria into the cytosol where they activate caspases, we extracted mitochondrial and cytoplasmic protein of all four groups and used Western blotting to determine the subcellular distribution of cytochrome *c* and the expression of procaspase-3, the precursor of caspase-3. The results showed that silencing of AFP in Huh7 cells (with HSS304, or the RNAi cocktail) is accompanied by cytosolic translocation of mitochondrial cytochrome *c* and sequential activation of caspase-3, as evidenced by reduced procaspase-3. We propose that AFP may negatively regulate Bax/Bcl-2 ratio by modulating P53 and leads to apoptosis resistance in tumorigenesis and tumor progression of HCC. Hence, by silencing the expression of AFP, Bax/Bcl-2 ratio is up-regulated via reducing mutant p53 protein expression which further triggers cytochrome *c* release from mitochondria sequentially activates caspase-3 to induce apoptosis. Our findings together with a previous report [27] support a model in which physiological relevant concentrations of AFP play a protective role against cytochrome *c* induced apoptosis.

Taken as a whole, our study demonstrate that (1) stealth RNAi against AFP can silence AFP gene effectively at both mRNA and protein levels in Huh7 cells; (2) silencing expression of AFP inhibits cell growth and induces apoptosis in Huh7 cells; (3) silencing AFP induces apoptosis may through the p53/Bax/cytochrome c/caspase-3 apoptosis signaling pathway in Huh7 cells, which needs further studies to confirm. These results imply that AFP may function as a hepatoma growth stimulator or apoptosis inhibitor. Thus, interference with AFP expression may serve as an option for suppressing tumor growth and improving prognosis of HCC patients with high AFP levels, although further *in vivo* studies are necessary.

Conflict of interest statement

Our research has no conflicts of interest with others.

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