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Regression of hepatocarcinoma cells using RNA aptamer specific to alpha-fetoprotein

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ABSTRACT

Alpha-fetoprotein (AFP) is a cancer-associated fetal protein and has long been utilized as a serum fetal defect/tumor marker to monitor distress/disease progression. In addition, AFP is closely associated with the proliferation of hepatocellular carcinoma. Thus, direct targeting of AFP has been recommended for a therapeutic strategy against hepatocellular carcinoma. In this study, we developed and characterized an RNA aptamer that specifically bound to the alpha-fetoprotein using SELEX technology. The aptamer interacted with the AFP with a K_D of ~33 nM. Importantly, the identified aptamer specifically and efficiently inhibited the AFP-mediated proliferation of hepatocarcinoma cells in a dose dependent manner. Moreover, the aptamer efficiently down-regulated AFP-induced expression of oncogenes in the cells. These results indicate that an AFP-specific RNA aptamer could be a useful therapeutic and diagnostic agent against AFP-related hepatocellular carcinoma.

1. Introduction

Alpha-fetoprotein (AFP) is the major serum glycoprotein that is normally produced in the fetal liver and yolk sac. AFP is highly expressed during the embryonic stage, but levels rapidly decline after birth, and serum AFP is almost undetectable in the adult serum [1]. However, AFP is re-expressed in regenerating and cirrhotic liver cells, hepatocellular carcinoma (HCC) cells, yolk sac tumor cells, and gastric cancer cells [2–4]. AFP is most commonly detected in HCC (almost 70% of tumors) and is implicated in the development of HCC. Moreover, abnormal serum levels of AFP are associated with neural tube defects, brain/spinal cord malformations, chromosomal abnormalities (aneuploidies), and various anatomic congenital disorders [5–8]. Therefore, AFP has been used both as a tumor monitoring agent and as an indicator for possible fetal neural tube defects and aneuploidies.

Due to their structural similarities, AFP is considered to assist the osmotic and carrier protein functions in the fetus as adult albumin does [9]. However, AFP has been known to be associated with the regulation of growth, differentiation, and tumorigenesis [1,10–12]. Several reports have shown that the molecular mechanism of AFP-mediated growth of human hepatocellular carcinoma cells is controlled by a transmembrane signaling transduction pathway. AFP receptors exist on the membranes of T lymphocytes, mouse hepatoma cells, and other tumor cells [13–16]. Interaction of AFP with AFP receptor can induce mRNA expression of the oncogenes *c-fos*, *c-jun*, and *N-ras*, and protein expression of mutative p53 and p21ras, which are connected with cell proliferation [17,18]. Therefore, inhibition of the function of AFP protein would be an effective approach to HCC-specific anticancer therapy.

Single-stranded RNAs which can recognize target molecules through folding into specific 3-dimensional structures are termed RNA aptamers. Such aptamers are evolved by an iterative selection method called systematic evolution of ligands by exponential enrichment (SELEX) [19,20]. RNA aptamers have been described as potentially very useful diagnostic and/or therapeutic agents against various human diseases due to their high target specificity and affinity, ease of homogeneous synthesis with chemical methods, easy modification and optimization, and poor immunogenicity, etc. [21,22].

In this study, we identified a specific RNA aptamer against AFP. We determined that the isolated aptamer specifically and efficiently inhibited AFP-induced oncogene mRNA overexpression and prevented HCC proliferation *in vitro*. These results suggest that the AFP specific aptamer can be a useful diagnostic and therapeutic agent against AFP-associated HCC.

2. Materials and methods

2.1. Protein purification

A recombinant protein of full length AFP was cloned into pET28a (+) expression vector (Invitrogen). The recombinant AFP

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was tagged with a hexahistidine at the N-terminus. Proteins were overexpressed in *Escherichia coli* BL21 (DE3) strain and purified with a nickel-chelate resin (Ni–NTA agarose, Qiagen). Purified AFP was purchased from Fitzgerald Industries.

2.2. Cell culture

Hepatocellular carcinoma cell lines, HepG2 (HB-8065 ATCC), Hep3B (HB-8064 ATCC), SK-Hep1 (HTB-52 ATCC), and Huh7 (JCRB0403 Japanese Collection of Research Bioresources) cell lines, and non-hepatocellular carcinoma cell lines, HT29 (HTB-38 ATCC), NIH-3T3 (CRL-1658 ATCC), AGS (CRL-1739 ATCC), HL-60 (CCL-240 ATCC), and MCF7 (HTB-22 ATCC), were incubated with each suitable medium (HepG2, Hep3B, SK-Hep1, MCF7: MEM/10% FBS/1% Antibiotic/Antimycotic Solution; Huh7, HT29, AGS, NIH-3T3, HL-60: DMEM/10% FBS/1% Antibiotic/Antimycotic Solution) at 37 °C, with 5% CO_2.

2.3. Selection procedure

In vitro selection was carried out essentially as described [23]. An RNA library of sequence 5′-GGGAGAGCGGAAGCGUGCU GGGCCN40CAUAACCCAGAGGUCGAUGGAUCCCCCC-3′ (where N40 represents 40 nucleotide sequences formed by equimolar incorporation of A, G, C, and U at each position) was produced by *in vitro* transcription of synthetic DNA templates with NTPs and T7 RNA polymerase (Takara). First, 5 μg of the RNA library was preincubated with 20 μL of Ni–NTA agarose beads in 100 μL of binding buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, and 1% BSA) for 20 min at room temperature. The RNA-bead complexes were then precipitated and discarded to remove RNAs nonspecifically bound to beads. The precleared supernatant was incubated with 5 μg of his-tagged AFP for 20 min at room temperature. AFP–RNA complexes were precipitated with beads, and pellets were washed three times with 0.4 mL of the binding buffer. RNAs were recovered, amplified with RT-PCR and *in vitro* transcription, and used for the next rounds of selection. Six subsequent rounds of selection were performed using the same procedure. In contrast, for rounds 7, 12 and 15, a more stringent condition was employed by reducing the AFP concentration: 1 μg (rounds 7–11), 0.5 μg (rounds 12–14), and 0.1 μg (rounds 15–16). After 16 rounds of selection, the amplified DNA was cloned and several clones were sequenced.

2.4. Assessment of binding activity of selected RNA aptamers

A semi-quantitative real-time RT-PCR analysis was performed to evaluate the binding activity of RNA aptamer. An RNA library, 16th SELEX RNA pool, or group I aptamer (300 fmole) was incubated in the absence or presence of AFP (30 pmole). RNA–protein complexes were then precipitated with Ni–NTA agarose beads and washed. Bound RNAs were extracted and reverse transcribed with 3′-primer (5′-GGGGGGATCCATCGACCTCTGGGTTATG-3′, 25 pmole), and then cDNA was amplified with 5′-primer (5′-GGGGGAGAGCGGAAGCGT GCTGGG-3′, 25 pmole) and the 3′-primer. All reagents except MMLV reverse transcriptase and Taq polymerase (Finnzymes) were obtained from the SYBRGreen core reagent kit (Molecular Probes). The conditions for the PCRs were 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s for 40 cycles. For standardization, a known concentration of the RNA aptamers was amplified by the same method.

2.5. Measurement of the binding affinity of RNA aptamers with SPR assay

We used a Biacore 2000 system for surface plasmon resonance experiments. Briefly, a CM5 chip (carboxymethylated sensor chip) was activated with 0.1 M NHS (*N*-hydroxysuccinimide) and 0.4 M of EDC (*N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide) to immobilize his-tagged AFP on the chip surface. After immobilization of the protein, the chip surface was deactivated with 1 M ethanolamine hydrochloride, pH 8.5, to protect the remaining activated groups. After stabilizing the base line, at least five different concentrations (50–800 nM) of the library RNA and three groups of selected RNA aptamer were injected into the flow cells to measure the binding constants and other kinetic parameters of the RNAs. The protein surface was regenerated with 50 mM NaOH after injection of each sample.

2.6. Cell proliferation assay

Aliquots containing 1×10^4 hepatocellular or non-hepatocellular carcinoma cells were plated into 96-well plates and cultured in each suitable medium for 24 h. The cultures were starved in each medium without FBS for another 16 h, and treated with different concentrations of AFP (5–80 mg/L) for 30 h. To analyze the inhibitory effect of the group I aptamer on cell proliferation, starved HepG2 cells were incubated with AFP (20 mg/L) or human serum albumin (HSA) (20 mg/L) for 30 h with library RNA (40 mg/L) or group I aptamer (10–40 mg/L). Prior to the experiment, complete media was treated with RNase inhibitor (400 unit/mL; Koschem) to block RNase activity. The proliferation of HepG2 cells was measured by the MTT assay as described [24].

2.7. Analysis of c-jun and c-fos expression

Aliquots containing 5×10^4 HepG2 cells were plated into 24-well plates and cultured in MEM for 24 h. The cultures were starved with MEM without FBS for another 16 h, and then treated with AFP (20 mg/L) or HSA (20 mg/L) for 12 h (*c-jun*) or 6 h (*c-fos*), respectively. To analyze the inhibitory effect of the group I aptamer on oncogene expression, starved HepG2 cells were incubated with AFP or HSA combined with library RNA (40 mg/L) or group I aptamer (10–40 mg/L). Prior to the experiment, complete media was treated with RNase inhibitor (400 unit/mL; Koschem). Total cellular RNA was isolated and reverse transcribed with a random hexamer (100 ng/L, Invitrogen) and MMLV reverse transcriptase (Finnzymes) according to the manufacturer's protocol. The real-time PCR analysis was performed by using the Rotor-gene (Roter-Gene 6000, Qiagen) and SYBRGreen core reagent kit (Molecular Probes). The quantity of *c-jun* or *c-fos* mRNA was normalized with 18s rRNA. For standardization, five concentrations of the each gene DNA were amplified using the same method. Reaction conditions were 5 min at 95 °C, 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C for 50 cycles. The sequences of primers used for human *c-jun*; forward: 5′-GACTGCAAAGATGGAAACGA-3′, reverse: 5′-GTTGCTGGACTGATTATCA-3′, human *c-fos*; forward: 5′-CCAACTTCATTCCCACGTC-3′, reverse: 5′-CTCCCTCCTCCGGTTGC-3′, human 18s RNA; forward: 5′-GTAAC CCGTTGAACCCCATT-3′, reverse: 5′-CCATCCAATCGGTAGTAGCG-3′.

2.8. Statistics

The significance of differences between the mean values within groups was tested by using a paired two group t test (Student's *t* test) with $P < 0.05$ as the criterion for significance.

3. Results

3.1. In vitro selection of RNA aptamer against alpha-fetoprotein

An RNA library of $\sim 10^{14}$ different molecules was generated, with each molecule containing 40 nucleotide long random

sequences flanked by defined sequences. To identify specific RNA aptamers that bound the AFP, we employed an *in vitro* selection technique using the RNA library as described in Section 2. After 16 iterative cycles of SELEX, the amplified cDNAs were cloned, and 19 different clones were sequenced (Fig. 1A). RNAs with very diverse sequences were then identified. However, several RNAs

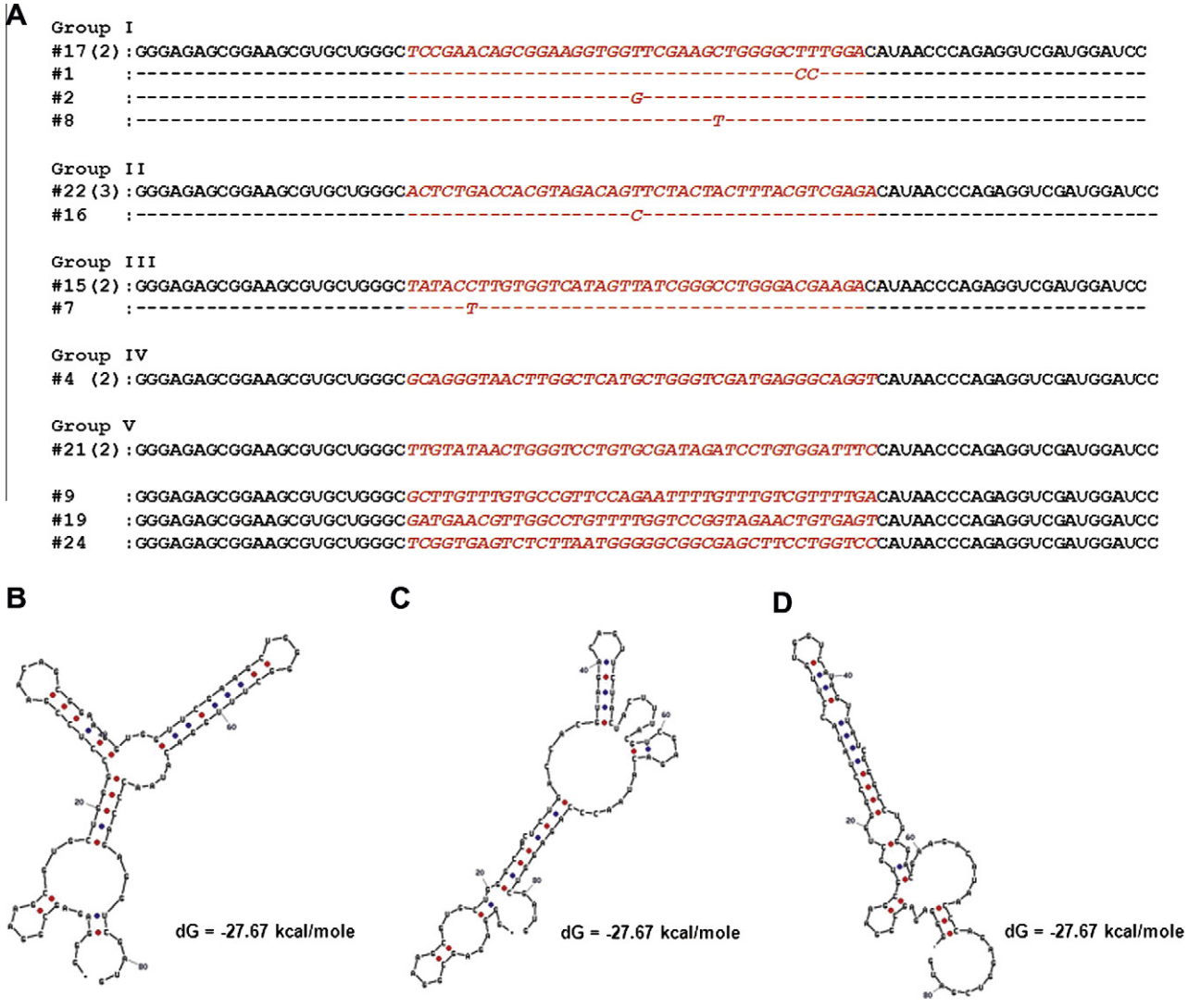

Fig. 1. Sequences and predicted structures of selected RNA aptamers. (A) After 16 rounds of *in vitro* selection, the sequences of 19 selected RNAs were determined. Selected RNAs can be divided into five different groups and three single clones, and some of them were present multiple times (numbers in parentheses). The lines drawn indicate that nucleotides at these positions were identical. Secondary structures of aptamer group I (B), group II (C), and group III (D) are depicted.

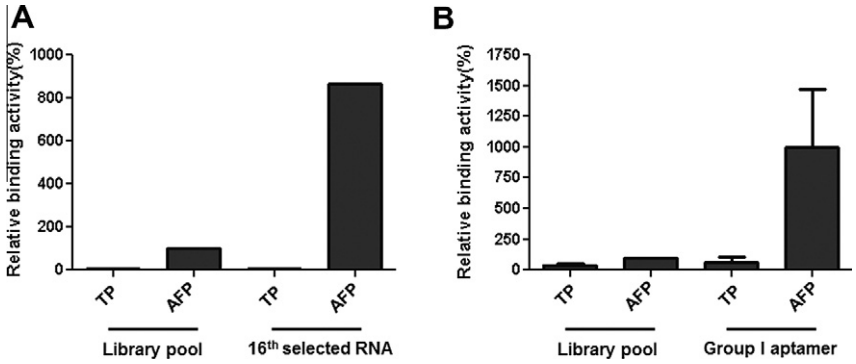

Fig. 2. Specific binding activity of 16th SELEX RNA pool and group I aptamer. (A) Library RNA or RNA pools after 16 rounds of selection were incubated in the presence of thymidine phosphorylase (TP) or AFP. TP was used as a nonspecific binding control. RNAs bound to each protein are represented as a percentage of library RNA bound to AFP. (B) Library RNA or group I RNA aptamer was incubated in the presence of TP or AFP. The amount of RT-PCR products from the RNAs bound to each protein were quantitated relative to the amount of library RNA bound to AFP. Averages of three independent measurements are presented with bars indicating standard deviations.

Table 1
Binding affinity of each group aptamer RNA to alpha-fetoprotein.

	k_a (1/Ms)	k_d (1/s)	K_A (1/M)	K_D (M)	Chi2
Group I aptamer	2.89E + 04 ± 2.16E + 04	9.73E-04 ± 7.17E-04	2.96E + 07 ± 3.54E + 05	3.39E-08 ± 4.95E-10	5.63E-01 ± 6.75E-01
Group II aptamer	8.32E + 03 ± 2.52E + 03	5.81E-04 ± 1.89E-04	1.44E + 07 ± 3.54E + 05	6.97E-08 ± 1.91E-09	1.26E + 00 ± 6.80E-01
Group III aptamer	5.31E + 04 ± 3.56E + 04	1.88E-03 ± 1.36E-03	9.16E + 07 ± 9.26E + 06	1.10E-08 ± 1.13E-09	2.09E + 00 ± 1.47E + 00
Library pool	1.16E + 04 ± 3.53E + 03	2.18E-03 ± 6.51E-04	5.34E + 06 ± 3.54E + 04	1.88E-07 ± 7.07E-10	1.45E + 00 ± 1.02E + 00

The above value is measured using BIA evaluation program. k_a, concentration of analyte binding to the target per an hour; k_d, concentration of analyte separating from the target per an hour; K_D, equilibrium constant showing binding strength; chi2, a value showing the difference between the calculation value by the BIA evaluation program and data obtained from actual experiment, which should be 10 or less.

had the same or very similar sequences and were found in multiple clones. As a working model, the secondary structures of group I, II, and III RNAs were predicted using the Mulfold program [25] (Fig. 1B–D).

Enrichment of RNA aptamers against AFP was determined using semi-quantitative real-time RT-PCR (Fig. 2). The selected RNA pool could not bind to control protein. In sharp contrast, the amount of RNAs that bound to AFP was noticeably increased by up to 8.6-fold in the 16th selected RNA pool, compared with the amount of library RNA bound to AFP (Fig. 2A). Moreover, the group I aptamer clone had a 10-fold higher binding activity compared to library pool RNA (Fig. 2B).

The binding affinity of the selected major group of aptamers against AFP was measured using the surface plasmon resonance

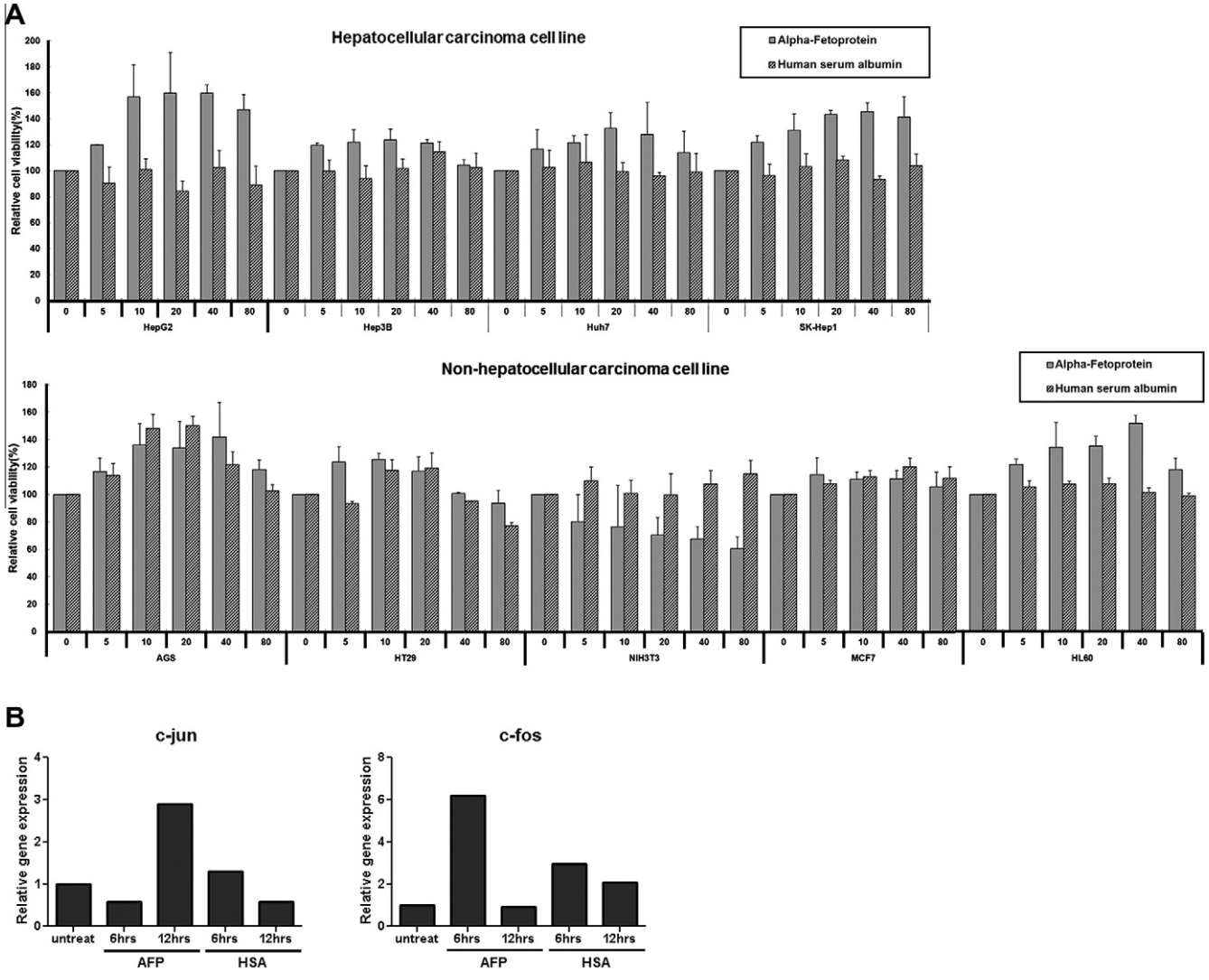

Fig. 3. Effect of AFP on the proliferation of hepatocellular carcinoma cells and their oncogene expression. (A) Various hepatocellular carcinoma or non-hepatocellular carcinoma cells were incubated with different concentrations (5–80 mg/mL) of AFP or HSA. The cell proliferation was measured by the MTT assay and expressed as the percentage of viability of untreated cells. Values represent averages of three independent experiments with standard deviation. (B) HepG2 cells were incubated with AFP or HSA for 6 and 12 h. Expression level of *c-jun* or *c-fos* mRNA was determined by quantitative real-time RT-PCR. The amount of *c-jun* or *c-fos* mRNA was normalized to the amount of 18s rRNA and quantitated relative to the amount in the untreated group.

assay (Table 1). Each group of RNA aptamers was 3- to 17-fold more tightly bound to AFP than library RNA, with a K_D of 33.9 nM (group I RNA), 69.7 nM (group II RNA), or 11 nM (group III RNA).

3.2. Effect of AFP on the proliferation of hepatocellular carcinoma cells

To test the effect of AFP on cell proliferation, we treated hepatocarcinoma or non-hepatocarcinoma cells with various concentrations of AFP for 30 h (Fig. 3A). HSA was used as a negative control. Proliferation of HCCs was increased with AFP in a dose dependent manner. Of note, HepG2 cells were the most significantly induced in their cell proliferation (up to almost 1.6-fold by AFP treatment), compared to the cell viability of untreated or HSA-treated controls. The cell proliferation of other hepatocellular carcinoma cells was also induced in an AFP-specific manner. Increase of cell proliferation was not observed in the HSA-treated groups. In contrast, cell proliferation was not changed by AFP treatment in most of the non-hepatocellular carcinoma cells. However, HL60 cells showed an almost 1.5-fold increase in cell proliferation at a dose of 40 mg/L as compared to the untreated control.

3.3. Effect of AFP on the expression of oncogene mRNA

Induction of oncogene mRNA expression in response to AFP was analyzed by quantitative RT-PCR analysis (Fig. 3B). The overexpression of *c-jun* or *c-fos* mRNA in HepG2 cells was quantitated at 6 or 12 h after treatment with AFP or HSA. AFP treatment for 12 h significantly increased the expression of *c-jun* mRNA in HepG2 cells by 2.9-fold, compared with the untreated group. Expression of *c-fos* mRNA after AFP treatment for 6 h was 6.2-fold higher than in the untreated group. However, HSA had no significant influence on the cellular expression levels of *c-jun* and *c-fos* mRNA.

3.4. Specific Inhibition of AFP-mediated cell proliferation by RNA aptamer

To test the effect of group I RNA aptamer on the AFP-mediated proliferation of HCCs, we added AFP and HSA to HepG2 cells with a library pool or group I aptamer (Fig. 4A). Cell viability was negligibly affected by treatment with either library RNA or RNA aptamer only, indicating there was no nonspecific RNA effect on cell survival. Treatment of AFP increased cell proliferation 1.55-fold compared to the untreated group. In contrast, the AFP-associated proliferation of HepG2 cells was significantly inhibited by group I aptamer in a dose dependent manner, but was not inhibited by library RNA. Cell proliferation was decreased by up to 63% at the highest concentration of aptamer (40 mg/L), compared with cells treated with AFP only.

3.5. Inhibitory effect of RNA aptamer on AFP-induced overexpression of oncogene mRNA

To determine the effect of group I aptamer on the AFP-induced oncogene overexpression, we analyzed the expression levels of *c-jun* and *c-fos* mRNA in HepG2 cells treated with AFP or HSA in the presence of library pool or group I aptamer (Fig. 4B). The AFP-mediated overexpression of *c-jun* mRNA was efficiently inhibited by the group I aptamer in a dose dependent manner, and was almost completely inhibited by the aptamer at a concentration of 40 mg/L, compared to the cells treated with AFP only. Group I

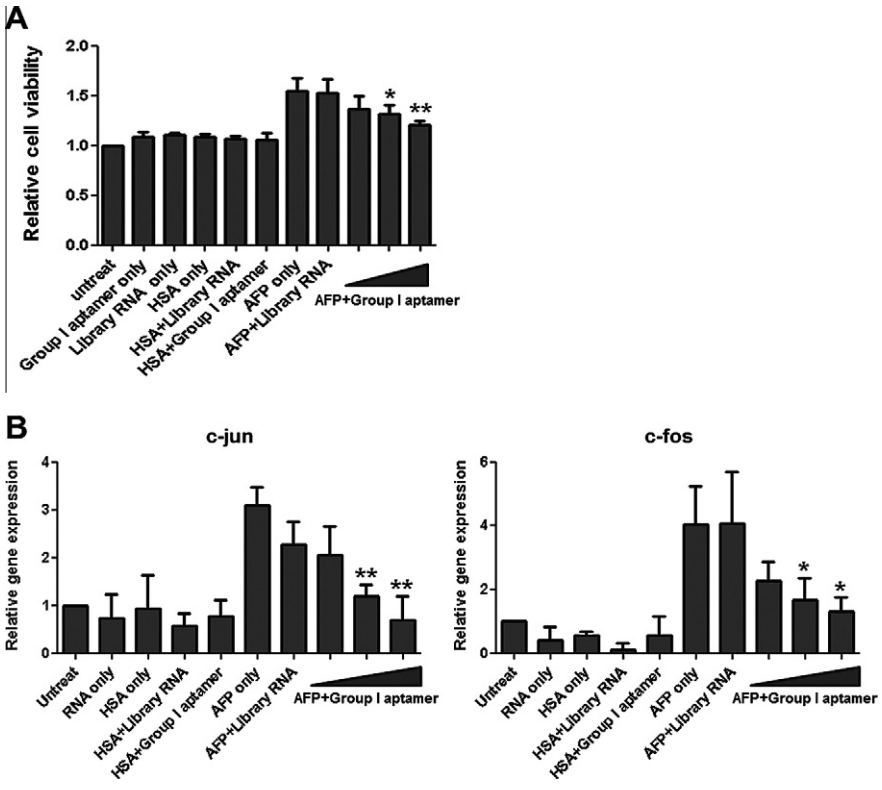

Fig. 4. Effect of RNA aptamer on AFP-induced proliferation of hepatocellular carcinoma cells and their oncogene expression. (A) The HepG2 cells were treated with AFP or HSA in the presence of library RNA (40 mg/mL) or various concentrations of group I aptamer (10, 20, 40 mg/L). The cell proliferation was measured by the MTT assay and expressed as relative to the viability of untreated cells. Values represent means of three independent experiments with standard deviation. (B) Expression level of *c-jun* or *c-fos* mRNA was determined by quantitative real-time RT-PCR of HepG2 cells treated with AFP or HSA for 12 h (*c-jun*) or 6 h (*c-fos*), with library (40 mg/mL), or with group I aptamer (10, 20, 40 mg/L). The level of *c-jun* or *c-fos* mRNA has been normalized to the amount of 18s rRNA and quantitated relative to the level in untreated cells. Values show averages of three separate experiments with standard deviation.

aptamer also significantly down-regulated *c-fos* mRNA overexpression by up to 67% at the concentration of 40 mg/L. However, library RNA had no effect on AFP-induced expression of both oncogenes. Groups treated with HSA or with RNA only did not show any influence on the *c-jun* or *c-fos* expression in HepG2 cells.

4. Discussion

AFP is an oncofetal glycoprotein which has long been used as a tumor marker for the diagnosis of hepatocellular carcinoma [26–28]. Moreover, AFP can promote cell proliferation [12,29–32] and overexpression of oncogenes [17]. Various tumor cells have AFP receptors on their membrane [16,33–35] and AFP could play an important role in regulating the growth of cells through receptor-mediated intercellular signal transduction [18,36]. In this study, we confirmed that AFP specifically and significantly induced proliferation of HCCs. On the other hand, AFP-specific induction of cell proliferation was not observed in most of non-hepatocellular carcinoma cell lines. However, proliferation of HL60 cell lines was significantly induced by AFP, most probably due to the presence of AFP receptors on the cell membrane [14]. Thus, AFP has been utilized as a therapeutic target for gene therapy, growth inhibitory peptides, and antibodies for drug delivery and immunotherapy [37–41]. However, no AFP-targeting ligands which can inhibit cancer cell growth have been reported.

In this study, we developed an RNA aptamer that specifically bound AFP with SELEX technology. The binding specificity and affinity toward the AFP were confirmed by real-time PCR analysis and a surface plasmon resonance experiment. The AFP-specific aptamer significantly decreased the AFP-associated proliferation of HCCs. Moreover, the specific RNA aptamer efficiently inhibited the AFP-induced upregulation of *c-jun* and *c-fos* gene expression. In contrast, HCC regression and upregulation of *c-jun* and *c-fos* mRNA were not influenced by library RNA. These findings indicate that the inhibition of AFP-induced HCC growth and oncogene expression was mainly due to the specific interaction of AFP with the specific aptamer.

Both *c-jun* and *c-fos* have the characteristics of early response genes. This study verified that expression of both oncogenes was efficiently and rapidly upregulated by AFP in HCCs. The *c-fos* gene responded more quickly to AFP treatment than did *c-jun*. These oncoproteins can control the expression of some later response genes through the activation of nuclear transcription factors. In addition, c-Fos and c-Jun proteins can cooperate with each other and form a heterodimer AP-1. The dimer can increase transcriptional activation of growth-related gene expression by binding to a gene transcription-regulated element [42]. Taken together, AFP-induced HCC proliferation will be caused by a cascading induction of expression of growth related oncogenes. The AFP-specific RNA aptamer developed in this study can suppress not only AFP-induced HCC proliferation but also AFP-mediated oncogene overexpression. This aptamer effect could be mainly due to sequestering of AFP, and hence due to inhibition of the initiation step of AFP-associated signal transduction. Identification and characterization of the AFP domain responsible for aptamer binding will be helpful to understand how the AFP induces HCC proliferation. Consequently, the AFP-specific aptamer can be a useful tool for conducting a mechanistic study of AFP-mediated HCC propagation as well as a potential therapeutic and diagnostic agent directed toward AFP-associated malignant disease.

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