Research Article

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# Alpha-fetoprotein acts as a novel signal molecule and mediates transcription of *Fn14* in human hepatocellular carcinoma

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**Background & Aims**: The function of cytoplasmic AFP as a regulatory factor in the growth of tumor cells has been well defined. However, its precise mechanism of action and its clinical significance remain to be worked out.

**Methods**: Specimens from HCC patients were analyzed by using immunohistochemistry, co-immunoprecipitation (CoIP), and chromatin immunoprecipitation (ChIP) assays to evaluate the role of AFP in RAR signaling-mediated carcinogenesis. Quantitative real-time reverse transcription PCR, Western blotting, confocal microscopy, CoIP, GST pull-down, siRNA, gene transfection, and ChIP assays were also used for analysis of cell lines.

**Results**: RAR is able to interact with cytoplasmic AFP and binds to the element of the regulatory region of the *Fn14* gene in the neoplastic tissue of HCC patients. An assay of hepatocyte cell lines of differing AFP expression showed that cytoplasmic AFP is able to block ATRA-induced nuclear translocation of RAR and expression of the *Fn14* gene. Knockdown of AFP in siRNA-transfected HepG2 and Bel7402 cells led to greater binding of RAR to its response element. The expression of the *Fn14* gene was therefore up regulated as reflected by increases in mRNA and protein levels. Conversely, transfection of HLE and L02 cells (AFP negative) with the *afp* gene resulted in apparent reduction of RAR binding to DNA and Fn14 protein.

**Conclusions**: Demonstration of the involvement of cytoplasmic AFP in RAR-mediated expression of the *Fn14* gene strongly indicates AFP plays a signal molecule-like role in the regulation of hepatocellular carcinoma growth.



Keywords: *Fn14*; Hepatoma; All *trans* retinoic acid; Nuclear receptor; Transcription regulation.

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*Abbreviations:* AFP, alpha fetoprotein; RAR, retinoic acid receptor; ATRA, all *trans* retinoic acid; HCC, hepatocellular carcinoma; *Fn14*, fibroblast growth factor-inducible 14; TWEAK, TNF-like weak inducer of apoptosis.

## Introduction

Alpha fetoprotein (AFP) is an oncofetal protein normally produced in the fetal liver and yolk sac, which is undetectable or found only in trace levels in adults. Although it has been widely used as a marker in clinical diagnosis, compelling progress made in determining the biological role of AFP has recently attracted considerable interest, as AFP shows a close association with HCC carcinogenesis and the high mortality rate of this tumor [1–3]. In addition, there has been increasing focus on the role of AFP in promoting cell growth in cultured cells, and this "growth factor" function is yet another intriguing aspect of AFP biology.

In recent years, our laboratory was the first to report that AFP functions as an intracellular signaling molecule which acts through binding to key proteins involved in growth or apoptosis signal pathways. Our studies have shown cytoplasmic AFP has the capability to disrupt the onward transmission of apoptotic signaling by blocking RA–RAR signaling and the caspase-3 cascade [4,5]. Subsequent studies showed that cytoplasmic AFP also blocks RA–RAR mediated expression of the growth arrest and DNA damage-inducible 153 gene (*GADD153*) and is responsible for the extent of cell survival [6]. In addition, cytoplasmic AFP was found to promote the PI3K/AKT pathway through interacting and interfering with PTEN protein which leads to aberrant growth of hepatocellular carcinoma cells [7]. These effects of cytoplasmic AFP were verified by knockdown of *AFP* mRNA with siRNA or overexpression of AFP by transfection of the *afp* gene in non-AFP producing cells. These results revealed a hitherto undiscovered role for cytoplasmic AFP in the maintenance of tumor cell growth and drug resistance. Based on these studies, ATRA resistance in the clinical therapy of AFP-producing hepatomas is at least in part attributable to high levels of cytoplasmic AFP. This suggests that ATRA chemotherapy for hepatocellular carcinoma should be combined with *afp* gene silencing to achieve maximum efficiency of this agent. The biological roles and clinical significance of AFP have been recently summarized and discussed [3]. However, findings to date have been insufficient to entirely clarify the intrinsic mechanism underlying the impact of cytoplasmic AFP, and in particular have been insufficient to clarify its clinical significance.

Our recent data from microarray analysis showed that hundreds of genes are up or down regulated in ATRA-treated HepG2

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cells [6]. One of the products of these genes includes fibroblast growth factor-inducible 14 (*Fn14*), which directly participates in the regulation of apoptosis or cell growth, and this finding stimulated our interest. Fn14 is the smallest TNFR superfamily member described up to now, and it is the only known signaling-competent receptor for the TNF-like weak inducer of apoptosis (abbreviated TWEAK), which has been shown to regulate cell death by mediation of TNF-α [8–11]. It has been shown that changes in the tumor microenvironment produced by TWEAK may contribute to cancer therapy resistance [12]. Sequence analysis of the proximal upstream region of the *Fn14* gene showed that a canonical RAR recognition site is present, raising the possibility that this gene is regulated by RAR signaling.

Evidence has thus now become convincing that AFP acts as a signal regulator involved in RAR signaling and affects the growth of hepatocellular carcinoma. However, there are issues still in question. Expression of the *Fn14* gene is up regulated in ATRA treated HepG2 hepatocellular carcinoma cells, as shown by microarray analysis [6]. In view of this, there is a question as to whether AFP is involved in ATRA-induced transcription of the *Fn14* gene by binding to RAR, which results in reduction of its nuclear translocation and binding to DNA. A second question is whether the regulatory effect of AFP in RAR-mediated expression of the *Fn14* gene identified in a cell-culture-based assay is of clinical significance, as the expression of the *Fn14* like *afp* gene is altered in HCC [13].

It is therefore of particular importance to determine whether intracellular AFP obstructs ATRA-induced apoptosis specifically by interfering with expression of the *Fn14* gene, and as a part of its overall effect AFP brings about uncontrolled proliferation and drug resistance.

Although the mechanisms involved in the capacity of AFP to affect cell death have not been well worked out, all evidence up to this point suggests these mechanisms have great potential importance. The current study was therefore undertaken to investigate the involvement of intracellular AFP in activation of the TWEAK-Fn14 cytokine-receptor axis, and also to further provide experimental support for clarification of the growth regulatory properties that have been ascribed to cytoplasmic AFP in carcinogenesis and ATRA resistance in HCC chemotherapy.

## Materials and methods

### *Specimens*

Human hepatocellular carcinoma tumor tissues obtained from 20 patients who recently underwent hepatectomy at Henan Cancer Hospital were divided into high (>1000 μg AFP/L, 10 cases) and low (<15 μg AFP/L, 10 cases) AFP groups based on the serum concentration of AFP. Tissue specimens from the cancerous liver were collected together with adjacent non-cancerous liver tissues in each case and stored in liquid nitrogen pending experimental use. All selected patients were biopsied to confirm the diagnosis of HCC by histopathologic evaluation. The study protocol was approved by the Ethical Committee of Henan Cancer Hospital. Informed consent was obtained from all patients.

### *Immunohistochemistry*

Immunohistochemical staining was performed by routinely used methods. Antibody against AFP and biotin-free HRP-labeled secondary antibody were purchased from Santa Cruze Inc., USA and Zhongshan Golden Bridge, Beijing, China, respectively.

### *Cell lines*

HepG2 and Bel7402 cells (both AFP-producing hepatocellular carcinoma cell lines), the HLE hepatoma cell line (hepatoma cell line which produces no detectable AFP), and L02 cells (normal human liver cell line which produces no detectable AFP) were gifts of the Department of Biology, Peking University Health Science Center and were maintained in DMEM medium supplemented with 10% FCS.

### *Quantitative real-time reverse transcription PCR (RT-qPCR)*

Expression of *Fn14* and *afp* mRNA was evaluated by quantitative real-time reverse transcription PCR (RT-qPCR) assay as previously described [14]. Primers used for RT-qPCR are listed in Table 1. Relative concentrations of *Fn14* or *afp* mRNA are presented as mean fold-change of samples to control.

### *Western blotting*

Western blotting was performed for analysis of expression of AFP, RAR and, *Fn14* in hepatocellular carcinoma specimens and cell lines as previously described [15]. Briefly, cells (or tumor and adjacent tissues) were lysed (or homogenized) in lysis buffer, and 40 μg of protein was utilized for each Western blot. Primary antibodies against AFP, RAR, Fn14, and β-actin were purchased from Santa Cruz Biotech Inc., USA. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG secondary Abs were purchased from Zhongshan Boil Tech Co., Beijing. Immunocomplexes were visualized by incubation of the filters with the Enhanced Chemiluminescence kit (Millipore Co., USA) and exposed with FUJI LAS 3000 (Japan).

### *Intracellular co-localization of RAR and AFP*

Cells treated with ATRA (80 μM) were fixed in paraformaldehyde solution (4%). Rabbit anti-RAR antibody and mouse anti-human AFP antibody (Santa Cruz Biotech Inc., USA) were added and incubated for 12 h. Secondary goat anti-mouse or anti-rabbit IgG antibodies conjugated with rhodamine (TRITC) or fluorescence isothiocyanate (FITC) (Zhongshan Boil Tech Co., Beijing) were applied for 2 h followed by addition of 100 μl DAPI (1 μg/ml). Cells were viewed and captured with a Laser Confocal Microscope (Leica TCS-NT SP2, Germany).

### *Co-immunoprecipitation*

Co-immunoprecipitation (CoIP) experiments for evaluation of the interaction of AFP and RAR were performed with hepatocellular carcinoma specimens and cell lines as described previously [16]. Antibodies were purchased from Santa Cruz Biotechnology, USA.

### *Purification of GST fusion protein and pull-down assay*

The GST pull-down assay was used for further verification of interaction of AFP and RAR as described previously [6]. GST–RAR clone was purchased from Beijng FunGenome Co., Ltd. For the GST pull-down assay, AFP was derived from the expression of pcDNA3.1-*afp* using the TNT T7 Quick Coupled Transcription/Translation System kit (Promega, USA). Interaction between the translated product of the TNT system and the GST–RAR fusion proteins was evaluated by Western blotting.

### *RNA interference assay*

The RNA interference technique with AFP-siRNA923 was used to assess the effect of AFP on the RAR signal pathway [5]. The specificity of AFP-siRNA923 was confirmed by examination of knockdown effect of AFP-siRNA923 on the expression of different proteins including STAT3, CCR5, p53 and, RAR (Supplementary Fig. 1). After transfection with AFP-siRNA923 for 24 h, the levels of AFP and Fn14 protein and the binding capacity of RAR to DNA in HepG2 and Bel7402 cells were examined with RT-qPCR or PCR (see primers in Table 1) and Western blotting. The effect of AFP knockdown was viewed and captured with a Laser Confocal Microscope as described above.