Elevated serum alpha fetoprotein levels promote pathological progression of hepatocellular carcinoma

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Abstract

AIM: To investigate the biological role of alpha fetoprotein (AFP) and its clinical significance in carcinogenesis of hepatocellular carcinoma (HCC).

METHODS: Clinical analysis of HCC patients and immunohistochemical examination were conducted to evaluate the relationship between serum AFP level and patient mortality. Confocal microscopy, Western blotting, dimethylthiazolyl-2,5-diphenyl-tetrazolium bromide, Cell Counting Kit-8 assays and flow cytometry were performed to explore the possible mechanism.

RESULTS: Among the 160 HCC patients enrolled in this study, 130 patients survived 2 years (81.25%), with a survival rate of 86.8% in AFP < 20 μg/L group, 88.9% in AFP 20-250 μg/L group, and 69.6% in AFP > 250 μg/L group, demonstrating a higher mortality rate in HCC patients with higher AFP levels. Surgical treatment was beneficial only in patients with low AFP levels. The mortality rate of HCC patients with high AFP levels who were treated surgically was apparently higher than those treated with conservative management. The results of immunohistochemistry showed that AFP and AFP receptor were merely expressed in tissues of HCC patients with positive serum AFP. Consistently, in vitro analysis showed that AFP and AFPS were expressed in HepG2 but not in HLE cells. AFP showed a capability to promote cell growth, and this was more apparent in HepG2 cells, in which the proliferation was increased by 3.5 folds. Cell cycle analysis showed that the percentage of HepG2 cells in S phase after exposure to AFP was modestly increased.

CONCLUSION: HCC patients with higher AFP levels show a higher mortality rate, which appears to be attributable to the growth promoting properties of AFP.

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Key words: Alpha fetoprotein; Receptor; Hepatocellular carcinoma; Mortality; Survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most frequent neoplasm worldwide, and accounts for 5.6% of all human cancers[1-3]. HCC in China has an increasing incidence, and it has become the second most frequent cause of estimated cancer-related death in the country[4]. Approximately 75%-80% of primary liver cancers are attributable to persistent viral infections with hepatitis B virus (HBV) or hepatitis C virus (HCV)[5]. HBV is the major etiologic factor of HCC in China[6]. Alpha fetoprotein (AFP) has served as a useful biomarker for diagnosis of HCC since the 1970s, when most patients with HCC were diagnosed at an advanced stage and had clinical symptoms. Serum levels of AFP above the reference value of 10 μg/L occur in approximately 75% of HCC cases[7]. Although AFP has a relatively low specificity in some patients with chronic non-neoplastic liver diseases, and it is non-diagnostic in some cases of small HCCs, AFP is still viewed as an important biomarker for the diagnosis of HCC[8,9].

AFP is an oncofetal protein normally produced in the fetal liver and yolk sac, and it is undetectable or found in trace amounts only in adults. In addition to its use in clinical diagnosis of liver cancer, the biological and pathophysiological functions of AFP have generated considerable interest. There has been compelling progress in these areas, particularly in regard to the role of this protein in cell growth and apoptosis. A recent study showed that cytoplasmic AFP may function as a regulator by interacting with phosphatase and tensin homolog deleted on chromosome ten protein and stimulating cell growth through the PI3K/AKT signal pathway[10]. Moreover, AFP co-localizes and interacts with retinoic acid receptors-β (RAR-β) in the cytoplasm, and plays a role in inhibiting translocation of RAR-β into the nucleus via competitive binding to RAR-β with all trans retinoic acid (ATRA) [11,12]. Thus, cytoplasmic AFP serves as an inhibitor in the retinoic acid-retinoic acid receptor signaling pathway and is most likely at least in part responsible for retinoid resistance in tumor chemotherapy[10]. By countering the effect of AFP, it may be possible to increase the sensitivity of tumor cells to ATRA. AFP also interacts with caspase-3 in the cytoplasm and blocks onward transmission of signaling from caspase-8[13]. Knockdown of AFP increases the sensitivity of hepatoma cells to tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and thereby triggers caspase-3 signaling. Therefore, it is possible that the combination of AFP gene silencing together with ATRA/TRAIL treatment will enhance the chemotherapeutic efficiency of these agents.

AFP therefore should be regarded not only as a marker for diagnosis of HCC, but also as a factor involved in ontogenetic and oncogenic growth. AFP receptors have been identified in various cell lines and tissues[14-20], and these receptors have been isolated and characterized[21-24]. Moreover, the intracellular events triggered by the binding of AFP to its receptor are being studied[25-27]. High mortality rates and poor clinical outcome are typically observed in patients with primary liver cancer as well as in other AFP-producing cancers. However, the relationship of AFP and its receptor with the mortality and patient outcome has not been fully clarified. Thus, understanding of the mechanism underlying this phenomenon may benefit the development of new clinical therapeutic strategies.

In the current study, we retrospectively analyzed the relationship between serum AFP levels and the mortality in 160 HCC patients and examined the in vitro effects of AFP on cell growth in order to support the assumption that elevated AFP is a significant risk factor in HCC mortality due to its capability of promoting growth of tumor cells.

MATERIALS AND METHODS

Subjects

One hundred and sixty HCC patients hospitalized in Beijing You’an Hospital between January 2006 and June 2009 were recruited to this study and the relationship between serum AFP levels and mortality was retrospectively assessed. In each case, a preliminary diagnosis of HCC was made based on the guidelines for clinical diagnosis and staging of primary HCC published by the Chinese Society of Liver Cancer (2001), fulfilling at least one of the following criteria[28]: (1) a hepatic space-occupying lesion with serum AFP ≥ 400 μg/L; (2) serum AFP < 400 μg/L but with a new hepatic space-occupying lesion, with arterial phase enhancement on computed tomography or magnetic resonance imaging. All the patients selected were confirmed by histopathological evaluation. Patient demographics and clinicopathological data are summarized in Table 1. The AFP cut-off value used in this study has been proved to be sensitive and specific, and defined by receiver operator characteristic curve as described before[29]. This study protocol (2006-LINSHEN-3) was approved by the Ethical Committee of Beijing You’an Hospital, Capital Medical University. Informed consent was obtained from all patients.

Of the 160 HCC patients, 88 underwent surgical resection and 72 were treated conservatively with only hetero-optimy. All the patients were followed up for 2 years at an interval of 6 mo. The survival rates of the patients with surgical and non-surgical management were retrospectively analyzed.

Immunohistochemistry

Immunohistochemical staining was performed on 4-μm formalin-fixed, paraffin-embedded tissue blocks. Tissue sections were deparaffinized and rehydrated, and heat-induced epitope retrieval was carried out in a 10 mmol/L citrate buffer (pH 6.0). After endogenous peroxidase was...
blocked with 3% H2O2, sections were incubated with primary antibodies against AFP (Santa Cruz Inc., United States, sc-8399) and AFPR (Santa Cruz Inc., United States, sc-51751) overnight at 4 °C. A biotin-free horseradish peroxidase-labeled secondary antibody (Zhongshan Golden Bridge, Beijing, China) was used for 60 min at room temperature. Coloration was performed with 3,3′-diaminobenzidzin.

**Cell lines**

HepG2 cells, an AFP-producing HCC cell line, were cultured in Dulbecco’s modified eagle media (DMEM) supplemented with 10% fetal calf serum (FCS). The HLE hepatoma cell line, which is an AFP non-producer showed no detectable amount of AFP, and was maintained in DMEM medium supplemented with 10% FCS.

**Western blotting**

Western blotting was performed for analysis of expression of AFP and AFP receptor (AFPR) in HepG2 and HLE cells as previously described[10]. Briefly, cell lysate (40 μg) from each sample was subjected to 10% sodium dodecyl sulfate-Polyacrylamide gel electrophoresis. Electrophoretic transfer of proteins from gels onto nitrocellulose membranes (Amersham, United Kingdom) was carried out in transblotting cells. Membranes were blocked by immersing in 5% nonfat milk (w/v)/PBS for 1 h, and then incubated with anti-AFP, AFPR and β-actin mAbs (Santa Cruz Biotech Inc, United States) at room temperature for 2 h. After rinsing with PBS/0.1% Tween-20, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse (Zhongshan Boil Tech Co, Beijing) IgG secondary Ab. Immunocomplexes were visualized by incubation of the filters with the Enhanced Chemiluminescence kit (Zhongshan Bridge, Beijing, China) was used for 60 min at room temperature. Coloration was performed with 3,3′-diaminobenzidzin.

**Intracellular localization of AFP and AFPR**

Localization of AFP and AFPR in HepG2 and HLE cells was analyzed with confocal microscopy as previously described[10]. Mouse anti-human AFP and mouse anti-human AFPR antibodies were purchased from Santa Cruz Biotech Inc, United States. Secondary goat anti-mouse IgG antibodies conjugated with rhodamine (Tetramethylrhodamineisothiocyanate) were purchased from Jackson Immun Res Lab, Inc, United States. Images of HepG2 and HLE cells were captured with a confocal laser microscope (Leica TCS-NT SP2, Germany).

**Determination of viability**

To assess the effect of AFP on cell proliferation, the dimethylthiazol-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay and analysis were performed with the Cell Counting Kit (CCK)-8 (Dojindo Laboratories, Kumamoto, Japan) as described previously[10]. The CCK-8 assay was used to verify the effect of AFP seen with the MTT assay[10]. Briefly, 5 × 10^4 HepG2 and human lens epithelial (HLE) cells were pipetted into 96-well microplates with various concentrations of AFP (0, 0.01, 0.1, 1, 10 and 100 mg/L) for the MTT assay, and were further subdivided into 0, 50, 100, 200 and 400 μg/L concentrations for both the MTT and CCK-8 assays. The absorbencies were measured at a wavelength of 570 nm for MTT assay and 450 nm for CCK-8 assay on a Universal Microplate Reader (EL×800). The percentage of cell proliferation for each treatment was calculated as % cell proliferation = [(A1/0 or 450 sample-background)/A1/0 or 450 control-background] × 100%.

**Cell cycle analysis**

Cell cycle measurement was performed by flow cytometry of DNA following propidium iodide (PI) staining[30]. Briefly, HepG2 and HLE cells were cultured in serum-free medium for 12 h to arrest the cell cycle. The supernatant was then replaced by fresh medium containing 10% FCS and cells were transferred into 6-well plates (3 × 10^5 cells/well). Cells were then treated with AFP (400 μg/L) for 24 h and stained by addition of 10 mg/mL PI at a final concentration of 50 mg/L. DNA content was analyzed with a FACScan-420 flow cytometer (Becton-Dickinson) as described previously. The distribution of cells in different cell cycle stages was determined according to the DNA content.

**Statistical analysis**

All data were statistically analyzed using χ2 test and the t test and SPSS16 software, and expressed as mean ± SD.

**RESULTS**

**Relationship between serum AFP level and mortality of HCC patients**

HCC patient survival was analyzed retrospectively. Table 2 shows the relationship between the serum AFP level and mortality among three groups of HCC patients. There were no significant differences in mortality among these groups in the first 6 mo of evaluation. However, after one year, a higher mortality rate was observed in the high AFP group (> 250 μg/L) as shown in Table 2. The mortality rate in these groups of patients showed a direct correlation with serum AFP level. At the end of 2 years, 130/160 (81.25%) patients were alive with a survival rate of 86.8% in AFP < 20 μg/L group, 88.9% in AFP 20-250 μg/L group, and 69.6% in AFP > 250 μg/L group. These results demonstrate that the HCC patients with AFP levels higher than 250 μg/L had a higher mortality rate.

Further analysis showed that the overall survival rate (88.9%, 48/54) in HCC patients with lower AFP levels (AFP < 20 μg/L and 20-250 μg/L) who were treated surgically, was apparently higher than in those with high AFP levels in both groups treated surgically (61.8%, 13/21) (P < 0.05) and nonsurgically (80.0%, 40/50) at 24 mo (P < 0.05) (Figure 1A). There were no significant differences in the survival rates between patients managed surgically and conservatively in the two groups with lower
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**Table 1** Clinical features of hepatocellular carcinoma patient cohort (n = 160) (mean ± SD)

| Concentration of AFP (μg/L) | < 20 | 20-250 | ≥ 250 |
|-----------------------------|------|--------|--------|
| Age (yr)                    | 51.6 ± 9.9 | 54.6 ± 10.3 | 51.2 ± 9.8 |
| Sex (male/female)           | 56/12 | 29/7   | 43/13  |
| ALT (U/L)                   | 580.6 ± 60.0 | 55.8 ± 48.2 | 58.7 ± 58.5 |
| Child                       | A     | B      | C      |
|                            | 52    | 12     | 4      |
| HBsAg (+)                   | 56    | 28     | 50     |
| HCV (+)                     | 1     | 1      | 1      |
| Non-viral                   | 11    | 7      | 5      |

AFP: Alpha fetoprotein; ALT: Alanine transaminase; HBsAg(+): Hepatitis B virus antigen positive; HCV: Hepatitis C virus; Non-viral: Non-viral etiology.

AFP levels. It is noteworthy that the survival rates of HCC patients with high AFP levels (> 250 μg/L) treated surgically (61.8%, 13/21) were lower than those treated conservatively (86.4%, 19/22) at 24 mo (P < 0.05). This suggests that surgical management confers survival benefit to HCC patients with lower AFP levels, but may be detrimental to the clinical course of HCC patients with AFP levels higher than 250 μg/L.

**Expression of AFP and AFPR in liver tissues of HCC patients**

We performed immunohistochemistry to confirm the existence of AFP/AFPR in tumor tissues of HCC patients. As shown in Figure 1B, neither normal liver tissues nor negative serum AFP showed any detectable AFP and AFPR. Interestingly, AFPR was expressed in AFP positive tissues but not in AFP negative tissues. The co-expression of AFPR and AFP in HCC patients indicated the functional relationship between these two proteins. Specific stronger staining of AFP and AFPR appeared in the cytosol and membrane, but not in the nucleus of tumor cells (Figure 1B). AFPR was not detected in fibrocytes surrounding the tumor cells. There was no qualitative relationship between serum AFP levels and the AFPR levels. In HCC patients with low serum AFP levels, immunohistochemistry still exhibited stronger AFPR staining. It is thus far unknown whether there is a clinical significance associated with expression of AFPR. Further clinical studies are needed to explore and establish the importance of AFPR in occurrence and progression of HCC.

**Confirmation of existence of AFP and AFPR in HepG2 and HLE cells**

To further evaluate the role of AFP in cell growth, cultured HepG2 and HLE hepatoma cell lines were used. Data from Western blotting showed that both AFP and AFPR were expressed in HepG2 but not in HLE cells (Figure 2A). Morphologic images under confocal microscope further confirmed that AFP and AFPR were present and scattered throughout the cytoplasm in HepG2 cells (Figure 2B and C). Although variable expressions of AFP in different cell lines have been reported previously, there has been no data regarding the presence and distribution of AFPR. It is of interest to note that AFPR is present only in cells with AFP. Consistent with the immunohistochemical observations noted above, HepG2 cells produced both AFP and AFPR, while HLE cells produced neither of these proteins. This suggests the possibility of co-transcription and functional interrelation of these molecules.

**Effect of AFP on cell proliferation**

To verify the effect of AFP on tumor growth, MTT and CCK-8 cell proliferation assays were performed. The MTT assay showed that the maximum effect of AFP in promoting growth of both HepG2 and HLE cells was achieved at a 24 h incubation, and the most effective AFP dosage was about 1 mg/L (Figure 3A). More significantly, proliferative effect was found in HepG2 cells, which showed a 3.5-fold increase in proliferation as compared with HLE cells at an AFP concentration of 1 mg/L. HepG2 cells are AFP and AFPR producing cells, and these cells showed a higher sensitivity in their response to AFP. When the AFP concentration range was limited to 0-400 μg/L, dose-dependent growth was observed in both cell lines (Figure 3C), however, HepG2 cells showed greater proliferation, similar to that shown in Figure 3A and B. The CCK-8 assay was used to further confirm the results obtained with MTT using AFP at a concentration range of 0-400 μg/L, which demonstrated a similar trend of growth (Figure 3D).

**Cell cycle analysis**

Flow cytometry was used to examine the effect of AFP on the cell cycle. Separation of cells in G0/G1, S phase and G2/M was based upon linear fluorescence intensity after staining with PI. Representative profiles are shown in Figure 4. The large initial peak (left) represents cells in G0/G1, the intervening area represents cells in S phase, and the final tail/small peak (right) represents cells in G2/M. These results showed that when HepG2 cells were exposed to AFP (400 μg/L), the percentage of cells in S phase was modestly increased (19.4%, P < 0.01) (Figure 4A and B). In contrast to HepG2 cells, the profile of the cell cycle in HLE cells did not show any significant change (Figure 4C and D).

**Table 2** Survival of hepatocellular carcinoma patients with different alpha fetoprotein levels over two years

| Time (mo) | Survival rates (%) (dead/alive cases) |
|-----------|--------------------------------------|
|           | < 20 μg/L | 20-250 μg/L | ≥ 250 μg/L |
| 0         | 100.0 (0/68) | 100.0 (0/36) | 100.0 (0/56) |
| 6         | 95.7 (3/65) | 97.2 (1/35) | 91.1 (5/51) |
| 12        | 94.1 (4/64) | 97.2 (1/35) | 80.4 (11/45) |
| 18        | 91.2 (6/62) | 97.2 (3/33) | 73.2 (15/44) |
| 24        | 86.8 (9/59) | 88.9 (4/32) | 69.6 (17/39) |

*P < 0.05 vs serum alpha fetoprotein < 20 μg/L and 20-250 μg/L groups.

AFPR was expressed in HepG2 but not in HLE cells.
HCC is one of the most common cancers worldwide, with a high mortality and prevalence rate in some countries, including China. Clinical studies have shown a potential relationship between AFP level and progression of HCC, and AFP may be used as a marker for monitoring treatment response in HCC patients [34-36]. As demonstrated in the retrospective analysis of 160 HCC patients in this study, patient mortality was apparently related to AFP levels. Patients with lower AFP levels (< 250 μg/L) showed a higher survival rate. Moreover, although surgical treatment has long been considered to be the primary therapeutic option for HCC [37], in this study surgery conferred survival advantage only in patients with lower AFP levels (< 250 μg/L). Surgical intervention in this study was associated with acceleration of death in patients with higher AFP levels (> 250 μg/L). These results may play a pathological role in carcinogenesis and progression of HCC.

There is extensive evidence showing that AFP is functionally an embryonic and fetal carrier/transport molecule for a multitude of ligands including fatty acids, bilirubin, heavy metals, steroids, retinoids, drugs, dyes and antibiotics [38]. However, the biological and pathophysiological roles of AFP associated with the occurrence and high mortality of HCC are still under study [22,39]. Silencing AFP expression by knockdown of its gene may play a role in growth arrest and apoptosis in human HCC cells [10,11,13,40]. Although AFP is widely used as a marker for diagnosis of HCC, some basic researches imply that AFP may be applicable in the treatment and prognostic monitoring of HCC patients [27,14]. Nevertheless, clinical observations supporting the biological role of AFP is still far from sufficient and additional data remains to be accumulated and evaluated.

**Figure 1** Survival rates of hepatocellular carcinoma patients and expression of alpha fetoprotein and alpha fetoprotein receptor in tumor tissues. A: Comparison of survival rates in hepatocellular carcinoma (HCC) patients with various alpha fetoprotein (AFP) levels who were treated surgically or non-surgically. Serum AFP levels of these HCC patients were divided into three groups: < 20 μg/L, 20-250 μg/L and > 250 μg/L. B: Immunohistochemical analysis of AFP and AFP receptor (AFPR) expressions in HCC tumor tissues. Membranous expression of AFPR is indicated with arrows. HE: Hematoxylin-eosin staining; AFP: Alpha fetoprotein; AFPR: Alpha fetoprotein receptor; HCC: Hepatocellular carcinoma.
Figure 2  Expression of alpha fetoprotein and alpha fetoprotein receptor in HepG2 and HLE cells. A: Western blotting; B and C: Confocal microscopy. The immunoblots and images captured by confocal microscopy are representative of experiments that were repeated at least three times. AFP: Alpha fetoprotein; AFPR: Alpha fetoprotein receptor.
Figure 3  Effects of alpha fetoprotein in proliferation of HepG2 and HLE cells. Different concentrations (0, 0.01, 0.1, 1, 10 and 100 mg/L) of alpha fetoprotein (AFP) were tested in cell culture. The proliferation of HepG2 (A) and HLE (B) cells was evaluated with the dimethylthiahzolyl-2,5-diphenyl-tetrazolium bromide (MTT) assay at 24 and 48 h. HepG2 and HLE cells were further tested with 0, 50, 100, 200 and 400 μg/L AFP and proliferation was evaluated by MTT (C) and a cell counting kit (cck)-8 assay (D) at 24 h. The differences in proliferation between HepG2 and HLE cells were statistically analyzed with SPSS16 software. Data are representative of experiments that were repeated three times and are presented as mean ± SD for 6-9 samples.

Figure 4  Effects of alpha fetoprotein on cell cycle progression of HepG2 and HLE cells. The distribution of cells in different cell cycle phases was determined according to DNA content. The data are representative of 3 independent experiments. Each panel represents a different treatment group. A: HepG2 cells without treatment; B: HepG2 cells treated with 400 μg/L alpha fetoprotein (AFP) for 24 h; C: HLE cells without treatment; D: HLE cells treated with 400 μg/L AFP for 24 h. In each panel, the number represents cell percentage in S phase. Data are presented as mean ± SD of 3 samples. AFP: Alpha fetoprotein.
The significance of AFPR in cell proliferation is of particular concern. However, until recently there has been little work involving AFPR in its clinical relation to HCC. Given the fact that AFP and its receptor are co-expressed in serum AFP positive but not in AFP negative tissues or cells as shown in this study, it is conceivable that cells expressing AFPR are more sensitive to AFP, thereby exhibiting greater proliferation and an associated increase in the proportion of cells in S phase. It has been inferred that AFP is secreted from hepatoma cells and acts on these cells through an autocrine mechanism. However, this does not occur in AFP negative tissues or cells as shown in this study. It is of particular interest that observations in HCC patients were consistent with findings from laboratory research, in that about one-third of HCC patients had similar AFP levels to healthy subjects, leading to a low mortality. Thus, based on these results and findings in cultured cell assays, the intrinsic mechanism underlying the role of AFP in the mortality of HCC patients might be implicated.

Taken together, these results emphasize the significance and importance of serum AFP level in HCC patient survival. It appears that AFP is not simply a marker for diagnosis, it is also a growth factor in tumor progression. This concept of the function of AFP is consistent with the observations in this study in that HCC patients with higher serum AFP levels appear to have a higher mortality. Incorporation of serum AFP level into the criteria for evaluating prognosis and determining therapeutic options in HCC patients will likely be of significant benefit in patient management. Nonetheless, further clinical studies are needed to confirm this conclusion.

**COMMENTS**

**Background**

Although alpha fetoprotein (AFP) has been used as a serum marker for the clinical diagnosis of hepatocellular carcinoma (HCC), the biological role of this molecule in relation to its clinical significance is still unclear. The goal of this study is to evaluate the relationship between elevated serum AFP levels and survival of HCC patients and possible underlying mechanisms.

**Research frontiers**

AFP is an oncogene protein and widely used as a marker in clinical diagnosis of HCC. In the last decade, compelling progress has been made in the two related research areas. First, AFP excreted into the circulation is able to promote cell growth and has been defined as a growth regulator in ontogenic growth and tumor progression. Second, it was recently demonstrated that intracellular AFP may function as a signal molecule through binding key proteins involved in growth or apoptosis signal pathways.

**Innovations and breakthroughs**

This study showed that AFP and AFPRI were merely expressed in tissues of HCC patients with positive serum AFP. HCC patients with higher AFP levels had a higher mortality rate, which appears to be attributable to the growth promoting properties of AFP.

**Applications**

The findings in this study emphasize the significance and importance of serum AFP level in HCC patient survival. It appears that AFP is not simply a marker for diagnosis, it is also a growth factor in tumor progression. Incorporation of serum AFP level into the criteria for evaluating prognosis and determining therapeutic options in HCC patients may significantly benefit the management of HCC patients.

**Peer review**

This is an interesting study which underlines the importance of AFP in hepatocellular carcinoma. The paper is well written. This is another study which shows that AFP must play a key role in the decision making in operative treatment for HCC patients. A prospective study with comparable groups of HCC patients (size and number of tumor nodules, differentiation, clinical parameters of patients) either with low or high AFP would be ideal in the future.

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