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Original Article

AFP specificity for HCC surveillance is increased by mitigating liver injury among treated chronic hepatitis B patients with elevated AFP

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Abstract: Objective: The aim of this study was to assess AFP response in chronic hepatitis B (CHB) patients with baseline positive AFP (≥7 ng/mL) who received antiviral therapy thereafter. Methods: A cohort study was conducted to assess AFP response in CHB patients who had baseline positive AFP and got antiviral therapy. Results: This retrospective study enrolled 302 antiviral-treatment-naïve CHB patients with positive AFP. After a 12-month antiviral treatment, 144 patients normalized AFP during follow-up while the rest remained AFP-positive. There were no significant differences in baseline characteristics and virologic and ALT responses to antiviral therapy between the two groups. During a mean follow-up of 34 ± 6 months, 16 patients (5.3%) in this cohort developed HCC, and 14 (8.9%) of them emerged in the AFP positive group. There was a significant difference (P=0.004) in HCC occurrence between AFP normalized and non-normalized groups after treatment. Univariate and multivariate analyses revealed that cirrhosis (HR=9.983, 95% CI=3.609-27.617, P<0.001), and non-AFP response to antiviral treatment (HR=6.517, 95% CI=1.475-28.784, P=0.013) were two independent factors associated with HCC occurrence. Conclusions: To our knowledge, this is the first investigator-initiated cohort study to assess the performance of on-treatment AFP in CHB patients with baseline positive AFP. In contrast to the criticism that AFP is neither sensitive nor specific, the current study has provided important evidence that on-antiviral-treatment AFP normalization is a specific protective marker for HCC in patients with HBV-related chronic liver diseases who started antiviral therapy thereafter.

Keywords: Chronic hepatitis B, AFP α-fetoprotein, HCC hepatocellular carcinoma, antiviral treatment

Introduction

Emergent hepatocellular carcinoma (HCC) can be accompanied by elevated serum alpha-fetoprotein (AFP). AFP is the most commonly used serum marker for HCC surveillance in China [1-3]. However, the American Association for the Study of Liver Disease does not recommend AFP for HCC surveillance, nor is AFP routinely included in evaluating responses to antiviral treatment because of the perceived poor AFP specificity in detecting early HCC [4]. Thus, there is a need to improve AFP specificity for HCC diagnosis through understanding underlying mechanisms for elevation of AFP in CHB patients [5-8].

Antiviral agents are known to effectively suppress not only HBV replication but also hepatitis activity, which may reduce the rate of false-positive AFP tests. However, some patients still develop HCC during antiviral therapy [9-11]. Therefore, it is clinically important to differentiate between high AFP levels associated with HBV infection and AFP elevation accompanying early HCC development, especially in antiviral-treatment-naïve patients with elevated AFP. Also, there has been no consensus regarding the clinical strategy for monitoring elevated AFP in these patients. Studies are needed to determine the risk of HCC after initialing antiviral treatment in treatment-naïve patients with baseline positive AFP. Thus, this study aimed to identify variables associated with AFP response and HCC risk after initialing antiviral treatment in CHB patients with baseline positive AFP.

Materials and methods

Patients

This single-center, retrospective investigator-initiated cohort study reviewed the medical records of 9964 chronic hepatitis B (CHB) patients with positive AFP (≥7 ng/mL, ULN) in Nanfang Hospital, Southern Medical University between January 2013 and February 2016. All patients were examined with abdominal ultrasound (US), computed tomography (CT) and/or magnetic resonance imaging (MRI) before enrollment to rule out possible underlying hidden HCC. Among them, 436 patients were confirmed to be antiviral-treatment-naïve upon their initial visit and subsequently received the antiviral treatment, 302 of whom were finally included in this study (**Figure 1**). Patients were excluded for: co-infection with human immunodeficiency virus, hepatitis C virus, or hepatitis D virus; a history of antiviral treatment within the preceding 5 years; or documented hepatocellular carcinoma (HCC). This study was approved by the Ethical Committee of Nanfang Hospital, Southern Medical University (NFEC-2014-079). The experiments were carried out in accordance with the approved guidelines and the informed written consent was obtained from all subjects.

Surveillance tests and patient follow-up

Patients visited the outpatient clinic every 1-3 months in the first year, every 3-6 months in the second year, and every 6 months thereafter; all patients were followed until 31 December 2017 unless they died earlier. HCC surveillance was conducted through monitoring AFP level and abdomen ultrasound (US) for at least 12 months after the initiation of antiviral therapy. Continuous AFP positive was defined as persistent AFP positivity at (≥7 ng/mL) upon tests during the first 12 months after antiviral therapy. The reason we chose 7 ng/ml as the cut-off of AFP level is that the serum AFP level was quantitatively measured with the Roche AFP kit, and its 95% confidence interval is 7 ng/mL. A dynamic imaging study (computed tomography or magnetic resonance imaging) was performed if (i) US surveillance detected >2 cm nodule(s) that had not been previously characterized or that grew significantly; or (ii) there were multiple (>3) cirrhosis-related nodules smaller than 2 cm; or (iii) we could not exclude infiltrative HCC; or (iv) a benign lesion was suspected by USG, such as hemangioma, focal fat-sparing area, or fat deposition in the liver or when the whole liver could not be visualized by USG examination; or (v) kinetic AFP level progressively increased on more than two occasions. Cirrhosis was diagnosed based on transient elastography (TE values ≥14.6 kPa), ultrasonography and MRI findings (such as signs of portal hypertension, ascites, heterogeneous echotexture, surface nodularity) or clinical evidence of liver cirrhosis (history of hepatic decompensation). HCC was diagnosed according to the American Association for the Study of Liver Diseases (AASLD) guidelines (1) [1].

Clinical and laboratory evaluation

Clinical data were collected including age, gender, Barcelona Clinic Liver Cancer (BCLC) stage, and abdominal computed tomography (CT) or magnetic resonance imaging findings of tumor size and vascular invasion. Laboratory data included platelet count (PLT), hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), HBV DNA, α-fetoprotein (AFP), albumin (ALB), total bilirubin (TBil), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The serum HBV DNA level was measured with the Cobras Taqman HBV Kit (Roche Diagnostics; lower limit of detection, 20 IU/mL). HBeAg, Anti-HBe, anti-HBc were detected using an Architect assay (Abbott Laboratories, Abbott Park, IL). Aminotransferases were measured according to standard procedures locally at the time of sampling. Serum AFP levels were determined by electro-chemiluminescence immunoassay (Roche Diagnostics; Positive boundary value, ≥7 ng/mL).

Statistical analysis

All statistical analyses were conducted using SPSS 20.0 software (SPSS Inc., Chicago, IL). Continuous variables were expressed in mean ± standard deviation or median (interquartile range, IQR), as appropriate. Continuous and categorical variables were analyzed using the Student's t-test, and the chi-square test (Pearson Chi-Square or Fisher's Exact Test), respectively. The cumulative rate of HCC was estimated using the Kaplan-Meier method. Kinetic changes in AFP, ALT and HBV DNA levels were analyzed by repeated measurement analysis of variance. Independent risk factors for HCC development were identified with the Cox proportional hazards regression model by

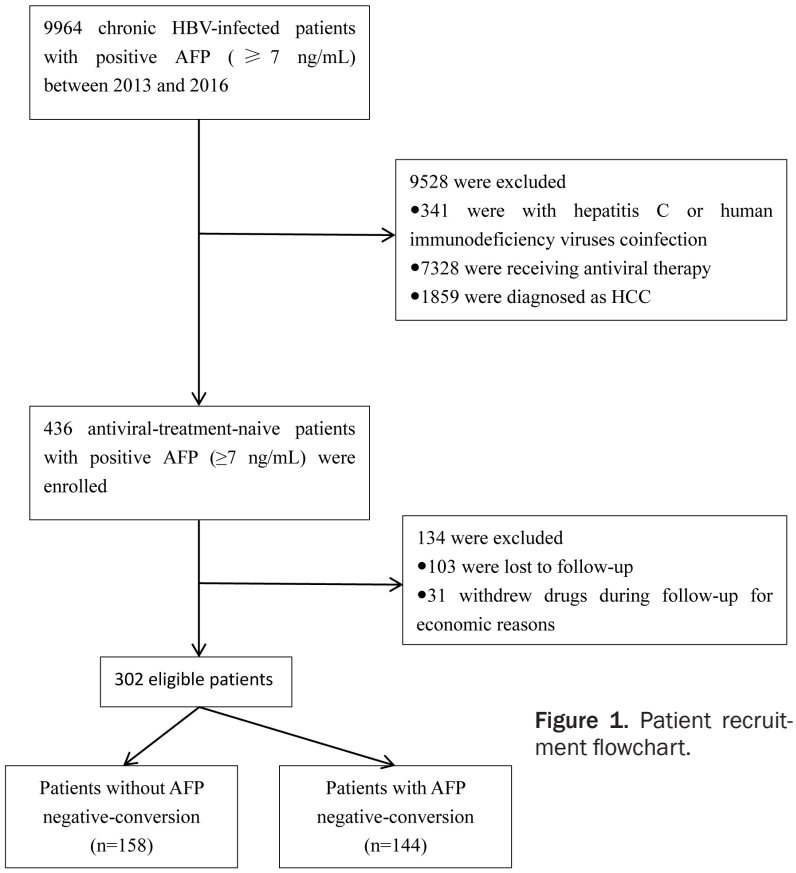

Figure 1. Patient recruitment flowchart.

means of a backward elimination procedure. Variables with *P* values <0.05 by univariate analysis were advanced for multivariate analysis. A *P* value less than 0.05 was considered significant.

Results

Baseline characteristics of patients

After screening, 302 patients fulfilled the inclusion criteria in the cohort. The median duration between their initial visit and anti-viral treatment was 1.1 month (range: 0.2 to 2.9 month). All patients had been followed for 34 ± 6 months (range: 25 to 69 months). The demographic, virologic, and clinical characteristics of the patients are summarized in **Table 1**. Their mean age was 40 ± 12 years, and 78.8% of them were male and 41 (13.6%) patients had cirrhosis.

Virology, ALT and AFP response

All patients initiated antiviral treatment with first-line antiviral drugs recommended by the guidelines after enrollment. The duration of therapy ranged from 6 to 69 months. Accordingly, 206 patients were treated with entecavir (68.2%), 74 with tenofovir (24.5%), 18 with Peginterferon alfa-2a (6.0%) and 4 with Peginterferon alfa-2a + tenofovir (1.3%).

After antiviral treatment, 144 patients achieved AFP normalization, while 158 remained positive. There were no significant differences in baseline characteristics and virologic and ALT response between AFP normalized and continuous positive groups. Serum HBV DNA levels declined significantly in response to the antiviral therapy in both groups. The 48-week virologic response rate was 94.44% (136/144) and 94.94% (150/158), respectively in AFP normalized and continuous positive group (*P*=0.849). The 48-week ALT response rate was 100% (73/73) and 96.10% (74/77), respectively (*P*=0.088). There were also no significant differences in virologic and ALT response between AFP normalized and continuous positive cirrhosis groups, as the 48-week virologic and ALT response rates were all 100% in cirrhosis patients.

Characteristics of HCC

Sixteen patients (5.3%) in this cohort developed HCC during follow-up. The median (range) time course for developing detectable HCC appearance was 17 months (range, 7-27) from the initiation of antiviral therapy. The 1-, 3-, and 5-year cumulative HCC incidence was 0.7% (95% CI=0.65%-0.75%), 7.2% (95% CI=5.6%-8.7%), and 16.6% (95% CI=14.9%-18.7%), respectively (**Figure 2**). Among these 16 patients who developed HCC, 6 (37.5%) had AFP levels at ≥20 ng/ml and 1 (6.3%) at ≥400 ng/ml. HCC occurred in 14 of 158 patients (8.9%) with continuous AFP positive, and 2 of 144 patients (1.4%) with AFP normalization during follow-up. On the other hand, HCC occurred in 10 of 41

Table 1. Baseline characteristics of the antiviral-treatment-naïve patients with positive AFP (≥7 ng/mL)

Characteristics	All patients (n=302)	Patients without AFP negative-conversion (n=158)	Patients with AFP negative-conversion (n=144)	*P* value
Gender				
Male (%)	238 (78.8%)	130 (82.3%)	108 (75.0%)	0.122
Age (years)				
>40 (%)	146 (48.3%)	82 (51.9%)	64 (44.4%)	0.195
HBsAg (IU/mL)	1900.43 ± 10631.64	2415.50 ± 13270.00	1335.29 ± 6631.18	0.379
HBeAg				
Positive (%)	231 (76.5%)	117 (74.1%)	114 (79.2%)	0.295
HBV DNA level, lg copies/mL	4.61 ± 1.94	4.58 ± 1.84	4.64 ± 2.04	0.786
AFP (ng/mL)	42.13 ± 182.16	47.13 ± 233.84	36.65 ± 98.75	0.618
AFP ≥20 ng/ml, n (%)	83 (27.5)	47 (29.8)	36 (25)	0.356
AFP ≥200 ng/ml, n (%)	9 (3.0)	4 (2.5)	5 (3.5)	0.743
AFP ≥400 ng/ml, n (%)	5 (1.7)	3 (1.9)	2 (1.4)	1.000
ALT (U/L)				
>40 (%)	150 (49.7%)	77 (48.7%)	73 (50.7%)	0.734
AST (U/L)				
>40 (%)	173 (57.3%)	86 (54.4%)	87 (60.4%)	0.294
PLT (10^9/L)				
<125 (%)	20 (6.6%)	7 (4.4%)	13 (9.0%)	0.109
ALB (g/L)				
<40 (%)	113 (37.4)	66 (41.8%)	47 (32.6%)	0.101
TBIL (umol/L)				
>20.5 (%)	109 (36.1%)	54 (34.2%)	55 (38.2%)	0.468
Cirrhosis				
Y (%)	41 (13.6%)	23 (14.6%)	18(12.8%)	0.602
Antiviral therapy				0.424
ETV	206 (68.2%)	110 (69.6%)	96 (66.7%)	
TDF	74 (24.5%)	40 (25.3%)	34 (23.6%)	
PEG-IFN	18 (6.0%)	6 (3.8%)	12 (8.3%)	
PEG-IFN+TDF	4 (1.3%)	2 (1.3%)	2 (1.4%)	

HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PLT, platelet count; ALB, albumin; TBIL, total bilirubin; ETV, entecavir; TDF, tenofovir; PEG-IFN, Peg-interferon.

patients (24.4%) with cirrhosis, and 6 of 261 CHB patients (2.3%) without cirrhosis at the time of the initial treatment (**Figure 3**). The tumor characteristics are listed in **Table 2**.

The HCC stage distribution at the time of diagnosis among the affected patients was as follows: BCLC stage 0 (n=10), BCLC stage A (n=4) and BCLC stage C (n=2). Of these 16 HCC patients, 14 (87.5%) were within the Milan criteria, defined as a solitary HCC<5 cm or with up to 3 nodules <3 cm. Of these patients, 11 (68.8%) were initially treated with surgical resection, 2 (12.5%) with radiofrequency ablation, 1 (6.3%) with percutaneous ethanol injection, and 2 (12.5%) with trans-arterial chemoembolization.

Kinetic changes in AFP, ALT and HBV DNA level in patients with continuous positive AFP

To evaluate the relationship between continuous elevated AFP and the incidence of HCC after antiviral therapy, we further analyzed the kinetic changes of AFP, virologic and biochemical response. After antiviral treatment, HBV DNA and ALT continues to decline in both the HCC and non-HCC group (P=0.002 vs. P<0.001; and P=0.03 vs. P<0.001, respectively), while AFP remains elevated in the HCC group and

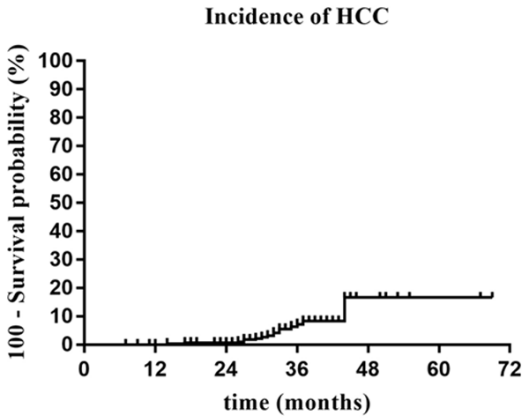

Figure 2. Cumulative incidence of hepatocellular carcinoma (HCC) in CHB patients with baseline positive AFP who started antiviral therapy thereafter.

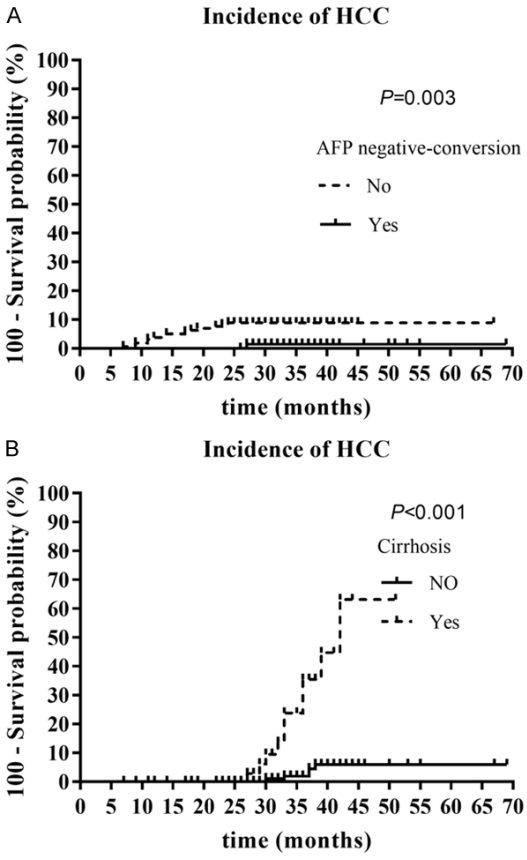

Figure 3. Kaplan-Meier estimates of the cumulative incidence of HCC in patients according to (A) the presence of AFP negative-conversion and (B) the presence of liver cirrhosis. The cumulative rates of HCC were significantly greater in patients without AFP normalization and with cirrhosis (P=0.003 and P<0.001, respectively).

declined in the non-HCC group (P=0.221 and P<0.001, respectively) (**Figure 4**).

Predictive factors for developing detectable HCC appearance

Predictive factors associated with HCC development were evaluated. Univariate analysis showed that both persistent AFP positivity (HR=0.151, *P*=0.014) and the presence of cirrhosis (HR=8.633, *P*<0.001) were significantly associated with the development of HCC (**Table 3**). We then entered these significant factors in multivariate analysis through Cox's proportional hazard model, and found that in addition to cirrhosis (HR=9.983, 95% CI=3.609-27.617, *P*<0.001), a non-AFP response to antiviral treatment (HR=6.517, 95% CI=1.475-28.784, *P*=0.013) was an indicator for possible emergence of cancer precursor cells/liver cancer cells in CHB patients (**Table 3**).

Discussion

To our knowledge, this is the first investigator-initiated cohort study to assess the performance of on-treatment AFP in CHB patients with baseline positive AFP. In contrast to the criticism that AFP is neither sensitive nor specific, the current study has provided important evidence that along with the presence of cirrhosis, on-antiviral-treatment AFP normalization is a rather specific protection indicator for HCC in patients with HBV-related chronic liver diseases who started antiviral therapy thereafter.

In this study, we retrospectively included a unique cohort of 302 antiviral treatment naive chronic hepatitis B patients who were AFP positive prior to 12-month antiviral treatment. Our investigative focus was AFP response in addition to virology and ALT response. We found that AFP normalized in 48% of treated patients while 52% did not normalize after the antiviral treatment though similar virology and ALT response was detected in both groups. HCC was detected in 8.9% of 158 patients with non-normalized AFP comparing 2 (1.4%) of 144 patients with normalized AFP during the follow up. Our results imply a CHB patient with persistent AFP positivity or non-AFP response who had an efficient virology and ALT response would have 6.35-fold (8.9% vs 1.4%) higher risk for developing HCC compared with CHB patients with normalized AFP. This risk was even higher in cirrhotic CHB patients with non-AFP response (43.48%, 10/23) comparing AFP normalized cirrhotic (0, 0/18) or CHB (1.59%, 2/126) patients (χ^2=52.739, *P*<0.005). The above data

Table 2. Characteristics of HCC

Tumor characteristics	HCC patients (n=16)
Maximum tumor diameter, mm	
≤20	10 (62.5%)
21-30	4 (25.0%)
31-50	1 (6.3%)
≥50	1 (6.3%)
Number	
Single	13 (81.3%)
Multiple	3 (18.8%)
Portal vein thrombosis, n (%)	2 (12.5%)
BCLC stage, n (%)	
Very early (0)	10 (62.5%)
Early (A)	4 (25.0%)
Intermediate (B)	0
Advanced (C)	2 (12.5%)
AFP	
Median (interquartile range) (ng/ml)	15.04 (2.68-474.80)
Mean ± SD (ng/ml)	53.64 ± 116.72
AFP ≥20 ng/ml, n (%)	6 (37.5%)
AFP ≥200 ng/ml, n (%)	1 (6.3%)
AFP ≥400 ng/ml, n (%)	1 (6.3%)
Treatment	
Surgical resection	11 (68.8%)
Radiofrequency ablation	2 (12.5%)
Percutaneous ethanol injection	1 (6.3%)
Transarterial chemoembolization	2 (12.5%)
Stereotactic body radiotherapy	0

AFP, alpha-foetoprotein; BCLC, Barcelona Clinic liver cancer; HCC, hepatocellular carcinoma; SD, standard deviation.

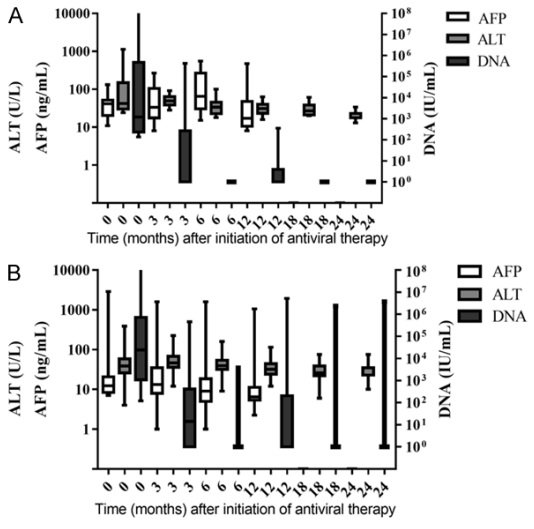

Figure 4. Box plot of AFP, ALT, and HBV DNA levels at different time points relative to time from starting antiviral therapy (A) among HCC patients with continuous positive AFP (n=14) and (B) among non-HCC patients with continuous positive AFP (n=144).

suggest the specificity of AFP-based HCC surveillance can be enhanced with kinetic monitoring of AFP response to antiviral treatment.

Our study from a clinical perspective also helped explain AFP production biology in CHB patients. To reduce AFP non-specificity, we must understand AFP production biology: which liver cells are responsible for AFP production. Clearly, cancer precursor cells/liver cancer cells produce AFP. There are additional liver cells that can also produce AFP. As reported, patients with hepatic necro-inflammation often have elevated levels of serum AFP, suggesting the liver cell regeneration process upon liver injury is accompanied by AFP production [12, 13]. However, such regeneration-based AFP production is contingent upon liver injury and is reversible if the liver injury is mitigated, while the cancer precursor cells/liver cancer cell-based AFP production is generally not reversible unless they are destroyed or removed. These differences may form a foundation to determine whether elevated AFP is produced by cancer precursor cells/liver cancer cells. As shown in this study, a persistent AFP positivity despite efficiently virology and ALT response in the treated CHB patients likely indicated the emergent cancer precursor cells/liver cancer cells. Our interpretation is consistent with recent reports that have focused on AFP levels for HCC diagnosis in patients undergoing ETV therapy. Elevated AFP levels at 6 months before or at the time of HCC incidence were shown to be useful in detecting existing HCC; and elevated AFP levels implied the presence of cancer cells [9, 14-17].

Our data may have two implications on HBV therapy and management: Call for starting antiviral therapy early among CHB patients. Guidelines for the management of chronic HBV infection issued by American Association for the Study of Liver Disease (AASLD) define the goal of antiviral treatment as blocking liver disease progression to delay or reduce HCC occurrence through inhibiting HBV DNA replication

Table 3. Univariate and multivariate analysis of variables affecting Hepatocarcinomagenesis in antiviral-treatment-naïve patients with positive AFP (≥7 ng/mL)

Factors	Univariate			Multivariate		
	HR	95% CI	*p* Value	HR	95% CI	*p* Value
Gender: M vs. F	1.466	0.344-6.245	0.600			
Age: >40 vs. ≤40	1.266	0.397-4.043	0.784			
Baseline HBsAg (IU/mL)	0.999	0.997-1.002	0.614			
Baseline HBeAg: P vs. N	1.650	0.333-18.185	0.416			
Baseline HBV DNA level, lg copies/mL	0.908	0.684-1.205	0.547			
AFP normalization: N/Y	9.297	1.949-44.358	0.005	6.517	1.475-28.784	0.013
ALT (U/L): ≤40/>40	0.708	0.199-2.514	0.646			
AST (U/L): ≤40/>40	0.879	0.283-2.727	0.869			
PLT (10^9/L): <125/≥125	0.000	0.000	0.980			
ALB (g/L): <40/40-55	1.249	0.402-3.875	0.843			
TBIL (μmol/L): 3.42-20.5/>20.5	0.755	0.211-2.697	0.502			
Cirrhosis: Y/N	11.293	3.700-34.137	0.000	9.983	3.609-27.617	0.000
Antiviral therapy: TDF/IFN/TDF+IFN vs. ETV			0.447			

HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PLT, platelet count; ALB, albumin; TBIL, total bilirubin.

[4]. In this study, the treated patients responded well regardless of AFP status, resulting in undetectable HBV DNA and normalization of ALT level in most patients. However, our study shows no close correlation between sustained biochemical and virologic responses and reduced HCC occurrence (unpublished data). In this study, both liver cirrhosis and the absent AFP response, but not the virology and ALT response to the antiviral treatment was independent risk factors associated with detectable HCC emergence. This is because current antiviral therapy may indirectly contribute to delayed or reduced HCC occurrence through mitigating liver injury and slowing down the progression of hepatic necro-inflammation and fibrosis to cirrhosis. However, it does not confer a direct anticancer function. If cancer precursor cells/liver cancer cells already emerged in a HBV infected liver, it probably cannot stop the progression of cancer precursor cells to cancer or the proliferation of the liver cancer cells no matter how potent the antivirals are. It seems to us that the key to achieving higher efficacy in reducing HCC incidence among CHB patients through antiviral treatment is to start the antiviral therapy early on or before the emergence of cancer precursor cells/liver cancer cells.

Include evaluation of AFP response to antiviral treatment. A routine kinetic monitoring of AFP may provide additional clinical value. Since AFP is notorious for the low specificity for HCC surveillance, especially with a single time point, an evaluation of AFP response is not required in treating CHB patients. However, this study demonstrated that the specificity of AFP could be significantly enhanced through kinetic monitoring of AFP response to the treatment. Most importantly, it can give us an earlier warning than imaging tests of likely emergent cancer precursor cells/liver cancer cells in HBV infected liver if AFP remains positive after an undetectable HBV DNA and normalized ALT are achieved, and enable physicians to closely monitor those CHB patients for early diagnosis and treatment of HCC.

This study has a few limitations. First, the retrospective nature of this study may have made the data collection selective. Fortunately, compliance to AFP testing was high because it was a routine test for almost all clinic visits. Second, no liver biopsies were performed among the included subjects. We diagnosed cirrhosis primarily based on imaging data. Third, only 16 HCC cases were detected in this study, which may have a negative impact on statistical power and may cause over-fitting of the multivariate model. Larger prospective studies are needed in the future. Fourth, 134 patients were excluded from the final analysis, which might have reduced the power of statistical analysis.

In conclusion, kinetic monitoring of AFP response to antiviral treatment of CHB patients who were AFP positive when starting therapy may eliminate non-specific confounders for elevated AFP and enhance the specificity of AFP-based HCC surveillance. A persistent AFP positivity despite sustained virology and ALT response, signals emergent cancer precursor cells/liver cancer cells in an HBV infected liver. Thus, a kinetic evaluation of AFP response may facilitate early HCC diagnosis and treatment.

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Disclosure of conflict of interest

None.

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