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ORIGINAL ARTICLE

Evaluation of Serum α-Fetoprotein Levels During Different Infection Phases of CHB Patients

Na Yang [*], Jin Feng [*], Zhuo R. Li, Kai H. Ming, Xiu X. Lei, Bang L. Xu

[*] *Co-first authors*
Department of Laboratory Medicine, Guangzhou First People's Hospital, Guangzhou Medical University, Guangzhou, China

SUMMARY

Background: **Chronic hepatitis B patients carry a high risk of developing hepatocellular carcinoma (HCC). α-Fetoprotein (AFP) is one of the most commonly used and reliable biomarkers for HCC. However, the AFP level during different phases of CHB is not well understood. We aimed to identify the AFP levels during the different infection phases of CHB patient and explore which phase is at high risk of developing HCC.**
Methods: **Three hundred and fifty-five CHB patients were divided into four groups: a. immune tolerant HBeAg-positive phase (IT); b. immune reactive HBeAg-positive phase (IR), c. inactive carrier state (IC), d. HBeAg-negative activation phase (ENA). The risk of development of HCC in different group is assessed by the serum AFP levels. An electrochemiluminescence assay was used to analyze serum AFP levels.**
Results: **Mean AFP levels were different in each phase of CHB ($p < 0.001$): IT (9.6 ng/mL), IR (33.7 ng/mL), IC (3.2 ng/mL), and ENA (71.6 ng/mL). The ENA phase had the highest AFP level and IC phase has the lowest. There was no correlation between serum AFP level and HBV viral load. A significant correlation between serum ALT levels and HBV viral load was observed ($r = 0.272$, $p < 0.01$).**
Conclusions: **These findings suggest that high levels of AFP during HBeAg-negative activation phase (ENA) may be associated with a high risk of developing of HCC. Furthermore, higher burden of HBV viral load is associated with more severe liver damage.**
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Correspondence:
Bang-Lao Xu
Department of Laboratory Medicine
Guangzhou First People's Hospital
Guangzhou Medical University
Guangzhou
China
Phone/Fax: +86 20-8104 8082
E-mail: banglaoxu@163.com

Xiu-Xia Lei
Department of Laboratory Medicine
Guangzhou First People's Hospital
Guangzhou Medical University
Guangzhou
China
Email: leixx999@163.com

KEY WORDS

AFP (α-fetoprotein), CHB (chronic hepatitis B), HBV (hepatitis B virus), HCC (hepatocellular carcinoma)

INTRODUCTION

Hepatitis B virus infection represents a major global health problem. Chronic hepatitis B virus (HBV) infection affects more than 400 million people globally, of whom 75% are Asians [1]. In China, there are an estimated 125 million hepatitis B carriers defined as persons positive for hepatitis B surface antigen (HBsAg) for more than 6 months [2]. People infected with the hepatitis B virus carries an increasing incidence and a high risk of developing hepatocellular carcinoma (HCC) [3-5]. Late HCC detection is associated with extremely

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poor outcome. Therefore, it is important to identify CHB patients who are at high risk for developing HCC.

Chronic hepatitis B infection is a state with dynamic alterations. Viral load or hepatic inflammation may change over time. The clinical course of chronic infection with HBV (CHB) is driven by the interplay between viral replication and the host immune response. Four phases of chronic HBV infection have been defined [6,7]: a. immune tolerant HBeAg-positive phase (IT), characterized by the presence of HBeAg and high burden of HBV DNA in the serum; b. immune reactive HBeAg-positive phase (IR): ALT levels fluctuate and the disease can advance to significant fibrosis; c. inactive carrier state (IC): loss of HBeAg and production of anti-HBe. During this phase serum HBV DNA levels are persistently low and ALT levels are below the upper limit of normal (ULN); d. HBeAg-negative activation phase (ENA): patients may have intermittent or persistent high HBV DNA and ALT levels with progressive liver injury. Previous studies showed that patients with a viral load ≥ 2000 IU/mL have an increased risk of HCC [8]. Others reported that HBeAg-negative patients who had lower HBV DNA levels (3 - 5 $\log_{10}$ IU/mL) may be associated with more progressive liver disease [9-11]. However, the risk of HCC during different CHB phases is not well understood.

Identifying the HCC-risk population of CHB patients provides important instructions for clinical CHB treatment. In clinical practice, serum alpha-fetoprotein (AFP) is the most commonly used surveillance tests for HCC [12,13]. Studies have shown AFP elevation is associated with greater long-term risk of HCC development [14]. The main objective of this study was to determine the AFP levels during different phases of CHB and analyze the HCC risk among different phases. We also assessed the correlation between serum AFP levels and HBV DNA load. Our study had important implications for evaluating the risk of HCC during different CHB phases.

MATERIALS AND METHODS

Patients

Three hundred and fifty-five CHB patients who attended the outpatient's Department of Infectious Diseases of the First People's Hospital, Guangzhou Medical College, during the period of June 2015 to June 2016, were chronically infected with HBV virus for at least 6 months. The study was conducted according to the guidelines of the Declaration of Helsinki. All patients were of Asian ethnicity and tested negative for markers of hepatitis C virus, hepatitis D virus, and human immunodeficiency virus (HIV). Informed consent was obtained from study subjects, and the study was approved by the local institutional ethics committee. Patient demographics, liver biochemistries, qualitative HBsAg, HBeAg status, and HBV DNA load were recorded. Biochemical and virological data were obtained from patient serum samples collected on the same day. Patients were classified into four groups: a. immune tolerant HBeAg-positive phase (IT); b. immune reactive HBeAg-positive phase (IR), c. inactive carrier state (IC), d. HBeAg-negative activation phase (ENA).

Serum HBsAg and HBeAg analysis

Serum HBsAg, HBeAg, and anti-HBe was analyzed using the Elecsys platform (Roche Diagnostics GmbH, Mannheim, Germany), as per the manufacturer's instructions (Roche, the Elecsys HBsAg II Screening Assay, Germany).

HBV DNA viral load

HBV DNA viral load testing was performed using the ABI7500 Real-Time qPCR System (USA), according to the manufacturer's instructions (GenWay, HBV Quantitative Real Time PCR Kit).

Biochemical parameters

Serum aspartate transferase, alanine transferase levels, and glutamyl transpeptidase were analyzed by an automated clinical biochemistry analysis system (Hitachi biochemistry analyzer 7080).

Statistical analysis

Continuous and categorical variables were compared between groups, using the Mann-Whitney test and one-way ANOVA for non-parametric continuous data. Pearson's correlation coefficient (r) was used to describe the correlation between two variables. Statistical analysis was performed using GraphPad and SPSS Statistic Software. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Patient classification

Serum samples from a total of 355 chronic hepatitis B virus carriers were analyzed by serological assays. All patients with chronic HBV infection were enrolled in the study from January 2015 to January 2016 and were classified into four groups: a. immune tolerant HBeAg-positive phase (IT); b. immune reactive HBeAg-positive phase (IR); c. inactive carrier state (IC); d. HBeAg-negative activation phase (ENA). As shown in Table 1, four phases were defined.

Clinicopathologic characteristics of four CHB phases

The baseline patient characteristics were presented in Table 2. The patient number of each phase was: IT (n = 69), IR (n = 102), IC (n = 126), ENA (n = 58), respectively. Overall there were more males in the whole study population (60.8%). The average AFP level was significantly higher in IR and ENA phase (IR: 33.7 ng/mL; ENA: 71.6 ng/mL) than in IT and IC (IT: 9.6 ng/mL; IC: 3.2 ng/mL) ($p < 0.05$). Notably, the ENA phase

Table 1. Definition of phases of CHB patients.

Phase	HBV-DNA (IU/mL)	HBeAg status	ALT (U/L)
IT	6-8 $\log_{10}$ IU/mL	+	≤ ULN
IR	> 2000 IU/mL	+	> ULN
IC	< 4 $\log_{10}$ IU/mL	-	< ULN
ENA	2-7 $\log_{10}$ IU/mL	-	> ULN

IT - immune tolerant HBeAg-positive phase, IR - immune reactive HBeAg-positive phase, IC - inactive carrier state, ENA - HBeAg-negative activation phase, HBeAg - hepatitis B e antigen, ULN - upper limit of normal, "+" – positive, "-" - negative.

Table 2. Baseline characteristics.

Parameters	IT	IR	IC	ENA	p-value
HBV DNA ($\log_{10}$IU/mL)	7.8 ± 0.79	7.1 ± 1.5	3.2 ± 0.3	5.1 ± 1.6	< 0.001
ALT (IU/mL)	24.8 ± 7.2	142.7 ± 227.2	23.4 ± 8.1	143.7 ± 236.9	< 0.001
AST (IU/mL)	25.8 ± 9.3	102.5 ± 177.3	24.6 ± 6.9	104.8 ± 182.5	< 0.001
γ-GT (IU/mL)	20.5 ± 16.7	49.6 ± 52.1	23.1 ± 7.0	95.6 ± 135.3	< 0.001
AFP (ng/mL)	9.6 ± 37.9	33.7 ± 175	3.2 ± 3.5	71.6 ± 258.1	< 0.001
Age (Years)	28.3 ± 6.7	33.4 ± 10.6	39.4 ± 11.2	45.8 ± 13.7	< 0.001
Gender (M/F)	32/37	70/32	70/56	44/14	

IT - immune tolerant HBeAg-positive phase, IR - immune reactive HBeAg-positive phase, IC - inactive carrier state, ENA - HBeAg-negative activation phase, HBV - hepatitis B virus, ALT - alanine transaminase, AST - aspartate aminotransferase, γ-GT - gamma-glutamyl transferase. Data are shown as mean ± SD or n (%).

Patient had the highest AFP levels while the IC phase had the lowest. The average age of patients in each phase was 28.3 (IT), 33.4 (IR), 39.4 (IC), 45.8 (ENA), respectively. Gradually increased average age was observed from IT phase to ENA phase. The patient in ENA phase was significantly older than other groups ($p < 0.001$). The levels of three liver function enzymes (ALT, AST and γ-GT) were significantly higher in IR and ENA phase.

Distribution of serum AFP levels among different CHB patients

The distribution of serum AFP levels across the CHB patients was evaluated. Patients were classified into 4 groups (IT, IR, IC, and ENA) according to the HBV DNA load and ALT level. As shown in Figure 1, the mean AFP levels in each group were 9.6 ng/mL (IT), 33.7 ng/mL (IR), 3.2 ng/mL (IC), 71.6 ng/mL (ENA), respectively. Notably, the ENA group had the highest AFP level while the IC group had the lowest. There was a significant difference between the ENA group and other groups ($p < 0.05$). The statistical differences between all groups were: IC vs. ENA: $p < 0.0001$; IT vs. ENA: $p = 0.0006$; IR vs. ENA: $p = 0.0199$; IR vs. IC: $p = 0.0002$; IT vs. IR: $p = 0.023$; IT vs. IC: $p = 0.6747$. There was no significant difference between the IT and IC group. A previous study had shown that HBeAg-negative patients who had lower HBV DNA levels (3 - 5 $\log_{10}$ IU/mL) may be associated with more progressive liver disease. Our results indicated that the HBeAg-negative reactivation phase is at a higher risk of developing HCC than other groups.

Correlation between AFP and serum HBV DNA load

The correlations between serum AFP levels and HBV DNA viral load in CHB patients were shown in Figure 2. There was no correlation between AFP levels and HBV DNA viral load in the whole population studied ($r = 0.038$, $p = 0.463$). Interestingly, there is a significant positive correlation between ALT and AFP levels (Figure 3).

Correlation between ALT and serum HBV DNA load

The correlation between ALT level and serum HBV DNA load was evaluated in 355 CHB patients. As shown in Figure 4, there was a significantly positive correlation between serum ALT levels and serum HBV DNA viral load in the whole population studied

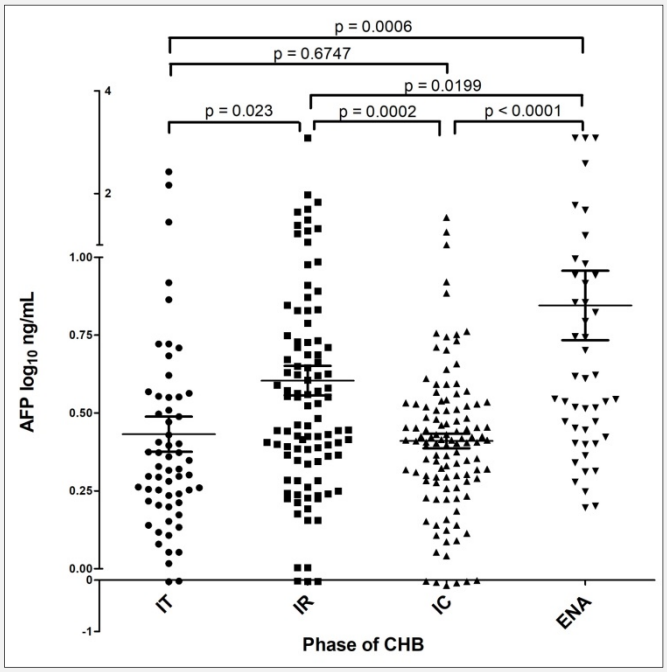

Figure 1. Distribution of AFP levels among four CHB groups.

Standard Mean error bar of each group are shown. IC vs. ENA - p < 0.0001, IT vs. ENA - p = 0.0006, IR vs. ENA - p = 0.0199, IR vs. IC - p = 0.0002, IT vs. IR - p = 0.023, IT vs. IC - p = 0.6747.
Short Communication for Figure 1.
AFP levels were different between groups. Among them, IT phase and IC phase were within the normal AFP level (< 20 ng/mL), whereas the IR phase and ENA phase were above the normal AFP level. Notably the ENA phase was significantly higher than other phases. Since elevated AFP levels is a risk factor for HCC development and can be used to help define at-risk populations, the higher AFP levels in patients of ENA phases indicated they were candidates for monitoring and prevention of developing HCC.

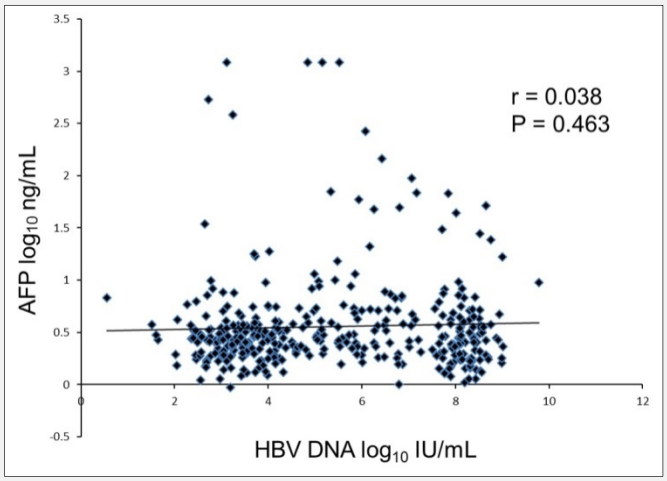

Figure 2. Correlation between serum AFP levels and hepatitis B virus DNA load in chronic hepatitis B patients.

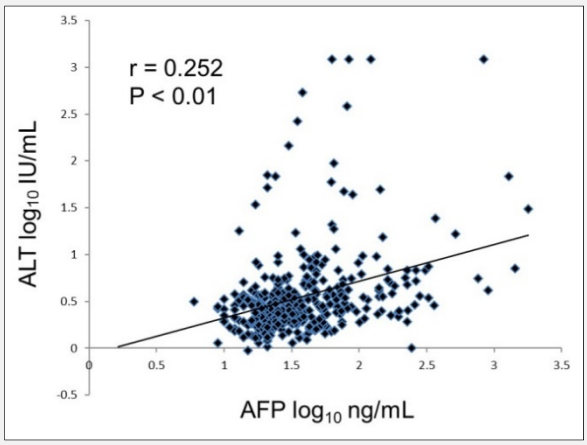

Figure 3. Correlation between serum AFP levels and ALT levels in chronic hepatitis B patients.

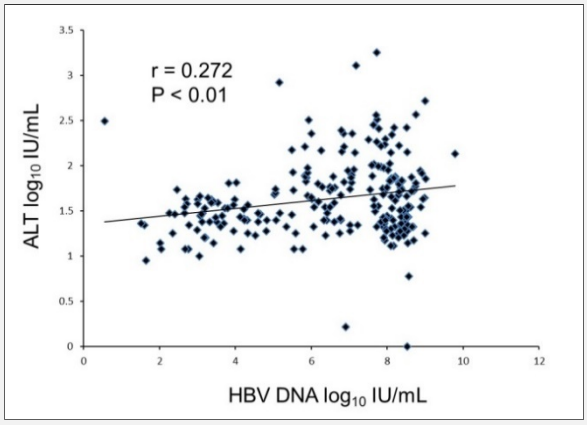

Figure 4. Correlation between serum ALT enzymatic activity and HBV DNA levels in chronic hepatitis B patients.

($r = 0.272$, $p < 0.01$). We also evaluated HBV viral load in two ALT level groups (ALT > 40 and ALT < 40). As shown in Figure 5, HBV viral load was significantly higher in ALT > 40 group ($P < 0.001$). There were some patients in ALT < 40 group that had high HBV viral load which indicated an immune tolerant state.

DISCUSSION

Serum alpha-fetoprotein (AFP) is the most widely used biomarker for HCC and is included in international guidelines for HCC surveillance [15,16]. Although it has its limitations as a screening test, elevated AFP level is a risk factor for HCC development and can be used to help define at-risk populations [17-20]. We retrospectively studied the distribution of AFP levels among the four phases of CHB patients and found that the mean AFP levels were significantly higher in HBeAg-negative reactivation phase than other phases. The mean AFP levels in each group were 9.6 ng/mL (IT), 33.7 ng/mL (IR), 3.2 ng/mL (IC), 71.6 ng/mL (ENA), respectively. Interestingly, the mean AFP level of IT phase and IC phase were within the normal AFP level

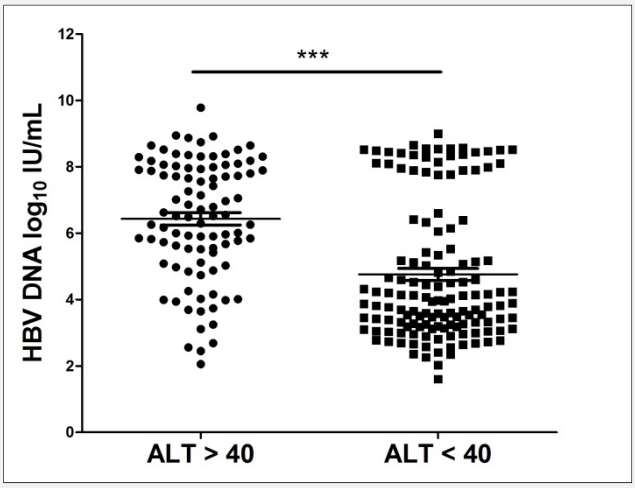

Figure 5. ALT levels and HBV viral load analysis in chronic hepatitis B patients.

Comparison of HBV viral load between two levels of ALT groups. * - $p < 0.001$.**

(< 20 ng/mL), whereas the IR phase and ENA phase is above the normal AFP level. In clinical practice, AFP levels of > 200 ng/mL lasting for two weeks and accompanied with liver solid masses are considered a high possibility of HCC. The higher AFP levels in patients of ENA phases indicated they were candidates for monitoring and prevention of developing HCC. In conclusion, our results indicated that HBeAg-negative activation phase had a higher risk of developing HCC compared with other phases.

HBeAg negative activation phase is characterized by negative HBeAg, HBV DNA levels > 2,000 IU/mL, and continued necroinflammation in the liver [9]. Patients with HBeAg-negative chronic hepatitis B are more likely to have fluctuating HBV DNA levels [21,22]. Previous study revealed that the HBeAg-negative activation phase represented a later stage in the course of chronic HBV infection and had more advanced liver disease such as cirrhosis [9,11]. In line with previous findings, we found the oldest age in this group. The high levels of AFP detected in this stage of patients implied the advanced liver disease. Since AFP is an embryonic protein, the increased AFP levels in this stage of patients also indicated active liver stem cell proliferation and transformation.

Previous studies revealed that HBV DNA was the major driver of disease progression in Asian population [8]. Patients with a viral load ≥ 2000 IU/mL have an increased risk of HCC, while patients with HBV DNA < 2000 IU/mL are at low-risk [8]. In our study, we found higher AFP levels in the immune active phase than immune tolerant phase and inactive carrier phase. Immune active phase is characterized by high levels of alanine aminotransferase (ALT), high HBV DNA load (> 2000 IU/mL), and active liver inflammation. The high AFP levels in immune active phase indicated an increased risk of HCC in the high viral load patients. Nevertheless, we found no correlation between HBV viral load and AFP levels in the whole study population. Moreover, it is interesting that there is a significant positive correlation between ALT and AFP levels, which implied the intimate association between liver inflammation and stem cell proliferation.

Alanine aminotransferase (ALT) is a reliable enzyme for liver inflammation. Despite the existence of the immune tolerant phase that is characterized by the high levels of serum HBV DNA and normal ALT levels, there was a significant positive correlation between ALT levels and HBV DNA load in the whole CHB study population (Figure 4, $r = 0.272$, $p < 0.01$). Consistently, despite the presence of the immune tolerant group, the HBV viral load was significantly higher in the ALT high group (> 40 IU/mL) (Figure 5). These results indicated that at least in the IR, IC, and ENA phases high HBV DNA load is associated with liver inflammation and active immune response.

CONCLUSION

Our results improved the understanding of the change of AFP levels among different CHB phases and provided evidence for identifying ENA phase patients as a high-risk population of developing HCC who are candidates

for early HCC prevention. A limitation of this study was AFP itself was suboptimal in its sensitivity and specificity for HCC detection and it will be useful to include other means such as ultrasound (US) to evaluate the HCC-risk population.

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Declaration of Interest:
All authors of this research paper have directly participated in the planning, execution, or analysis of the study. All authors of this paper have read and approved the final version submitted. The contents of this manuscript have not been copyrighted or published previously. The contents of this manuscript are not now under consideration for publication elsewhere. The contents of this manuscript will not be copyrighted, submitted, or published elsewhere while acceptance by the Journal is under consideration. There are no directly related manuscripts or abstracts, published or unpublished, by any author(s) of this paper.

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