The Association of N-Glycan and Alpha Fetal Protein in Hepatitis B Associated Hepatic Disease

Li Guo  
Second Affiliated Hospital of Nanchang University

Lijun Wan  
Second Affiliated Hospital of Nanchang University

Youwen Hu  
Second Affiliated Hospital of Nanchang University

Bo He  
Second Affiliated Hospital of Nanchang University

Moran Zhang  
Second Affiliated Hospital of Nanchang University

Kedong Xu  
Second Affiliated Hospital of Nanchang University

Fengfei Zheng  
Second Affiliated Hospital of Nanchang University

Gejirifu De  
Second Affiliated Hospital of Nanchang University

Zhengqiang Wu  
Second Affiliated Hospital of Nanchang University

Chungen Hu  
Second Affiliated Hospital of Nanchang University

Cheng Yuan  
Second Affiliated Hospital of Nanchang University

Zixiang Huang  
Second Affiliated Hospital of Nanchang University

Ting Liu  
Second Affiliated Hospital of Nanchang University

Shanwen Shanwen Chen  
Second Affiliated Hospital of Nanchang University

Zhili Wen (wenzhili@126.com)  
Second Affiliated Hospital of Nanchang University
Abstract

Objective: Chronic hepatic disease caused by hepatitis B virus (HBV) is a serious threat to health in worldwide. There is evidence that the change of N-glycan is involved in the mechanism of hepatic fibrosis, liver cirrhosis (LC) and hepatocellular carcinoma (HCC) in patients with hepatitis B. The level of serum alpha-fetoprotein (AFP) is elevated in many patients with hepatitis B infection. AFP is not only a fetal carrier protein and tumor marker, but also participates in the regulation of a variety of important cellular functions, such as cell growth, differentiation, apoptosis, angiogenesis and immune regulation. However, the mechanism between AFP and N-glycan is not clear. The study investigated the association of N-glycan and AFP in Hepatitis B associated hepatic disease.

Patients and Methods: Sixty patients with Hepatitis B associated hepatic diseases and twenty healthy individuals were selected in this study. Serum AFP, N-glycan, hematological parameters, and clinical data were assessed in this cohort. The Spearman rank method was used to evaluate the associations among them. The study was designed as a retrospective cross-sectional study.

Results: Serum levels of N-glycan and AFP were significantly higher in Patients with hepatic disease compared with the controls; levels of both were elevated with development of the disease. In patients with hepatic disease, N-glycan was positively correlated with AFP, Age, AST, GGT, PT, CA125, while negatively correlated with ALB, CHE, RBC ($P < 0.05$ for all). AFP was positively correlated with HBV DNA, TBIL, DBIL, AST, ALT, ALP, GGT ($P < 0.05$ for all), while negatively correlated with ALB and CHE in hepatic disease patients ($P < 0.05$ for both). In addition, there was a trend of increasing N-glycan with elevated AFP level in the combined hepatic disease group. In LC, the level of N-glycan in the decompensatory was significant higher than in the control ($P = 0.007$), and AFP level in the compensatory increased than the controls ($P = 0.003$). In HCC, levels of both N-glycan and AFP in the compensatory and decompensatory groups elevated than those in the control ($P < 0.001$ and $P = 0.004$ for N-glycan and $P < 0.001$ for both in AFP, respectively).

Conclusion: Our data suggest that high AFP levels in Hepatitis B related hepatic disease are closely related to the development of liver disease through interaction with N-glycan.

Introduction

Hepatitis B virus (HBV) infection is a major public health problem, causing high mortality and social burden worldwide. The WHO estimated in 2015 that 257 million individuals (3.5% of the global population) are chronically infected with HBV, most of who were born before the widespread use of the vaccine. There are about 130 million HBV carriers in China, accounting for 10% of the country's total population, of which 30 million are chronic infections and 300000 die of HBV-related diseases every year. HBV infection is the most common cause of chronic liver disease, and there may be persistent low-grade liver inflammation, accompanied by transient high liver inflammation and activation of fibrotic processes, leading to liver fibrosis and cirrhosis or even progressing to HCC. However, the
pathophysiology of liver disease is not fully understood, and it has been reported that glycosylation can occur in serum proteins of liver disease. Glycosylation is a complex biological modification. The changes of glycosylation can affect protein isoelectric point, PH stability, thermal stability, and mutual recognition with lectins. N-glycan and O-glycan are the most common protein glycosylation modifications, which are composed of galactose, N-acetyl galactosamine, N-acetylgalactosamine, fucose, mannose, sialic acid, and other monosaccharides and present in most proteins in human blood circulation. The type and quantity of N-glycan is much more than that of O-glycan in human body, and Abnormal N-glycosylation involved in the development and progression of cancer, such as cell signaling and communication, tumor cell division and invasion, cell matrix interaction, and immune regulation. Therefore, unique changes in tumor-related N-glycosylation can provide unique biomarkers. In recent years, the changes of N-glycan related to precancerous lesions also have attracted more and more attention. N-glycan are affected by many factors in vivo, not only related to age, sex and body weight, but also related to metabolic syndrome (MetS) such as BMI, SBP, DBP, fasting blood glucose (FBG) and so on. It is reported that human serum glycoproteins are mainly produced by the liver, while immunoglobulins are produced by the immune system, such as IgG, IgM, IgA, IgE and IgD are secreted by B lymphocytes in the process of immune response. Therefore, the abnormality of the structure and quantity of N-glycosylation can often reflect the pathological changes of liver and B lymphocytes. HCC patients often develop from HBV infection and liver cirrhosis in China. HBV receptor-mediated endocytosis can mediate the entry of HBV into hepatocellular carcinoma cells, and the key molecule is core-fucosylation. In addition, in areas with high prevalence of HBV, N-glycosylation mutations in the main hydrophilic region of specific HBSAg participated in host HBV immune escape. Therefore, understanding the expression of N-glycan in HBV-related liver diseases will facilitate to a better understand of the molecular mechanisms of HBV-related liver injury and tumor, and provide new clues for diagnosis, prognosis, and treatment.

At present, AFP is considered to be the most important biomarker for screening and diagnosis of HCC. If combined with imaging methods, AFP can become a reliable marker for early detection of HCC. Clinically, high serum AFP level usually means a high risk of HCC, while in HCC patients treated with surgical resection, elevated serum AFP level indicates a poor prognosis after operation. In recent years, it has been found that the biological function of AFP is not only a tumor marker, but also can be used as a therapeutic target for HCC. In addition, it is reported that AFP has the ability to regulate cell differentiation, proliferation and tumorigenesis. In HCC cells, AFP can activate PI3K/AKT signal pathway by binding to PTEN (a tumor supressor), which not only participates in the proliferation of HCC cells, but also stimulates the expression of tumor metastasis-related protein EpCAM, CXCR4 to promote the metastasis of HCC cells. AFP can also form an AFP-caspase-3 complex with the pro-apoptotic protein caspase-3, inhibiting the transduction of caspase-8 signals and caspase-3 entering the nucleus and antagonizing apoptosis. Recently, it has been found that acetylated AFP increases its protein stability, which can further block PTEN and bind to the pro-apoptotic protein caspase-3 to promote tumor proliferation, metastasis and inhibit its apoptosis. In addition, it was also found that high levels of
Acetylated AFP in HBV infection-related HCC tissues were associated with poor prognosis and low survival rate.\(^{34}\)

Interestingly, both of N-glycan and AFP are not only used as early detection markers for HCC\(^{27,35,36}\), but also involved in the regulation of cell differentiation, proliferation, and tumorigenicity\(^{14,15}\). However, there is little clinical data to elucidate the relationship between AFP/ N-glycan and the pathogenesis of chronic liver disease. To further investigate the relationship between N-glycan and AFP in HBV-infected patients with chronic liver disease and analyze their clinical characteristics, serum samples from a total of 60 HBV-infected patients with chronic liver disease (CHB, LC, HCC: 20 each) and 20 healthy subjects were selected in this study. The serum N-glycan value was detected by fluorescence capillary electrophoresis (DSA-FACE) based on DNA sequencing and the serum AFP level was detected by electrochemiluminescence (ECL).

**Patients And Methods**

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University and conducted from October 2019 to February 2020. Eighty individuals were selected for this study, including 20 cases of chronic hepatitis B (CHB), 20 cases of liver cirrhosis (LC), 20 cases of hepatocellular carcinoma (HCC), and 20 age-matched healthy controls. All patients and the controls gave written informed consents to the project's aims. Patients with chronic HBV infection who were treated in the outpatients of the Department of Gastroenterology and the Department of infection were included in the study. The inclusion criteria were over 18 years old, non-pregnant women, and no previous history of treatment for hepatitis B. Patients with acute HBV infection, or with HCV or HIV co-infection, and those with long-term alcohol consumption were excluded from the study. Chronic HBV infection was defined as HBsAg positive lasting for more than 6 months, HBe antigen (+/-), ALT or AST increasing continuously or intermittently. Cirrhosis was confirmed by ultrasound with associated hypoproteinemia and prolonged prothrombin time. Hepatocellular carcinoma was diagnosed by ultrasound or computed tomography (CT) scan of the liver for a mass and alpha-fetoprotein (AFP > 500 ng/mL)\(^{37,38}\). Liver biopsy was not done in the evaluation of the hepatitis as well as in the diagnosis of the patient with hepatic mass. Since 2010, Child-Pugh score (CP score) has been used as a prognostic indicator through the Clinical and treatment guidelines for chronic HBV and co-infection with HCV, including consideration of indications for liver transplantation. CP score is considered to be a simple and adaptable tool to assess the health status of patients with LC and HCC\(^{39,40}\). According to the staging criteria of CP score, both of LC and HCC patients were divided into compensatory group (Child A, the low-risk group) and decompensatory group (Child B/C, the high-risk group: ascites, hemorrhage and sepsis).

To study the association of AFP and N-glycan in patients with hepatitis B liver disease, we redivided the 60 patients into three groups according to serum levels of AFP: Group A, AFP levels < 10 ng/mL; Group B, AFP levels 10–200 ng/mL; and Group C, AFP levels > 200 ng/mL. Hepatitis B patients with other malignant tumors and metastatic liver cancer were excluded because radiotherapy and/or chemotherapy...
may change the metabolism of patients. In addition, liver diseases caused by HCV/HIV co-infection, alcohol or drugs are excluded. None of the subjects had undergone surgery or medication or received blood transfusions before the start of the study.

**Collection and evaluation of samples**

Blood samples were collected using three tubes: EDTA anticoagulant, sodium citrate anticoagulant (1:9), and no anticoagulant. Blood in the EDTA tube was analyzed by an automatic cell counter (model Sysmex XE-2100 hematology analyzer; Sysmex Corporation, Kobe, Japan) for the determination of complete blood count, including red blood cells (RBC), hemoglobin (Hb), platelet (PLT) and white blood cells (WBC). Plasma separated from the test tube of sodium citrate (1:9) anticoagulant was analyzed by an automatic hemagglutination analyzer (model Sysmex CA-7000 hemagglutination Analyzer, Sysmex Company, Kobe, Japan), including prothrombin time (PT) and activated partial thromboplastin time (APTT). Blood without anticoagulant was kept for 2 h at room temperature for biochemical detections including liver function (TBIL: total bilirubin, DBIL: direct bilirubin, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, GGT: γ-glutamyl transferase, TBA: total bile acid, CHE: cholinesterase, AFU: alpha-L-fucosidase, CG: cholyglycine and ALB: albumin) and renal function (BUN: blood urea nitrogen, CRE: creatinine and RBP: Retinol binding protein) by an automatic biochemical analyzer (model Beckman CX7; Beckman Coulter, Fullerton, CA, USA). Serum levels of AFP, CEA (carcinoembryonic antigen), CA199 (glucoprotein antigen 19-9), and CA125 (glucoprotein antigen 125) were measured by the chemiluminescent immunoassay method (model Roche cobas e601, Roche, Basel, Switzerland). Serum N-glycan was measured by fluorescence capillary electrophoresis (model ABI 3500 Analyzer; Thermo Fisher, New York, USA).

**Statistical analyses**

Data were given as mean ± SD values based on the normal distribution or as median (25th–75th percentile) values when data were non-normally distributed. Statistical differences among groups were assessed by one-way analysis of variance of post hoc multiple comparisons: least significant difference (normal distribution data) or nonparametric statistics. The Kruskal–Wallis H, Mann–Whitney U, and Wilcoxon rank tests were used to assess non-normal distribution data. The associations among serum levels of N-glycan, AFP and other clinical laboratory parameters were tested with the Spearman rank correlation. Bivariate correlations were used to evaluate the factors that regulated N-glycan and AFP production in each group. SPSS for Windows version 25.0 software was used for statistical analyses (SPSS Inc., Chicago, IL). Values of $P<0.05$ were considered to be statistically significant.

**Results**

**General clinical data in the patient groups and the controls**

Data were obtained from 80 (41 men, 39 women) participants. General characteristics of the three hepatic disease groups and the controls are summarized in Table 1. In terms of liver function, AST and
ALT levels were elevated significantly in the patients groups compared with the control group (all \( P \leq 0.001 \)). Similarly, GGT, TBA and CG levels were significantly increased in the hepatic disease groups (GGT index: \( P = 0.033 \) in CHB, \( P < 0.001 \) in LC and HCC; TBA index: \( P = 0.001 \) in CHB, \( P < 0.001 \) in LC and HCC; CG index: \( P < 0.001 \) for all). In addition, among three hepatic disease groups, GGT in HCC group was significantly higher than that in CHB group (\( P = 0.009 \)), but there was no significant difference between HCC and LC groups as well as between LC and CHB (\( P > 0.05 \) for both); TBA in LC group was significantly higher than that in CHB and HCC groups (\( P = 0.017 \) and \( P = 0.001 \), respectively); CG in LC group was significantly increased than that in CHB and HCC groups (\( P = 0.001 \) and \( P = 0.037 \), respectively). Although the levels of DBIL, ALP, and GLU in LC and HCC group were significantly higher than those in control group and CHB group (\( P < 0.05 \) for all), no difference existed between CHB group and the controls (\( P > 0.05 \) for all). Except that TBIL in LC group and HCC group was significantly higher than that in control group (\( P < 0.001 \) and \( P = 0.001 \), respectively), it was found that LC group was higher than CHB group (\( P = 0.01 \)). AFU in CHB and HCC group was higher than that in control group (\( P = 0.042 \) and \( P = 0.005 \), respectively), but there was no difference between LC group and control group (\( P > 0.05 \)) as well as among hepatic disease groups (\( P > 0.05 \) for all). Interestingly, ALB, CHE and RBP in LC group and HCC group were significantly lower than those in CHB group and control group (\( P < 0.05 \) for all), while no significant difference existed between CHB group and control group (\( P > 0.05 \) for all). In addition, no difference in BUN and CRE levels (renal function index) were found between the hepatic disease patients and the controls. Although WBC in LC group was significantly decreased than that in CHB, HCC and control group (\( P < 0.01 \) for all groups), no differences were found among CHB, HCC and the controls. RBC in LC and HCC groups were lower than that in CHB and the controls (\( P < 0.05 \) for all groups), no differences were found between CHB and the controls as well as LC and HCC groups (\( P > 0.05 \) for both). Although Hb levels in CHB group was significantly increased than that in LC and HCC groups (\( P = 0.005 \) and \( P = 0.003 \), respectively), no differences were found among LC, HCC and the controls. PLT in LC group was significantly lower than that in HCC, CHB and control group (\( P < 0.001 \) for all groups), while PLT in HCC group was lower than that in CHB and control group (\( P < 0.001 \) for both), but there was no difference between CHB and control group. PT in LC and HCC groups were higher than that in CHB and control group (\( P < 0.001 \) for all), while there was no difference between LC and HCC as well as CHB and control group. APTT in patients groups were higher than in the control (\( P = 0.028 \) for CHB, \( P = 0.002 \) for LC, \( P = 0.007 \) for HCC, respectively). In addition, the examination of tumor markers showed that there were no significant differences in CEA among the patients groups and the controls. In particular, CA199 and CA125 in the patients groups were higher than those in the control group (\( P < 0.01 \) for all), except that there was no difference in CA199 among the patients groups, while CA125 in the LC and HCC groups was higher than that in the CHB group (\( P = 0.002 \) and \( P = 0.009 \), respectively).
Table 1
Clinical and laboratory data in the hepatitis B virus associated liver disease groups and the controls.

|            | CHB            | LC             | HCC            | Control      |
|------------|----------------|----------------|----------------|--------------|
| n (M/F)    | 20(11/9)       | 20(10/10)      | 20(12/8)       | 20(8/12)     |
| Age (years)| 43.55 ± 10.34f | 48.90 ± 12.57  | 54.90 ± 9.31a  | 47.15 ± 10.99|
| ALB (g/L)  | 44.27 ± 5.09df | 36.21 ± 7.19b  | 35.70 ± 6.12b  | 42.39 ± 3.99 |
| TBIL (umol/L)| 14.05 (11.17–17.89)c | 22.50 (15.24–39.00)b | 18.09 (14.45–20.85)b | 11.87 (9.15–14.01) |
| DBIL (umol/L)| 2.78 (1.93–3.75)de | 5.22 (3.92–13.78)b | 3.66 (2.91–5.61)b | 2.18 (1.36–2.67) |
| AST (U/L)  | 39.39 (30.52–74.93)b | 55.36 (39.59–76.92)b | 48.81 (36.55–84.68)b | 18.95 (17.76–21.55) |
| ALT (U/L)  | 48.87 (24.29–113.19)b | 42.44 (25.89–57.13)b | 41.58 (31.94–62.19)b | 16.86 (11.29–24.34) |
| ALP (U/L)  | 90.13 (73.43–99.26)ce | 114.87 (89.01–157.54)b | 109.66 (81.71–175.17)b | 70.82 (58.99–83.40) |
| GGT (U/L)  | 29.16 (17.24–61.90)af | 43.86 (28.24–96.01)b | 67.98 (34.40–136.46)b | 19.77 (13.96–28.09) |
| TBA (umol/L)| 7.77 (5.16–22.34)bc | 26.62 (18.74–38.33)bf | 5.80 (3.45–12.65)b | 2.96 (1.93–2.96) |
| CHE (U/L)  | 8601.27 (7135.69–10563.73)def | 5159.38 (4064.57–5502.13)b | 5198.48 (5168.74–5198.48)b | 8969.05 (8890.09–8969.05) |
| AFU (U/L)  | 28.37 (19.49–31.85)a | 29.30 (22.36–39.67) | 30.45 (23.25–33.30)b | 22.75 (21.69–24.04) |
| CG (ug/ml) | 4.70 (1.69–5.71)bd | 13.11 (5.93–16.40)be | 5.66 (2.25–10.70)b | 1.27 (1.16–1.27) |
| GLU (mmol/L)| 5.14 (4.66–5.39)ce | 6.61 (5.03–7.54)a | 5.99 (5.51–6.83)a | 5.36 (4.68–5.51) |
| BUN (mmol/L)| 4.71 (3.81–5.44) | 5.11 (4.23–6.59) | 5.10 (3.03–6.79) | 4.96 (3.35–5.54) |
| CRE (mmol/L)| 66.60 (59.13–71.97) | 64.14 (56.54–69.65) | 65.62 (55.02–77.44) | 61.83 (54.28–67.41) |
| RBP (mg/L) | 35.13 (21.34–45.33)ce | 26.59 (20.49–34.77)a | 26.60 (23.16–26.60)b | 39.70 (35.64–41.61) |
|                   | CHB        | LC          | HCC         | Control    |
|-------------------|------------|-------------|-------------|------------|
| **WBC (x 10^9/L)**| 5.89 ± 1.32^d | 3.59 ± 1.61^bf | 5.53 ± 2.04 | 6.13 ± 2.03 |
| **RBC (x 10^12/L)** | 4.60 ± 0.56^df | 3.85 ± 0.93^a | 3.81 ± 0.74^b | 4.43 ± 0.50 |
| **Hb (g/L)**      | 142.05 ± 17.60^df | 121.55 ± 29.24 | 120.05 ± 23.17 | 129.60 ± 18.47 |
| **PLT (x 10^9/L)**| 220.25 ± 55.00^df | 89.35 ± 55.87^bf | 158.35 ± 60.28^b | 222.60 ± 42.62 |
| **PT (sec)**      | 11.55 (11.17–11.90)^df | 12.80 (12.25–15.45)^b | 13.25 (12.28–14.45)^b | 11.50 (11.33–11.93) |
| **APTT (sec)**    | 27.25 (25.90–27.88)^a | 28.50 (26.50–30.62)^b | 28.05 (26.22–30.40)^b | 26.00 (25.53–26.25) |
| **CEA (ng/mL)**   | 1.40 (0.87–1.50) | 1.61 (0.54–2.20) | 1.75 (0.84–2.09) | 1.24 (0.73–1.31) |
| **CA199 (U/mL)**  | 16.84 (9.02–28.19)^b | 23.23 (15.73–31.61)^b | 17.03 (10.95–22.33)^b | 6.93 (4.38–7.18) |
| **CA125 (U/mL)**  | 15.06 (14.37–15.06)^bd | 40.01 (18.38–40.01)^b | 89.05 (12.78–154.59)^b | 9.10 (9.10–9.10) |

Data are mean ± SD values or median (25th – 75th percentile) values as indicated.

- \(^{a}P<0.05, \({}^{b}P<0.01\) when patients groups (chronic hepatitis B \([CHB]\), liver cirrhosis \([LC]\), and hepatocellular carcinoma \([HCC]\)) were compared with control subjects.
- \(^{c}P<0.05, \({}^{d}P<0.01\) when the CHB group was compared with the LC group.
- \(^{e}P<0.05, \({}^{f}P<0.01\) when the CHB and LC groups were compared with the HCC group.

Levels of AFP and N-glycan in the patient groups and the controls

Serum AFP concentrations were higher in the hepatic disease groups than in the controls (CHB, \(P=0.011\); LC, \(P=0.029\); HCC, \(P<0.001\)). A significant difference was found between the CHB and HCC groups (\(P=0.024\)), as well as between the LC and HCC groups (\(P=0.048\)), but no marked difference was found between the CHB and LC groups (\(P=0.892\)) (Fig. 1a).
Serum N-glycan levels were elevated significantly in LC and HCC groups compared with the controls ($P = 0.013$ for LC and $P < 0.001$ for HCC). The N-glycan level in the HCC group were significantly higher than that in the CHB group ($P < 0.001$) and LC group ($P = 0.005$), but there was no difference between the CHB and control groups ($P = 0.534$), as well as between the CHB and LC groups ($P = 0.055$) (Fig. 1b).

The influencing factors on AFP and N-glycan expression in the hepatic disease patient groups and the controls

To analyze the association that existed between AFP and N-glycan in hepatic disease patients, the correlation between AFP and N-glycan was studied, as well as the associations among N-glycan / AFP with other parameters in the hepatic disease patients and the controls. To minimize the statistical error caused by small samples, we put all hepatic disease patients into one group. Spearman rank correlations were used to evaluate these associations in this study. The levels of N-glycan / AFP in the combined hepatic disease patients group (CHB, LC, and HCC) and the controls are shown in Figs. 2–8. In the combined hepatic disease patients groups, a significant positive correlation was found between N-glycan and AFP ($R = 0.351, P = 0.006$), as well as N-glycan and age ($R = 0.273, P = 0.034$), N-glycan and AST ($R = 0.303, P = 0.019$), N-glycan and GGT ($R = 0.429, P = 0.001$), N-glycan and PT ($R = 0.378, P = 0.003$), N-glycan and CA125 ($R = 0.349, P = 0.006$) (Fig. 2a-f). Meanwhile, negative correlation was found between N-glycan and ALB ($R = -0.319, P = 0.013$), as well as N-glycan and CHE ($R = -0.459, P < 0.001$), N-glycan and RBC ($R = -0.288, P = 0.026$) (Fig. 2g-i). In the CHB group, positive correlation existed between N-glycan and ALT ($R = 0.513, P = 0.021$), N-glycan and TBA ($R = 0.547, P = 0.012$), N-glycan and APTT ($R = 0.450, P = 0.047$), N-glycan and CRE ($R = 0.455, P = 0.044$), while negative correlations between N-glycan and BUN ($R = -0.460, P = 0.041$) was found (Fig. 4a-e). In the LC group, a positive correlation between N-glycan and GGT ($R = 0.456, P = 0.043$) was found (Fig. 6). Although positive correlation existed between N-glycan and AFU ($R = 0.489, P = 0.029$) and negative correlation was found between N-glycan and WBC ($R = -0.589, P = 0.006$), No relationship was found between N-glycan and AFP in the controls ($R = 0.170, P = 0.407$) (Fig. 8a-b).

In the combined hepatic disease patients groups, a significant positive correlation was found between AFP and HBS DNA ($R = 0.315, P = 0.014$), as well as AFP and TBIL ($R = 0.257, P = 0.047$), AFP and DBIL ($R = 0.314, P = 0.014$), AFP and AST ($R = 0.482, P < 0.001$), AFP and ALT ($R = 0.465, P < 0.001$), AFP and ALP ($R = 0.327, P = 0.011$), AFP and GGT ($R = 0.471, P < 0.001$), while a negative correlation existed between AFP and ALB ($R = -0.295, P = 0.022$), as well as AFP and CHE ($R = -0.293, P = 0.023$) (Fig. 3a-i). In the CHB group, positive correlation existed between AFP and AST ($R = 0.579, P = 0.007$) and between AFP and ALT ($R = 0.496, P = 0.026$), while negative correlations between AFP and CHE ($R = -0.624, P = 0.003$), AFP and BUN ($R = -0.457, P = 0.043$), AFP and GLU ($R = -0.662, P = 0.001$) were found (Fig. 5a-e). In the LC group, positive correlation between AFP and HBS DNA ($R = 0.686, P = 0.001$) was found as well as AFP and TBIL ($R = 0.515, P = 0.020$), AFP and DBIL ($R = 0.487, P = 0.030$), AFP and AST ($R = 0.820, P < 0.001$), AFP and ALT ($R = 0.773, P < 0.001$), AFP and GGT ($R = 0.533, P = 0.016$), AFP and AFU ($R = 0.567, P = 0.009$), AFP and RBC ($R = 0.579, P = 0.007$), AFP and Hb ($R = 0.529, P = 0.017$) (Fig. 7a-f). In addition, negative relationship were found between AFP and Age ($R = -0.756, P < 0.001$), AFP and BUN ($R = -0.544, P = 0.001$).
0.013), AFP and CEA (R = -0.489, P = 0.029) in the LC group (Fig. 7g-l). In the control group, a negative correlation existed between AFP and DBIL (R = -0.477, P = 0.034) (Fig. 8c). In addition, no correlation was found between N-glycan / AFP level and those of the laboratory parameters above in the HCC group.

**Effect of AFP and N-glycan expression in the diabetes patients and the controls**

To study whether there is a dose–response relationship between AFP and N-glycan, the 60 hepatic disease patients were redivided into three groups according to AFP level (Group A, AFP levels < 10 ng/mL; Group B, AFP levels 10–200 ng/mL; and Group C, AFP levels > 200 ng/mL) (Table 2). AST, ALT, GGT and HBV DNA in Group A were lower than in Groups B and C (P < 0.05 for all), and no difference were found between Groups B and C (P > 0.05 for all). In addition, AFU level in group A was decreased than in Group C (P = 0.002), while no difference were found between group A and group B (P = 0.052), group B and group C (P = 0.207).
Table 2
Clinical and laboratory data in the hepatitis B virus associated liver disease patients with different levels of AFP.

| AFP groups     | Group A(< 10 ng/mL) | Group B(10–200 ng/mL) | Group C(> 200 ng/mL) |
|----------------|---------------------|-----------------------|----------------------|
| n (M/F)        | 36(18/18)           | 14(11/3)              | 10(4/6)              |
| Age (years)    | 50.97 ± 11.84       | 47.29 ± 11.67         | 45.00 ± 10.30        |
| ALB (g/L)      | 41.37 (35.74–46.02) | 37.98 (35.91–41.30)   | 32.58 (30.97–33.53)  |
| TBIL (umol/L)  | 15.71 (12.52–20.32) | 15.71 (12.52–20.32)   | 19.27 (16.66–32.63)  |
| DBIL (umol/L)  | 3.29 (2.35–4.36)    | 5.54 (2.57–7.44)      | 5.78 (3.57–10.31)    |
| AST (U/L)      | 39.38 (30.20-54.34) | 92.81 (47.68-123.34)  | 69.50 (42.94-180.43) |
| ALT (U/L)      | 34.19 (20.55–49.52) | 90.35 (47.62-181.27)  | 80.64 (36.93-183.11) |
| ALP (U/L)      | 90.78 (75.80-119.97)| 107.03 (96.98-151.38) | 148.57 (87.49-345.52)|
| GGT (U/L)      | 29.16 (19.58–53.77) | 67.30 (34.27-100.66)  | 123.23 (68.31-148.53)|
| TBA (umol/L)   | 9.95 (4.56–32.41)   | 19.71 (8.68–28.62)    | 5.80 (4.96–12.25)    |
| CHE (U/L)      | 5975.98 (5198.48-9139.79) | 5595.95 (5225.55–7630.00) | 5198.48 (4902.99-5198.48) |
| AFU (U/L)      | 26.38 ± 7.55        | 34.04 ± 13.42         | 40.51 ± 21.61        |
| CG (ug/ml)     | 5.71 (2.32–11.27)  | 5.88 (3.38–12.25)     | 5.85 (2.55–10.70)    |
| GLU (mmol/L)   | 5.66 (5.10–6.60)    | 4.95 (4.32–6.61)      | 5.91 (5.79–7.05)     |
| BUN (mmol/L)   | 5.27 (4.19–6.32)    | 4.26 (3.29–4.85)      | 5.45 (2.81–6.95)     |
| CRE (mmol/L)   | 64.90 (55.79–74.63) | 67.23 (62.47–72.07)   | 59.88 (50.18–74.98)  |

Data are mean ± SD values or median (25th – 75th percentile) values as indicated.

\(^{a}P<0.05, ^{b}P<0.01\) when Group A, Group B were compared with Group C.

\(^{c}P<0.05, ^{d}P<0.01\) when when Group A was compared with Group B.

M/F, males/females; ALB, albumin; TBIL, total bilirubin; DBIL, direct bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, \(\gamma\)-glutamyl transferase; TBA, total bile acid; CHE, cholinesterase; AFU, alpha-L-fucosidase; CG, cholyglycine; GLU, glucose; BUN, urea nitrogen; CRE, creatinine; RBP, Retinol binding protein; WBC, white blood cell. RBC, red blood cell; Hb, hemoglobin; PLT, platelets; PT, prothrombin time; APTT, activated partial thromboplastin time; CEA, carcinoembryonic antigen; CA199, glucoprotein antigen 19–9; CA125, glucoprotein antigen 125; HBV, hepatitis B virus.
|          | Group A     | Group B     | Group C     |
|----------|-------------|-------------|-------------|
| RBP (mg/L) | 31.39 ± 17.44 | 29.49 ± 9.13 | 24.26 ± 6.11 |
| WBC (× 10^9/L) | 5.09 ± 2.00   | 4.52 ± 1.51   | 5.38 ± 2.30   |
| RBC (× 10^{12}/L) | 4.05 ± 0.85 | 4.21 ± 0.77   | 4.02 ± 0.90   |
| Hb (g/L)   | 127.72 ± 26.28 | 133.50 ± 25.10 | 120.60 ± 23.66 |
| PLT (× 10^9/L) | 157.11 ± 84.32 | 159.43 ± 79.50 | 147.10 ± 53.01 |
| PT (sec)   | 11.95 (11.38–13.30) | 12.55 (12.07–13.47) | 13.40 (12.65–16.82) |
| APTT (sec) | 27.40 (25.98–28.60) | 27.50 (26.10–32.15) | 29.60 (27.33–35.85) |
| CEA (ng/mL) | 1.50 (0.62–1.85) | 0.94 (0.50–1.73) | 1.52 (0.58–2.06) |
| CA199 (U/mL) | 19.48 (8.69–26.55) | 19.73 (14.89–30.66) | 17.77 (15.29–28.96) |
| CA125 (U/mL) | 18.08 (10.73–40.01) | 30.45 (15.06–40.01) | 24.25 (14.30–125.94) |
| HBV DNA (log_{10}IU/mL) | 2.70 (2.70–3.34)^ac | 4.34 (2.70–6.93) | 4.39 (2.86–6.09) |

Data are mean ± SD values or median (25th – 75th percentile) values as indicated.

^aP < 0.05, ^bP < 0.01 when Group A, Group B were compared with Group C.

^cP < 0.05, ^dP < 0.01 when when Group A was compared with Group B.

M/F, males/females; ALB, albumin; TBIL, total bilirubin; DBIL, direct bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; TBA, total bile acid; CHE, cholinesterase; AFU, alpha-L-fucosidase; CG, cholyglycine; GLU, glucose; BUN, urea nitrogen; CRE, creatinine; RBP, Retinol binding protein; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; PLT, platelets; PT, prothrombin time; APTT, activated partial thromboplastin time; CEA, carcinoembryonic antigen; CA199, glucoprotein antigen 19–9; CA125, glucoprotein antigen 125; HBV, hepatitis B virus.

Specifically, N-glycan levels were elevated significantly in group C compared with the group A (P = 0.026), while no differences existed between group A and group B (P = 0.050) and between group B and group C (P = 0.653) (Fig. 9). There was a trend of increasing N-glycan with elevated AFP level in the combined hepatic disease group (Fig. 2a).

**Levels of N-glycan and AFP in patients with the different developmental stages of LC and HCC**

According to the staging criteria of liver function Child-Pugh (CP) score, we divided the LC and HCC patients into two groups respectively: the compensatory group and decompensatory group. In the patients with LC, The levels of N-glycan in the decompensatory was significant higher than in the control (P = 0.007), but no differences were found between the decompensatory and the compensatory groups (P = 0.389) as well as between the compensatory and the controls (P = 0.178). Although there was a
significant difference in AFP levels between the compensatory and the controls ($P=0.003$), no differences were found between the decompensatory and the compensatory groups ($P=0.092$) and between the decompensatory and the controls ($P=0.109$) (Fig. 10a). The results indicated that the levels of N-glycan were more valuable in reflecting the disease severity in patients with LC compared with the levels of AFP. In the patients with HCC, Levels of both N-glycan and AFP in the compensatory and decompensatory groups were significant higher than those in the control ($P<0.001$ and $P=0.004$ for N-glycan and $P<0.001$ for both in AFP, respectively), but no difference were found between the compensatory and decompensatory groups ($P=0.155$ for N-glycan and $P=0.422$ for AFP) (Fig. 10b). Our data suggested that levels of N-glycan and AFP in patients with HCC were closely related to the stage of disease development.

**Discussion And Conclusions**

Chronic hepatitis B is a common clinical multiple liver disease, which has the characteristics of great harmfulness, high incidence and strong infectivity. In the case of long-term infection and replication of HBV, it can lead to the continuous development of liver inflammation and increase the risk of liver cirrhosis and liver cancer. HBV infection can cause changes in serological indexes when liver damage is caused. Examination of serological indexes in patients with HBV can effectively reflect their liver injury and inflammation, so as to guide clinical treatment. In this study, our results showed that there were varying degrees of hepatocyte damage (increased AST, ALT and GGT), cholestasis (increased TBA and CG), decreased catabolism (increased TBIL and DBIL) and insufficient synthesis (decreased ALB, CHE and RBP, but increased PT and APTT) in patients with liver disease, especially in patients with LC and HCC. At the same time, the tumor non-specific markers (CA199 and CA125) were also abnormal (Table 1). Our data suggest that abnormal liver function is related to the development of the disease, and it is necessary to closely monitor the changes of liver function indexes in patients.

AFP is a specific serological biomarker of HCC. Clinically, AFP is used to diagnose, predict recurrence and judge prognosis of HCC. AFP is the main protein in embryonic plasma and is usually produced in fetal liver and yolk sac, but its transcription is inhibited by methylation of AFP gene after birth, so that it is not be detected or trace level in healthy adults. However, the level of AFP will change significantly under pathological conditions. Our study showed that the level of serum AFP in patients with liver disease was significantly higher than that in the control group ($P<0.05$). Significantly elevated AFP level is a sign of malignant progression of chronic liver disease. In patients with HCC, AFP levels are significantly increased than CHB and LC ($P=0.024$ and $P=0.048$, respectively) (Fig. 1a). In addition, our study showed that AFP level in LC compensated group was higher than that in control ($P=0.003$), especially in HCC group, AFP in compensated and decompensated group was higher than that in control group ($P<0.001$ for both) (Fig. 10a-b). Our data suggest that AFP may play an important role in the occurrence and development of HCC.
Glycosylation is the process of addition of glycans to glycoproteins and is one of the most important post-translational modifications of proteins. Oligosaccharides attached to proteins are involved in a variety of biological processes, such as protein-protein recognition, receptor interactions, cellular communication, adhesion, immune defense and inflammation. Abnormal glycosylation is considered to be an important feature of tumorigenesis and development, including HCC, which can interfere with cell adhesion, migration and proliferation. Most of the glycoproteins in serum are produced by hepatocytes, and the changes of serum N-glycome spectrum mainly reflect the physiological changes of liver. Our results show that the value of serum N-glycan of patients with LC and HCC were higher than that of healthy people. In patients with liver disease, especially in patients with HCC, the value of serum N-glycan was the highest. Our data suggest that the abnormal expression of N-glycan in patients with liver disease may play a key role in the progression of liver disease. At present, some studies have shown that glycosylation is related to liver fibrosis, liver cirrhosis and liver cancer. Our results demonstrated that N-glycan value in the decompensated stage of LC was higher than that in the control group, while N-glycan value in the compensated and decompensated stage of HCC were higher than that in the control group (P<0.001 and P=0.004), suggesting that the value of N-glycan is also related to the development of the disease.

It is intriguing that positive correlation between N-glycan and AFP, Age, AST, GGT, PT, CA125, respectively, and negative correlation with ALB, CHE and RBC were been found in our cohort study, indicating that there may be interactions between AFP, Age, AST, GGT, PT, CA125, ALB, CHE, RBC and N-glycan. Unlike our study, sialylated glycans was found to be negatively correlated with age in breast cancer patients, which may be due to differences in glycans expression levels in different diseases. In CHB group, N-glycan was positively correlated with ALT, TBA, APTT and CRE, and negatively correlated with BUN, which suggested that N-glycan was correlated with ALT, TBA, APTT, CRE and BUN in CHB. In LC group, there was a positive correlation between N-glycan and GGT. In patients with liver disease, AFP was positively correlated with HBSDNA, TBIL, DBIL, AST, ALT, ALP and GGT. There was a negative correlation between ALB and CHE, which indicated that the level of AFP in patients with liver disease was correlated with liver injury, cholestasis and synthetic function. In CHB group, AFP was positively correlated with AST and ALT, and negatively correlated with CHE, BUN and GLU. In LC group, AFP was positively correlated with HBSDNA, TBIL, DBIL, AST, ALT, GGT, AFU, RBC and Hb, but negatively correlated with Age, BUN and CEA. Our data suggest that the expression of N-glycan and AFP in the occurrence and development of liver disease may be regulated by many factors.

In order to further test whether the increase of serum N-glycan in patients with liver disease is related to AFP, 60 patients with liver disease were re-divided into three groups according to the level of AFP: low level of AFP, medium level of AFP, and high level of AFP. Our results showed that high levels of AFP and high levels of N-glycan occurred at the same time, and it was found that there was a strong positive correlation between serum AFP and N-glycan in patients with liver disease. In addition, our data demonstrated that N-glycan and AFP levels in both LC and HCC patients increased as the disease progresses.
progressed to decompensation. Our data suggest that elevated levels of AFP and N-glycan may be involved in the occurrence and development of liver disease.

In conclusion, our data suggested a close correlation between AFP and N-glycan levels, which may be interwoven with the development of liver disease. Elevated levels of AFP and N-glycan may be one of the pathogenesis mechanisms of liver disease progression. At present, serum AFP detection is a routine test for laboratory monitoring of chronic liver disease. Given the close relationship between AFP and N-glycan, these biochemical markers will help predict the likelihood of cirrhosis and liver cancer during disease progression and treatment. The limitations of the study are the absence of hepatitis C and other non-infectious liver diseases and that the numbers of patients in this study are not big enough to show statistical significance. A large population should be needed to minimize statistical variance. In addition, we only analyzed the serum levels of N-glycan and AFP in patients with HBV related; although our data showed the close relationship between them, more experiments would be required to investigate the molecular mechanisms between N-glycan and AFP at the cellular level.

Declarations

Acknowledgments

This work is supported by the National Natural Science Foundation of China (grant number 81960440) and the Financial Support Program for Academic Leaders of Major Disciplines in Jiangxi Province, China (grant number 20182BCB22018).

Compliance with ethical standards

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

This article is conducted in accordance with the Ethical Guidelines of the Declaration of Helsinki.

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Figures
Levels of alpha fetal protein (AFP) and N-Glycan in hepatic disease subgroups compare with the control group.
Figure 2

(a–i) Correlations among N-Glycan and alpha fetal protein (AFP), Age, aspartate aminotransferase (AST); γ-glutamyl transferase (GGT), prothrombin time (PT), glucoprotein antigen 125 (CA125), albumin (ALB), cholinesterase (CHE), red blood cell (RBC) in the hepatic disease groups.
Figure 2

(a–i) Correlations among N-Glycan and alpha fetal protein (AFP), Age, aspartate aminotransferase (AST); \(\gamma\)-glutamyl transferase (GGT), prothrombin time (PT), glucoprotein antigen 125 (CA125), albumin (ALB), cholinesterase (CHE), red blood cell (RBC) in the hepatic disease groups.
Figure 3

(a–i) Correlations among alpha fetal protein (AFP) and hepatitis B virus DNA (HBV DNA), total bilirubin (TBIL), direct bilirubin (DBIL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), albumin (ALB), cholinesterase (CHE) in the hepatic disease groups.
Figure 4

(a–i) Correlations among alpha fetal protein (AFP) and hepatitis B virus DNA (HBV DNA), total bilirubin (TBIL), direct bilirubin (DBIL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), albumin (ALB), cholinesterase (CHE) in the hepatic disease groups.
Figure 5

(a–e) Correlations among N-Glycan and alanine aminotransferase (ALT), total bile acid (TBA), activated partial thromboplastin time (APTT), creatinine (CRE), urea nitrogen (BUN) in the chronic hepatic B (CHB) group.
Figure 6

(a–e) Correlations among alpha fetal protein (AFP) and aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholinesterase (CHE), urea nitrogen (BUN), glucose (GLU) in the chronic hepatic B (CHB) group.
Figure 6

Correlation between N-Glycan and γ-glutamyl transferase (GGT) in the liver cirrhosis (LC) group.
Figure 7

(a–e) Correlations among alpha fetal protein (AFP) and aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholinesterase (CHE), urea nitrogen (BUN), glucose (GLU) in the chronic hepatic B (CHB) group.
Figure 7

(a–l) Correlations among alpha fetal protein (AFP) and hepatitis B virus DNA (HBV DNA), total bilirubin (TBIL), direct bilirubin (DBIL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), alpha-L-fucosidase (AFU), red blood cell (RBC), hemoglobin (Hb), Age, urea nitrogen (BUN), carcinoembryonic antigen (CEA) in the liver cirrhosis (LC) group.
Figure 7

(a–l) Correlations among alpha fetal protein (AFP) and hepatitis B virus DNA (HBV DNA), total bilirubin (TBIL), direct bilirubin (DBIL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), alpha-L-fucosidase (AFU), red blood cell (RBC), hemoglobin (Hb), Age, urea nitrogen (BUN), carcinoembryonic antigen (CEA) in the liver cirrhosis (LC) group.
Figure 8

(a–c) Correlations among N-Glycan and alpha-L-fucosidase (AFU), white blood cell (WBC), direct bilirubin (DBIL) in the controls.
Figure 8

(a–c) Correlations among N-Glycan and alpha-L-fucosidase (AFU), white blood cell (WBC), direct bilirubin (DBIL) in the controls.
Figure 8

Correlation between N-Glycan and γ-glutamyl transferase (GGT) in the liver cirrhosis (LC) group.

\[
R = 0.456 \\
p = 0.043
\]
Figure 9

Levels of N-Glycan in different concentrations of alpha fetal protein (AFP) subgroups.
Figure 10

Levels of N-Glycan and alpha fetal protein (AFP) in hepatic disease subgroups with compensatory or decompensatory groups versus the control: (a) liver cirrhosis (LC) and (b) hepatocellular carcinoma (HCC). CI: confidence interval.

**p < 0.001
**p < 0.001
**p = 0.003
**p = 0.007
Figure 12

Levels of N-Glycan and alpha fetal protein (AFP) in hepatic disease subgroups with compensatory or decompensatory groups versus the control: (a) liver cirrhosis (LC) and (b) hepatocellular carcinoma (HCC). CI: confidence interval.