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Alterations of glycan branching and differential expression of sialic acid on alpha fetoprotein among hepatitis patients

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Abstract The level of serum glycoproteins and their glycosylation pattern change in liver diseases including hepatocellular carcinoma (HCC). Some of them, especially alpha fetoprotein (AFP), serve as useful biomarkers for HCC. The present investigation showed high level of AFP in hepatitis B cirrhosis (HBV-LC) and hepatitis C cirrhosis (HCV-LC) patients. However, increase of AFP level was not significantly high in chronic hepatitis B (HBV-CH) as determined by ELISA using monoclonal anti-human AFP (mAb-AFP). The differential expression of sialic acid linkage was observed in HBV-CH and HCV-LC by ELISA; the former bound strongly with *Sambucus nigra* agglutinin (SNA), which has exclusive binding specificity for NeuAcα2-6-, whereas HCV-LC reacted preferably with *Maackia amurensis* agglutinin (MAA) which recognizes NeuAcα2-3-. There was significantly high glycan branching in HBV-LC and HCV-LC in comparison to controls as illustrated by concanavalin A. This was further confirmed by *Phaseolus vulgaris* erythroagglutinin (E-PHA) and *Datura stramonium* agglutinin (DSA). Enhanced fucosylation of AFP was observed in HBV-LC, HCV-LC and HCC patients by ELISA using fucose binding *Aleuria aurantia* lectin; however, maximum binding was found in HCC. Fucosylation with α1-6 linkage was further confirmed by *Lens culinaris* agglutinin (LCA). From the above results it is concluded that the changes in concentration of AFP, differential expression of sialic acid, increase of glycan branching and fucosylation have a prognostic value of hepatitis and it could be possible that lectin-based assay with AFP can aid in diagnosis of hepatitis diseases besides clinical examination and routine laboratory investigation.

Keywords Alpha fetoprotein · Aleuria aurantia lectin · Fucosylation · Liver cirrhosis · Liver biopsy

Abbreviations

AFP	Alpha-fetoprotein
AGP	Alpha1- acid glycoprotein
ALL	*Aleuria aurantia* lectin
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
ConA	Concanavalin A
HBV-CH	Chronic hepatitis B
HBV-LC	Hepatitis B cirrhosis
HCV-LC	Hepatitis C cirrhosis
MAA	*Maackia amurensis* agglutinin
SNA	*Sambucus nigra* agglutinin
Tf	Transferrin

Introduction

To search for accurate biomarkers for early diagnosis of chronic liver diseases is a global demand [1]. Routinely

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used methods for diagnosis of chronic hepatitis (HBV and HCV) are clinical examination and biochemical tests such as hematologic indices, measurement of prothrombin time, serum proteins including albumin, immunoglobulins (IgM-HAV, IgM-HDV, IgM-HEV) and bilirubin. Besides assay of liver enzymes, *e.g.,* aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) are critically important. However, about 40% chronic hepatitis patients later progressed into liver cirrhosis in which about 10% of the patients developed liver cancer (HCC). Liver biopsy is the present gold standard to assess structural liver damage caused by liver cirrhosis. However, this has several disadvantages such as lack of sensitivity, risk of complication and discomfort to patients. No reliable non-invasive diagnostic approach is available for chronic hepatitis induced cirrhosis. Therefore, a suitable biomarker of liver cirrhosis is urgently needed. However, the AST/ALT ratio has previously been suggested as marker for cirrhosis [2, 3]. These tests cannot be useful for differential diagnosis of viral hepatitis with cirrhosis and among viral hepatitis *viz.,* HBV and HCV patients. The altered glycosylation of serum protein in patients with various pathogenesis was reported [4]. Glycosylation change in patients' glycoproteins such as fucosylation in alpha-1 acid glycoprotein (AGP) in ascetic fluid with liver cirrhosis [4, 5], haptoglobin (Hp) in alcoholic liver disease [6, 7], serum cholinesterase in LC [8, 9]. Previously, we observed that enhanced fucosylation of AGP in chronic hepatitis B (HBV-CH) and hepatitis B cirrhosis (HBV-LC) patients [10]. Alpha-fetoprotein (AFP), which is an embryo-specific glycoprotein composed of approximately 580 amino acid (MW~72 kDa) and containing 3–5% of carbohydrate [11–13]. It is the mostly used serum biomarker for cirrhosis and HCC worldwide. This is a major serum protein of human and is normally expressed in the fetal liver, gastrointestinal tract (GI), and yolk sac [14]. In human it is synthesized by the yolk sac and liver (1–2 months) and is subsequently predominant in the liver [15]. In early fetal life the AFP level can be 100 to 1,000—fold higher than that in normal individuals and decreases gradually after birth. The normal range of AFP in adults and children is reported to show variation from nearly 50 to 5 ng/ml [16, 17]. However, it starts up again under the stimulus of some diseases in the liver and pathological condition.

In both benign liver disease and primary hepatic cancer most of the serum AFP binds to Con A [18–21]. A significant reduction in AFP binding to Con A occurs when the cancer is in another site such as ovary or testicle [22] or is present as metastases in the liver [23, 24]. It is thought that this change is caused through the presence of bisecting *N*-acetylglucosamine residues of the type described above for yolk sac tumours, as the affinity of glycopeptides for Con A is reduced if a bisecting *N*-acetylglucosamine is present [25]. However, it has been shown that the enzyme that adds the bisecting *N*-acetylglucosamine to glycoproteins is elevated in sera and liver tissue from patients with hepatomas and liver cirrhosis [26]. There were many reports that AFP is well established as a specific and sensitive marker of HCC. High levels of serum AFP may be responsible for the development of HCC [27]. The binding property of lectin with AFP was originally described by Breborowicz [28] and Miyazaki [29] They used *Lens culinaris* agglutinin (LCA), which recognizes α1-6 fucosylation on N-glycans [30] in crossed immune affino-electrophoresis and demonstrated that AFP in the serum of patients with HCC had increased proportions of LCA-reactive AFP, whereas AFP in the serum of patients with chronic liver disease contained less LCA-reactive AFP. More sensitive methods of distinguishing AFP between HCC and chronic liver disease have been established by using antibody affinity blotting for the detection of AFP bands separated by LCA affinity electrophoresis [31, 32]. Using this method, LCA reactive AFP is a good marker for the differential diagnosis of HCC in the case of chronic liver disease [33–39]. In addition, it represents a good marker for the early diagnosis of HCC [30]. Furthermore, LCA-reactive AFP is a possible indicator for a poor prognosis for patients with HCC [40, 41]. However, in some HCC patients abnormal glycoprotein of AFP was observed, which was confirmed by LCA-affinity column chromatography [42].

The present investigation monitors the alteration of AFP concentration in chronic hepatitis B (HBV-CH), hepatitis B cirrhosis (HBV-LC) and hepatitis C cirrhosis (HCV-LC) patients' sera with respect to that of AFP of healthy individuals by estimating the concentration of AFP by ELISA. This study also presents the change in fucosylation of AFP by ELISA using fucose binding *Aleuria aurantia* lectin and *Lens culinaris* agglutinin. Change of sialic acid linkage was assayed by using two sialic acid specific lectins, *viz., Maackia amurensis* agglutinin and *Sambucus nigra* agglutinin, respectively. Increase in glycan branching evaluated by Concanavalin A, *Phaseolus vulgaris* erythroagglutinin (E-PHA), *Phaseolus vulgaris* leukoagglutinin (L-PHA) and *Datura stramonium* agglutinin (DSA) is also described herein.

Materials and methods

Materials

Maackia amurensis agglutinin (MAA), *Sambucus nigra* agglutinin (SNA), Concanavalin A, *Phaseolus vulgaris* erythroagglutinin (E-PHA), *Phaseolus vulgaris* leukoagglutinin (L-PHA), *Datura stramonium* agglutinin (DSA),

Lens culinaris agglutinin, rabbit monoclonal antihuman AFP, biotin 3-sulfo-*N*-hydroxysuccinimide ester and streptavidin horse-radish peroxidase (HRP) conjugate were purchased from Sigma (USA). *Aleuria aurantia* lectin (AAL) was procured from Vector Laboratories. EIAgen AFP kit was the product of Adaltis Italia (S.p.A.) All other materials and reagents used were of high analytical grade and obtained from commercial sources.

Patients' sera

Sera from 20 chronic hepatitis B (HBV-CH), 25 hepatitis B induced liver cirrhosis (HBV-LC), 14 hepatitis C virus induced liver cirrhosis (HCV-LC) and 10 hepatocellular carcinoma (HCC) patients were collected from the Department of Hepatology, Post Graduate Institute of Medical Education and Research, Chandigarh. The diagnosis was based on clinical examination and biochemical tests (measurement of serum ALT, AST, ALP, gammaglutamyltransferase (γ-GT), bilirubin, albumin, IgA, prothrombin time, proaccelerin, and haemoglobin) and virological investigations. Hepatitis B and hepatitis C virus infected patients were tested for hepatitis-B surface antigen (HBsAg) and Anti-HCV respectively. Blood from 15 age and sex-matched healthy individuals were served as control. All sera were stored at −20°C. Informed consent was obtained from each patient and healthy individuals. Ethical committee of the University approved the study. The clinical features of different groups of patients are summarized in Table 1.

Quantification of AFP by ELISA

The concentration of AFP in serum samples was measured by ELISA using AFP kit following manufacture's instructions. Different concentrations (0, 0.5, 2.5, 12.5, 25.0 and 50.0 ng/100 μl) of AFP as standard and sera from different groups of patients *viz.,* HBV-CH, HBV-LC, HCV-LC as well as healthy individuals (100 μl each) were added into the wells of a 96 well microtiter plate (NUNC) previously coated with streptavidin. 100 μl of monoclonal biotin-AFP conjugate was added into each well and left for 30 min at 37°C. The wells were washed with citrate-borate buffer containing 0.1% Tween-20 and Amphotericin-B (2.5 μg/ml), followed by addition of 100 μl of HRP conjugated mAb-AFP to each well and incubated at 37°C for 30 min. To each well 3′,3′,5′,5′ tetramethylbenzidin (TMB) (100 μl), and 0.01% H_2O_2 in citrate buffer was added and the plate was left for 30 min at room temperature. The absorbance was measured at 450 nm in an ELISA reader after adding 100 μl of 1.5 M H_2SO_4 to each well. AFP concentration in each sample was calculated using the standard curve prepared by AFP. All experiments were done in triplicate and data presented were their mean values.

Distribution of α2-3-/α2-6- linked sialic acid in AFP

To understand the distribution of α2-3- and α2-6-linked sialic acid present in N-linked glycans of AFP, ELISA was performed using two sialic acid specific lectins, *viz*., *Maackia amurensis* agglutinin (MAA, specificity: NeuAcα2-3Galβ1-4GlcNAc) and *Sambucus nigra* agglutinin (SNA, specificity: NeuAcα2-6Galβ1-4GlcNAc). The wells of a microtiter plate were coated with 100 μl of mAb-AFP (10 μg/ml, 100 μl/well) in 0.01 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.6 and left for 24 h at 4°C, washed with 100 μl 0.01 M PBS, pH 7.4, containing 0.05% Tween-20 and incubated with 100 μl of PBS containing 1% BSA at room temperature for 1 h. To each well diluted sera of different patients, the AFP concentration of which was adjusted to 500 pg/ml, was added and allowed to react with the mAb-AFP for 2 h at 25°C. After washing 100 μl of biotinylated MAA and SNA (1:100 in PBS) were added separately and incubated for 1 h followed by addition of 100 μl of HRP-labeled streptavidin (1:1000 in PBS). To each well 0.1% *O*-phenylenediamine dihydrochloride (OPD) (100 μl), and 0.05% H_2O_2 in 0.05 M citrate phosphate buffer, (pH 5.0) were added. The plates were left for 1 h at room temperature. The absorbance of each well was measured after adding 50 μl of 1.5 M H_2SO_4 to

Table 1 Clinical features of patients with chronic hepatitis B (HBV-CH), hepatitis B induced liver cirrhosis (HBV-LC) and hepatitis C induced liver cirrhosis (HCV-LC)

Groups	Control (*n*=15)	HBV-CH (*n*=20)	HBV-LC (*n*=25)	HCV-LC (*n*=14)
Age (years)	25–64	28–70	16–72	34–69
Gender (M/F)	11/4	17/3	20/5	10/4
AFP (ng/ml)	26±2	42±17	93±59	94±65
ALT (U/l)	5–40	13–114	31–89	63–138
AST (U/l)	5–40	17–140	26–186	63–138
ALP (U/l)	35–125	75–218	57–531	98–526
Bilirubin (g/dl)	3.6–5.0	3.1–5.0	2.5–5.0	2.7–4.8
Albumin (g/dl)	3.5–5.5	2.9–5.0	2.5–5.0	3.1–4.8
Prothrombin time (s)	12–16	14–16	14–18	16–21

the wells. The ratio of absorbances at 450 nm of AFP recognized by MAA and SNA were calculated (MAA/SNA ratio). All experiments were done in triplicate and data presented were their mean values.

Branching of AFP glycan

ConA binds to the biantennary glycan chain more strongly than tri- and tetra-antennary glycan because of easy access of the lectin to the core mannose units. To establish alteration in the antennary glycan chain of AFP during disease state ELISA was performed with ConA following the same procedure as above except addition of rabbit mAb-AFP-HRP conjugate, 100 µl of HRP-conjugated ConA (1:1000) were added to each well and incubated for 2 h at room temperature. The absorbance was measured at 450 nm using a microtiter plate reader. Similarly, HRP-conjugated E-PHA, L-PHA and DSA were used to asses the alteration of glycan branching of AFP. All experiments were done in triplicate and data presented are their mean values.

Quantification of fucose by ELISA

A lectin sandwich immunoassay was used to quantitate the change of fucosylation in AFP of different groups of patients including HCC using fucose-specific *Aleuria aurantia* lectin (AAL). Following the same procedure as above, 100 µl of biotin-conjugated AAL (1:100 in PBS) was added to each well, and the plate was incubated at room temperature for 2 h. After washing three times with PBS-T, 100 µl of streptavidin-HRP conjugate (1:1000 in PBS) were added to the wells and the rest of the experiments were followed as above. To ascertain fucosylation in HBV-LC and HCV-LC through α1-6 linkage to the innermost GlcNAc residue, HRP-conjugated LCA was used following the same procedure as above. All experiments were done in triplicate and data presented are their mean values.

Statistical analysis

The STATISTICA 6.0 computer program was used for statistical analysis. The results are presented as mean±SD. The statistical analysis was performed by unpaired Student's *t* test. For the statistical significance two-tailed *p* value of less than 0.05 was considered significant.

Results

Serum AFP levels

Serum AFP levels in HBV-CH (n=20), HBV-LC (n=25), HCV-LC (n=14) patients and in healthy individuals group (n=15) were assayed by ELISA. Figure 1 showed that the level of AFP in the patient groups were significantly higher ($p<0.05$) as compared to that of healthy individuals. In patient groups HCV-LC (94±65) showed highest level of AFP, which was recorded lowest in HBV-CH (42±17). An elevated AFP level (>100 ng/ml) was demonstrated in most patient groups, whereas in all HBV-CH patients AFP level was found to be less than 100 ng/ml.

Reactivity of AFP with sialic acid specific lectins

AFP in all patients sera reacted with SNA and MAA, which indicates that oligosaccharide chains in AFP contain sialic acids, which are glycosidicaly linked through α2-6 or α2-3 to penultimate galactose in glycan chain. Figure 2 shows that there was almost no change in the distribution of sialic acid linkage in AFP in HBV-LC patients. However, a significant change was observed in HCV-LC ($p<0.001$) and HBV-CH ($p<0.05$) in respect to healthy individuals. Table 2 illustrates that HBV-CH showed lower MAA/SNA ratio, compared to controls indicating the predominance of α2-6 linked sialic acid in AFP oligosaccharide chains. On the other hand higher MAA/SNA ratio in HCV-LC patients indicates a major change in the sialic acid linkage, which is due to predominance of α2-3 linked sialic acid.

Change in the degree of branching

To investigate any possible change in the antennaric oligosaccharide chains in AFP, ELISA was performed using ConA. This could be correlated with the disease state and thus used as a prognosis of the disease. The absorbance at

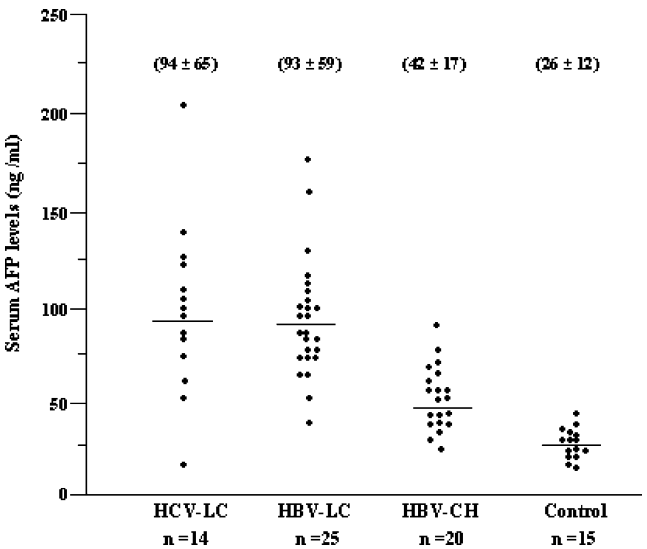

Fig. 1 Serum AFP concentration among different groups of hepatitis patients and healthy individuals. Each horizontal line indicates the mean value of AFP. Data is presented as mean±SD ($p<0.05$).

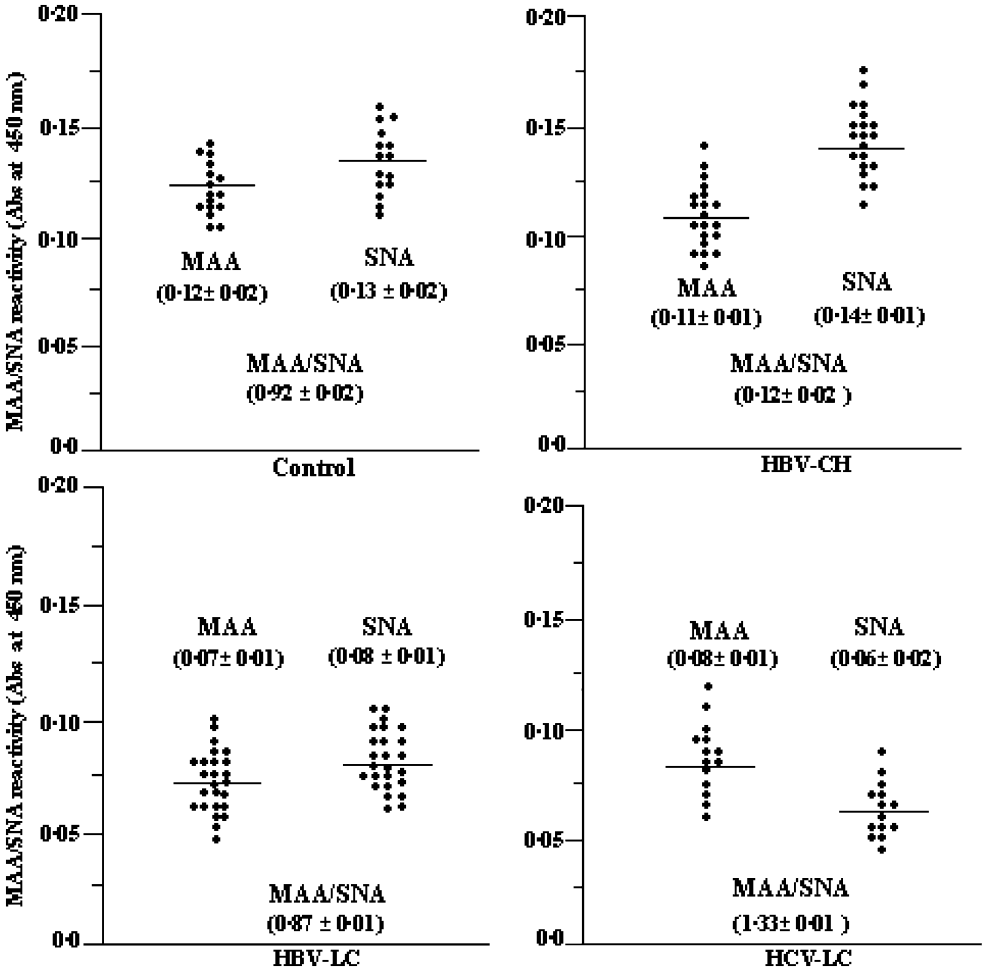

Fig. 2 Graphical representation of MAA and SNA reactivities with serum AFP of different hepatitis patient groups and healthy individuals. Data is presented as mean±SD ($p<0.001$)

450 nm was an indirect measure of the extent of ConA binding to AFP. Figure 3 shows a change in absorbance in HBV-LC and HCV-LC patients in respect to healthy individuals, which indicates there was alteration in the antennaric oligosaccharide chain. HBV-LC showed major change among the different groups of patients, which indicates an increase in the glycan branching, this might give an insight into the progression of liver disease. Almost no change was observed in HBV-CH patients. This result was confirmed by using E-PHA, L-PHA and DSA. E-PHA bound stronger with AFP than L-PHA. HBV-LC patients showed maximum binding with E-PHA (Fig. 4a and b). Similarly, DSA like E-PHA also showed highest binding with HBV-LC suggesting tri- and tetraantennary complex glycan structure in AFP (Fig. 4c).

Concentration change of fucose

To investigate the change in concentration of fucose in AFP of different patients groups including HCC, ELLSA was performed using *Aleuria aurantia* lectin (AAL). Figure 5a shows fucose was significantly elevated in all patients compared to normal individuals. In the patient groups, the highest fucose level was found in the HCC (0.064±0.02) ($p<0.001$) and lowest level was observed in HBV-CH (0.011 ±0.002). AFP is known to contain fucose, which is attached

Table 2 Relative distribution of AFP-glycoform with different reactivity towards MAA and SNA

Groups	AFP reactivity with sialic acid specific lectins		Ratio of MAA/SNA
	MAA	SNA	
HBV-CH (n=20)	0.11±0.01	0.14±0.01	0.78±0.01
HBV-LC (n=25)	0.07±0.01	0.08±0.01	0.87±0.01
HCV-LC (n=14)	0.08±0.01	0.06±0.02	1.33±0.01
Control (n=15)	0.12±0.02	0.13±0.01	0.92±0.01

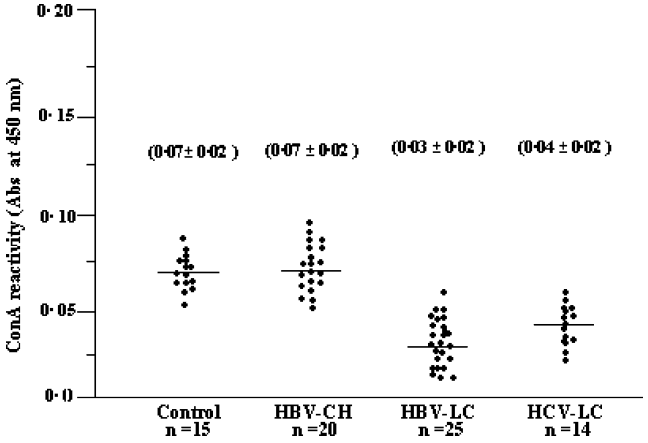

Fig. 3 Reactivity of ConA with AFP in different groups of patients. Data is presented as mean±SD ($p<0.005$)

to innermost GlcNAc through α1-6linkage. Fucosylation level of AFP in both HBV-LC and HCV-LC was compared using LCA; it showed HCV-LC (0.256±0.18) bound with the lectin more intensely compared to HBV-LC (0.175± 0.12) ($p<0.005$) (Fig. 5b).

Discussion

Infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) is the major etiology of hepatocellular carcinoma [43–46]. Both HBV and HCV cause acute and chronic infections in liver and most chronically infected individuals remain asymptomatic for many years [47]. About 40% of all chronic HBV carriers eventually develop liver cancer, and it is estimated that over 1 million people worldwide die because of HBV/HCV-associated liver

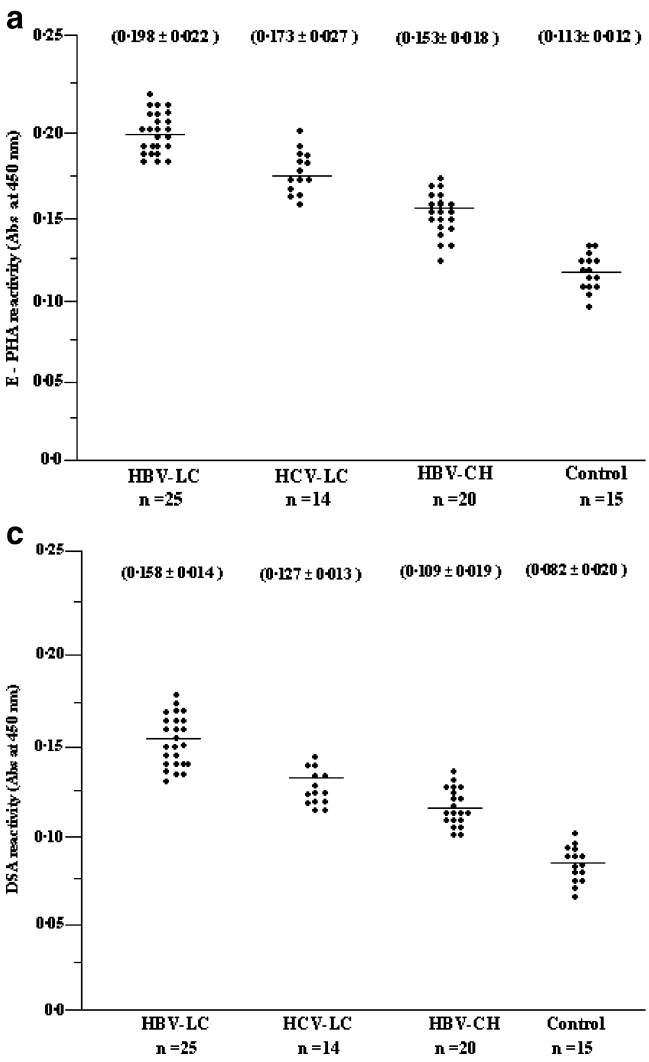

Fig. 4 **a** Reactivity of E-PHA with AFP in different groups of patients. Data is presented as mean±SD ($p<0.007$). **b** Reactivity of L-PHA with AFP in different groups of patients. Data is presented as mean±SD ($p<0.014$). **c** Reactivity of DSA with AFP in different groups of patients. Data is presented asmean±SD ($p<0.01$).

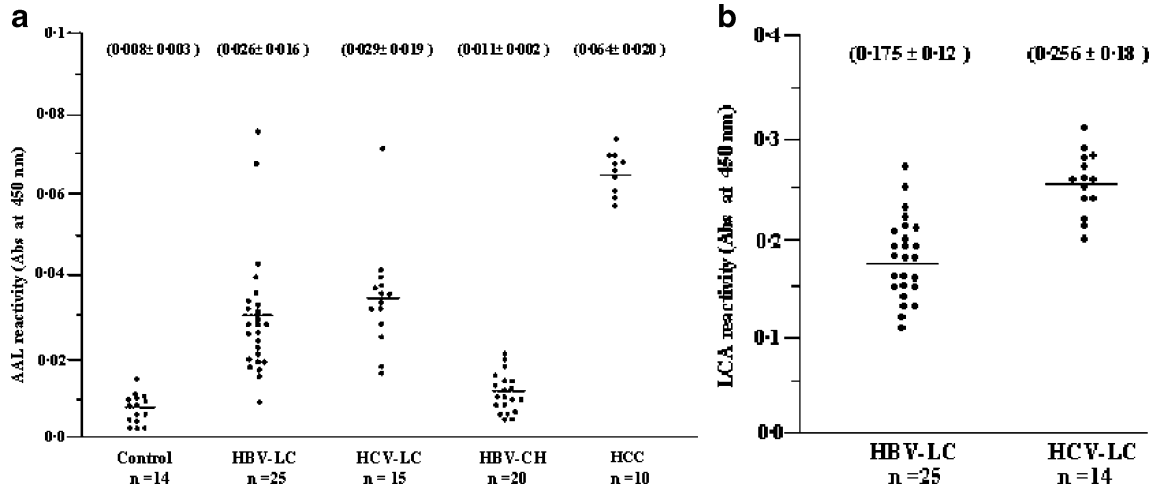

Fig. 5 **a** Fucose concentration of AFP in different groups of liver disease patients evaluated by ELISA using *Aleuria aurantia* lectin. Data is presented as mean±SD ($p<0.001$). **b**. Fucose concentration of AFP in HBV-LC and HCV-LC patients evaluated by ELISA using *Lens culinaris* agglutinin. Data is presented as mean±SD ($p<0.005$)

cancer [48, 49]. Indeed, HBV and HCV infections are associated with >80% of all HCC cases worldwide and can be as high as 96% in regions where HBV is endemic. The progression of liver disease into liver cancer is primarily monitored by serum levels of the oncofetal glycoprotein, α-fetoprotein (AFP), or the core fucosylated glycoform of AFP (AFP-L3). Liver diseases such as hepatitis, liver cirrhosis, hepatocellular carcinoma are the ones that have the largest number of patients. When an acute hepatitis transformed into chronic one followed by liver cirrhosis and a tumor of the liver, or from hepatitis into hepatocellular carcinoma, it is necessary to establish an effective diagnostic system and prognosis of the disease progression. It is also necessary to make an early diagnosis for treating these diseases effectively and preventing the disease from developing into fatal liver cirrhosis or hepatocellular carcinoma. Therefore, an accurate diagnostic marker can be searched for a hepatic disease, which can reflect the prognosis of the disease of the patient. When disease is present, glycan structure changes and these changes in serum glycoprotein provide a record of the pathological processes that are occurring. With the future development of efficient diagnostic methods to analyze glycan structure there are possibilities for developing new methods for the diagnosis, prognosis and the monitoring of the disease.

The current study suggests that the level of AFP in different patient groups have significantly increased in comparison to healthy individuals. Distribution of sialic acid linkages (α2-3-/α2-6-) was considered an important factor in differential diagnosis of liver diseases. Unlike hepatitis C virus induced cirrhosis (HCV-LC) and chronic hepatitis B (HBV-CH) a small but non-significant change in sialic acid linkage in AFP was noted in HBV-LC patients. There was a high percentage of α2-3 linkage containing sialic acid present in HCV-LC and α2-6 sialic acid linkage in HBV-CH. In addition, change in the glycan branching of AFP was observed in most patients by using ConA lectin. In both benign liver disease and primary hepatic cancer most of the serum AFP binds to Con A. Like α-2-HS-glycoprotein [50], transferrin (Tf) [51] and α1-antichymotrypsin (ACT) [52] in liver disease, our present study also showed increased branching. There was significant decrease in ConA binding (*i.e.*, higher content of tri- and tetraantennary chains) in HBV-LC patients in respect to other patients. However, almost no change was observed in HBV-CH. DSA confirmed the presence of tri- and tetraantennary complex type glycans in all groups of patients; of them highest branching was observed in HBV-LC. Triantennary complex-type glycans with 2,6 or 2,4 branched was differentiated by E-PHA, which suggested the presence of 2,4 branched complex type glycan structure in AFP as well as triantennary with bisecting GlcNAc, whereas very little amount of 2,6 branched complex type glycan structure in AFP was found to be present in HBV-LC as determined by L-PHA. Fucosylation of AFP is also considered to be a most important biomarker in accurate diagnosis of liver diseases. There are number of reports of changes in the fucosylation of other serum glycoproteins in liver diseases namely Tf, AGP [10] and haptoglobin. In our study high amount of fucose content was found in all patients' groups; of them HCC showed highest and HBV-CH lowest amount of fucose. Enhancement of fucose attached to innermost GlcNAc was proved to be α1-6 linked.

From the foregoing results of the above study the following conclusions are derived. Chronic hepatitis B (HBV-CH) can be diagnosed from those of other hepatitis by preferential binding of AFP with SNA. Hepatitis B

cirrhosis (HBV-LC) can be diagnosed from those of other hepatitis by considering high glycan branching. The prevalence of α2-3-linked sialic acid in AFP can be an index for diagnosis of HCV-LC. Addition of the above results to the conventional liver function tests may indeed enhance the diagnostic accuracy of liver diseases.

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