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Serum levels of glycosaminoglycans (GAGs) and insulin like growth factor-1 (IGF-1) as diagnostic markers for early hepatocellular carcinoma in cirrhotic patients with or without diabetes

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The significance of serum levels of glycosaminoglycans (GAGs) and insulin like growth factor-1 (IGF-1) in early screening of hepatocellular carcinoma in cirrhotic patients was evaluated. The effect of diabetes on GAGs and IGF-1 levels was also estimated. Fifty cirrhotic patients with early stage Hepatocellular carcinoma (HCC) (22 were diabetic), thirty control cirrhotic patients without HCC (11 were diabetic) and twenty normal control subjects, were enrolled to the study. Serum α-fetoprotein (AFP), the commonly used marker for HCC, was measured in all HCC patients. Serum GAGs increased significantly, while IGF-1 was reduced in patients with cirrhosis and early stage HCC compared to normal control ($P < 0.001$). There was a significant reduction in GAGs and IGF-1 levels in control cirrhotic group compared to HCC group ($P < 0.05$). HCC patients who had normal AFP showed significantly increased GAGs and reduced IGF-1 levels compared to normal control. In comparison with corresponding non-diabetic patients, diabetic patients showed a significant increase in serum GAGs in both cirrhotic control and HCC ($P < 0.01$), while a significant decrease was observed in serum IGF-1 only in HCC ($P < 0.05$). Concomitant determination and monitoring of serum GAGs and IGF-1 could be used as a simple, low cost and non-invasive marker for HCC in cirrhotic patients.

Key words: Hepatocelluar carcinoma, liver cirrhosis, alpha fetoprotein, glycosaminoglycans, insulin like growth factor-1.

INTRODUCTION

Hepatocellular carcinoma (HCC) is currently the fifth most common cancer worldwide and is the third leading cause of cancer-related mortality (Thomas et al., 2010). Liver transplantation has been reported as a promising treatment for patients with small tumors, while it lacks effectiveness when patients with HCC become symptomatic (Kakodkar and Soin, 2012; Yuen et al., 2001). There-fore, early diagnosis is a critical point in management of patients with HCC.

Cirrhosis is the most important risk factor in the development of HCC (Fattovich et al., 2004); therefore, it is recommended that patients with cirrhosis undergo surveillance. Current guidelines recommended abdominal ultrasound (US) as the main surveillance test in patients with cirrhosis (Bruix and Sherman, 2005) however, the level of recommendation was low. AFP is the most utilized surveillance biomarker for HCC worldwide.

Recent systematic reviews of the literature show that the quality of evidence supporting the use of AFP as a

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diagnostic and screening test for hepatitis C virus (HCV)-related HCC is limited (Colli et al., 2006). Another systematic review indicated that studies evaluating AFP as surveillance test suffered from variable study design, patient characteristics, sample size and verification bias (Gupta et al., 2003). Although, elevated serum AFP value in HCC patients was a common finding; however still, others had low or normal values (Tangkijvanich et al., 2000). In addition, both false positive and false negative results are obtained when AFP is used as a simple serum marker for HCC; therefore, better studies are needed to determine AFP's performance, particularly in early stages.

GAGs are unbranched heteropolysaccharides which, with the exception of hyaluronic acid, are covalently attached to a core protein, forming what is known 'proteoglycans'. There are at least seven GAGs: hyaluronic acid, chondroitin sulfate, keratan sulfates I and II, heparin, heparan sulfate and dermatan sulfate (Hardingham and Fosang, 1992). GAGs are produced by fibroblasts, and are quickly cleared almost exclusively in the liver by a very efficient and specific receptor mechanism in SEC (Testa et al., 2006; Parés et al., 1996; Nanji et al., 1996). Since GAGs are important component of extracellular cell matrix, therefore SEC dysfunction may result in increased deposition of extracellular matrix, which leads to liver fibrosis and cirrhosis (Parés et al., 1996).

IGF-1 is an active somatomedin that is produced mainly by the liver. It is a basic polypeptide chain with three disulfide bridges and approximately 50% amino acids homology to insulin (Rinderknecht and Humbel, 1976). IGF-1 is the mediator of the anabolic and mitogenic activity of GH and possesses several roles in initiating puberty, cell proliferation and differentiation, and stimulating erythropoiesis (Froesch et al., 1996). Acute effects of IGF-1 are the same as those elicited by insulin (Froesch et al., 1996). Abnormalities in growth factors secretion are possibly involved in the promotion and/or progression of tumor growth. IGF-1 has been shown to be a mitogen for cancer cell lines by acting as an autocrine factor. Deregulation of IGF system has been implicated in the pathogenesis of several malignancies. In many epidemiologic studies, high serum IGF-1 levels have been associated with an elevated risk of prostate carcinoma (Mucci et al., 2010), breast carcinoma (Baglietto et al., 2007) and colorectal carcinoma (Ma et al., 2001). Although experimental studies have demonstrated an important role of IGF-1 in hepatocarcinogenesis, the clinical data regarding IGF-1 in patients with HCC are scarce and controversial.

Diabetes is associated with a 2 to 3-fold increase in the risk of HCC, regardless of the presence of other major HCC risk factors (Davila et al., 2005). Diabetic patients are at risk for HCC, probably as a result of the hepatic injury, fibrosis and eventual cirrhosis resulting from fatty liver disease associated with diabetes (Yu and Yuan, 2004). Moreover, type 2 diabetes is characterized by insulin resistance and compensatory hyperinsulinemia that is associated with an elevated proportion of proinsulin and split products of proinsulin molecules with some homology to IGF-1 (Davies et al., 1993). Insulin or its precursors have been shown to interact with liver cells by IGF receptor I or hybrid receptors to stimulate mitogensis and carcinogensis (Kahn and White, 1995).

The objectives of this study were to investigate: (1) whether serum GAGs and IGF-1 measurements are significant diagnostic markers for early stage HCC in cirrhotic patients particularly those with normal AFP levels and (2) whether diabetes affects the levels of these two markers in cirrhotic HCC patients.

MATERIALS AND METHODS

Patients

Fifty newly diagnosed cirrhotic patients with HCC from Oncology Center, Mansoura University, Egypt were enrolled into this study. Among these, 22 patients were diabetic. Since HCC patients in this study were also cirrhotic, a control group of thirty cirrhotic subjects without HCC (11 diabetic and 19 non-diabetics) was selected from the out- and in-patient clinics of the Specialized Internal Medicine Hospital, Mansoura University, Egypt and used to nullify the effect of cirrhosis on GAGs and IGF-1 level. The normal control group comprised twenty apparently healthy subjects. Informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Hospital Ethics Committee.

HCC was defined by histological examination or by the appropriate imaging characteristics as defined by accepted guidelines (Bruix et al., 2001). Staging was determined by the Barcelona Clinic Liver Cancer staging system (Bruix and Sherman, 2005). Early stage HCC was defined by a single lesion between 2 to 5 cm or ≤ 3 lesions each < 3 cm, without portal vein thrombosis or extrahepatic metastasis. Patients who had prior treatment of their tumor or history of other solid tumors were excluded. The presence of cirrhosis was defined histologically or non-histologically by evidence of portal hypertension in the presence of chronic liver disease. Evidence of portal hypertension included: (1) a cirrhotic-appearing liver on ultrasound or computed tomography examinations with splenomegaly and no vascular thrombosis, (2) thrombocytopenia with a platelet count < 120 mm^{-3}, and/or (3) presence of esophagogastric varices on endoscopic examination. All investigations necessary for diagnosis were carried out in the Oncology Center and Specialized Internal Medicine Hospital, Mansoura University, Egypt.

Blood sample collection and processing

Blood samples were collected from patients and control subjects and then divided into two portions: One portion was collected into sodium citrate (in a ratio of 0.9 ml of blood to 0.1 ml of 3.8% sodium citrate solution), and used for determination of prothrombin time and

concentration within 3 h. The second portion was allowed to co-agulate and centrifuged to obtain sera for immediate determination of routine liver function tests and glucose levels. The rest of serum was divided into aliquots and kept deeply frozen at -20°C for investigations of AFP, GAGs and IGF-1.

Assessment of liver function

Serum levels of ALT, AST, albumin (A), total protein and total bilirubin were estimated using commercially available kits obtained from Randox Company. Serum globulin (G) is the difference between total protein and albumin; consequently A/G ratio is determined. Prothrombin time and subsequent prothrombin concentration were determined on citrated blood using DiaPlastin reagent purchased from DiaMed Company.

Measurement of fasting blood glucose

Fasting blood glucose was estimated according to glucose oxidation method using kits from Randox Company.

Determination of serum AFP

AFP was determined according to the method of Cattini et al. (1993) using DSL-10-8400 ACTIVE AFP ELISA assay kits (an enzymatically amplified "two-step" sandwich-type immunoassay). The assay was performed according to the manufacturers' instructions.

Determination of serum total GAGs

Isolation of total GAGs from serum samples

All serum samples were analyzed for GAGs after their precipitation from serum according to Ohkawa et al. (1977). Briefly, 1 ml of each sample was dehydrated by adding 10 ml chilled acetone. The mixture was stirred for 30 min at room temperature and centrifuged at 1,500 rpm for 15 min at 4°C. The dehydrated sample was delipidated by washing twice with 10 ml of ether and the precipitate was air-dried. The defatted dry powder was re-suspended in 1 ml of 0.5 M chilled NaOH and stirred at 4°C overnight. Then, it was neutralised with 1 M HCl to pH 6 to 8, and then an equal volume of 0.1 M Tris-HCl buffer was added and the sample was heated for 30 min in a boiling bath. The sample solution was digested with pronase E in two doses of 1 mg at 24 h intervals, at 50°C. After being acidified with 1 M HCl to pH 5 to 6, GAGs were precipitated by adding 2 ml cetylpyridinium chloride (final concentration 2.5 g/L) and kept at 4°C overnight. The precipitate was collected by centrifugation at 4,000 rpm for 30 min at 4°C and then washed twice with 5 ml of 98% ethanol. The final precipitate was dissolved in 1 ml of 0.05 Tris-HCl buffer.

Determination of total GAGs by carbazole assay

The GAGs uronic acid was quantified using the carbazole-borate reagent according to photometric method of Bitter and Muir (1962). Briefly, 0.5 ml of the isolated GAG sample was added to 3 ml of reagent A (25 mM $Na_2B_4O_7$ in concentrated H_2SO_4) and heated at 100°C for 10 min. After the reaction was cooled in ice, 100 µl of

reagent B (carbazole 1.25 g in 1 L 100% ethanol) was added to the reaction which was heated at 100°C for another 15 min. The absorbance was read by spectrometry at 525 nm and compared with that of reference solutions of glucuronic acid. Serum GAGs were expressed as µg uronic acid per ml of serum (Bitter and Muir, 1962). All chemicals were purchased from Sigma-Aldrich (S. Louis, USA).

Determination of serum IGF-1

IGF-1 was determined by DSL-10-2800 ACTIVE Non-Extraction ELISA assay according to the method of Hall and Sara (1983). The DSL-10-2800 ACTIVE Non-Extraction IGF-1 ELISA is an enzymatically amplified "two-step" sandwich-type immunoassay. The procedure was preceded according to the manufacturers' instructions provided with the kits.

Statistical analysis

Statistical analysis was performed by GraphPad Prism (GraphPad Software San. Diego, CA, USA). Data were presented as means ± standard error of mean (SEM) and analyzed with one way Analysis of variance (ANOVA), followed by Bonferroni testing. Correlation coefficient [r] was used to measure the mutual correspondence of two quantitative variables in the same studied group. $P < 0.05$ was considered statistically significant.

RESULTS

Liver function

Liver function of HCC and cirrhotic groups compared to control subjects are shown in Table 1.

Serum AFP

Serum concentrations of AFP in HCC group and control subjects are shown in Table 2. Serum AFP was not increased in all HCC patients and showed variations among patients. According to the serum concentration of AFP, the patients with HCC in this study were sub-classified into three subgroups: (1) HCC patients with a normal concentration of serum AFP (<15 ng/ml), including 22 patients, (2) patients with a questionable positive (below the cut off) levels of serum AFP (15 to 400 ng/ml), including 18 patients and (3) those with serum AFP concentration above the cut off value (> 400 ng/ml), including 10 patients. Patients in the second and third subgroups showed a significant increase in serum concentration of AFP ($P < 0.001$) compared to the control subjects.

Serum GAGs

Patients in both HCC and cirrhotic groups showed a sig-

Table 1. Liver function in normal control group (n = 20), cirrhotic control (n = 30) and early stage HCC group (n = 50) (mean ± SEM).

Liver Function	Normal control	Cirrhotic control	Early stage HCC
ALT (U/L)	16.8±1.9	59.1[a]***±6.47	61.7[a]***±5.9
AST (U/L)	12.7±1.4	64.0[a]***±5.94	81.0[a]***[b]***±6.7
Total bilirubin (mg/dl)	0.53±0.04	1.2[a]*±0.095	1.7[a]*±0.28
Prothrombin time (s)	12.0±0.21	20.57[a]***±1.2	19.96[a]***±1.3
Prothrombin concentration (%)	96.2±7.6	35.21[a]***±3.9	37.2[a]***±3.2
Serum albumin (gm/dl)	4.717±0.16	3.05[a]**±0.101	2.96[a]***±0.071
Serum total protein (gm/dl)	7.152±0.123	7.27±0.191	7.58±0.179
Serum globulin (G) (gm/dl)	2.45±0.16	3.91[a]*±0.22	4.2[a]*±0.201
A/G ratio	1.93±0.15	0.84[a]***±0.042	0.79[a]***±0.034

a: significantly different from control, b: significantly different from cirrhotic patients, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 2. Serum levels of α-fetoprotein (AFP) in early stage HCC patients compared to normal control group (mean ± SEM).

Group	Serum AFP (ng/ml)
Normal control (n=20)	5.990±0.384
HCC patients with normal levels of AFP (<15 ng/ml) (n=22)	7.47±1.6
HCC patients with questionable positive levels of AFP (15-400 ng/ml) (n=18).	125.839[a]***±24.46
HCC patients with serum AFP levels above the cut off value (>400 ng/ml) (n=10)	4547.8[a]***±789.6

a: significantly different from control, ***$P < 0.001$.
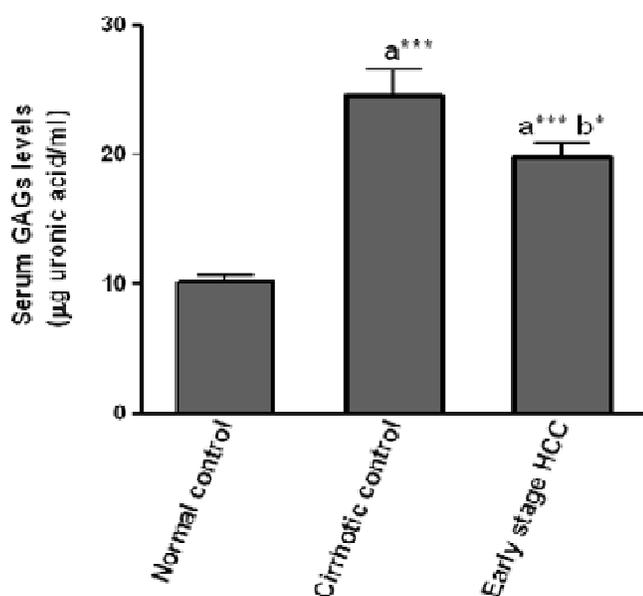
Figure 1. Serum glycosaminoglycans (GAGs) concentration in normal control, cirrhotic control and early stage HCC patients(mean ± SEM). a: significantly different from control, ***$P < 0.001$; b: significantly different from cirrhotic patients, *$P < 0.05$.

nificant increase ($P < 0.001$) in serum GAGs concentration as compared to control subjects (19.8 ± 1.05 and 24.6 ± 1.93 µg uronic acid/ml versus 10.06 ± 0.6 µg uronic acid/ml, respectively), with a significant increase ($P < 0.05$) in cirrhosis group as compared to HCC group (Figure 1). Figure 2 showed serum GAGs in HCC patients with variable levels of AFP. All HCC patients including those who had normal AFP (< 15 ng/ml) showed significantly increased GAGs compared to normal control subjects. To evaluate the effect of diabetes existence on serum levels of GAGs in cirrhosis and early stage HCC, patients were further sub-classified into diabetic and non diabetic patients, and the GAGs levels in diabetic patients were compared to the levels in corresponding non diabetic patients (Figure 3). Diabetic cirrhotic (n = 11) and HCC (n = 22) patients exhibited a significant increase ($P < 0.01$) in serum GAGs in comparison with corresponding non diabetic patients(36.8 ± 4.07 versus 22.03 ± 2.3 µg uronic acid/ml in cirrhosis and 29.36 ± 2.62 versus 18.2 ± 0.95 µg uronic acid/ml in HCC).

There was a significant positive correlation (r = 0.471, $P < 0.001$) between serum GAGs and serum glucose levels in HCC patients (Figure 4). Also, there was a significant
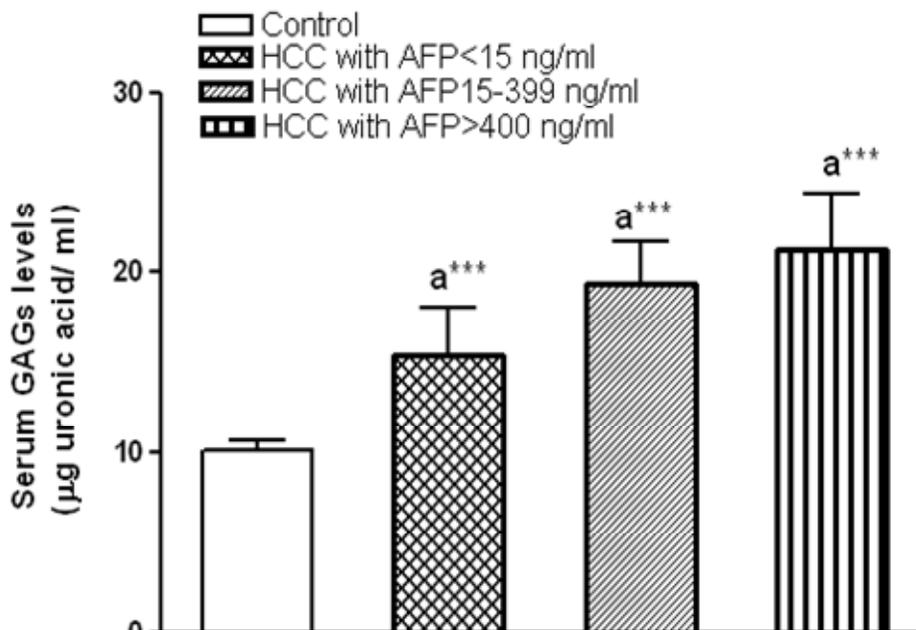
Figure 2. Serum glycosaminoglycans (GAGs) level in early stage HCC patients with AFP < 15 ng/ml, those with AFP (15 to 399 ng/ml) and those with AFP > 400 ng/ml (mean ± SEM). a: significantly different from control, ***$P < 0.001$.
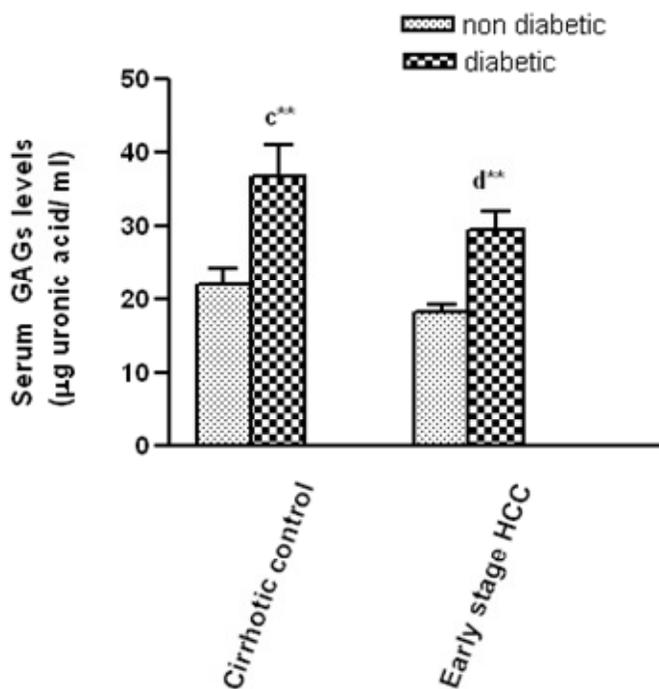
Figure 3. Serum glycosaminoglycans (GAGs) concentration in diabetic and non diabetic patients of cirrhotic control and early stage HCC groups (mean ± SEM). c: significantly different from non diabetic control cirrhotic patients, **$P < 0.01$; d: significantly different from non diabetic HCC patients, **$P < 0.01$.

positive correlation (r = 0.48, $P < 0.01$) between serum GAGs and serum glucose in cirrhotic patients (Figure 5).

Serum IGF-1

In contrast to GAGs, serum IGF-1 concentration showed a significant decrease ($P < 0.001$) in patients with cirrhosis and HCC as compared to control group (35.73 ± 5.6 and 21.87 ± 2.78 ng/ml versus 76.39 ± 12.2 ng/ml, respectively), with a significant reduction ($P < 0.05$) in HCC patients as compared to cirrhotic group (Figure 6). Figure 7 showed serum IGF-1 in HCC patients with variable levels of AFP. All HCC patients including those who had normal AFP (< 15 ng/ml) or below cut off values (15 to 400 ng/ml) showed a significantly decreased IGF-1 as compared to normal control subjects. To estimate the effect of diabetes existence on serum levels of IGF-1 in cirrhosis and early stage HCC, patients were further sub-classified into diabetic and non diabetic patients, and the IGF-1 levels in diabetic patients were compared to the levels in corresponding non diabetic patients. Diabetic patients with HCC (n = 22) exhibited a significant decrease in serum concentration of IGF-1 in comparison with non diabetic patients (11.98 ± 4.42 versus 26.05 ± 1.85 ng/ml, $P < 0.05$), while insignificant difference was observed between diabetic and non diabetic cirrhotic patients (36.56 ± 8.62 versus 35.37 ± 6 ng/ml (Figure 8).
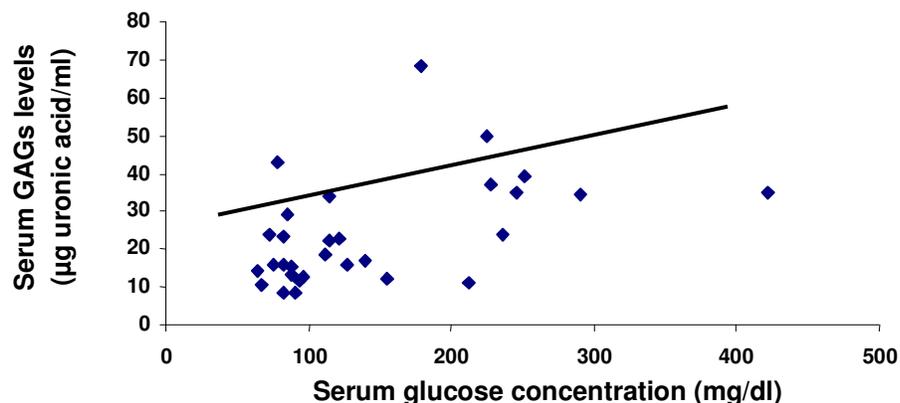
Figure 4. Significant positive correlation between levels of serum glucose (mg/dl) and serum glycosaminoglycans (GAGs) (µg uronic acid/ml) in cirrhotic control patients (r = 0.48, *P*< 0.01).
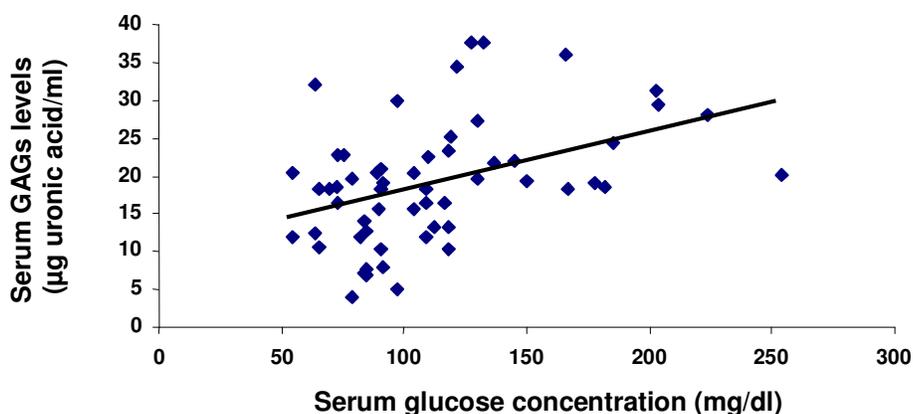
Figure 5: Significant positive correlation between levels of serum glucose (mg/dl) and serum GAGs (µg uronic acid/ml) in early stage HCC patients (r = 0.471, *P* < 0.001).

There was a significant negative correlation between serum levels of IGF-1 and GAGs (r = -0.311, *P* < 0.05) in HCC patients (Figure 9). On the other hand, serum IGF-1 showed a significant positive correlation with serum albumin level in cirrhotic group (r = 0.49, *P* < 0.01) (Figure 10) and in HCC patients (r = 0.45, *P* < 0.001) (Figure 11).

DISCUSSION

HCC is one of the most malignant tumors affecting humans, with increasing incidence worldwide (Thomas et al., 2010). The precocious diagnosis is a critical point in management of patients with HCC, since early detection of the cancer increases the chance of treatment (Yuen et al., 2001). In spite of great use of AFP and its importance as a tumor marker for HCC, there are some limitations of its usefulness as being not sensitive as a screening mark in general population (Colli et al., 2006; Tangkijvanich et al., 2000). Elevated serum AFP value in HCC patients was a common finding; however still, others had low or normal values (Tangkijvanich et al., 2000), particularly in early stage. These findings coincide with our results where not all pathologically proven early stage HCC cases in this study have elevated level of AFP. There was a wide variation in their AFP levels, in which 22 (44%) of patients had normal AFP (< 15 ng/ml), 18 (36%) had moderately elevated levels (15 to 400 ng/ml) and only 10 patients (20%) were with markedly elevated AFP
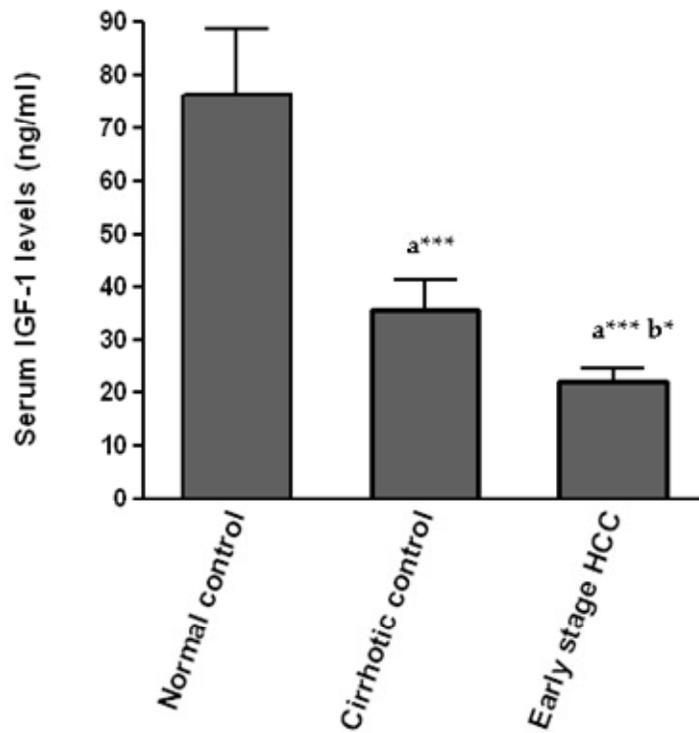
Figure 6. Serum levels of insulin-like growth factor (IGF-1) in normal control, cirrhotic control and early stage HCC patients (mean ± SEM). a: significantly different from control, ***$P < 0.001$; b: significantly different from cirrhotic patients, *$P < 0.05$.
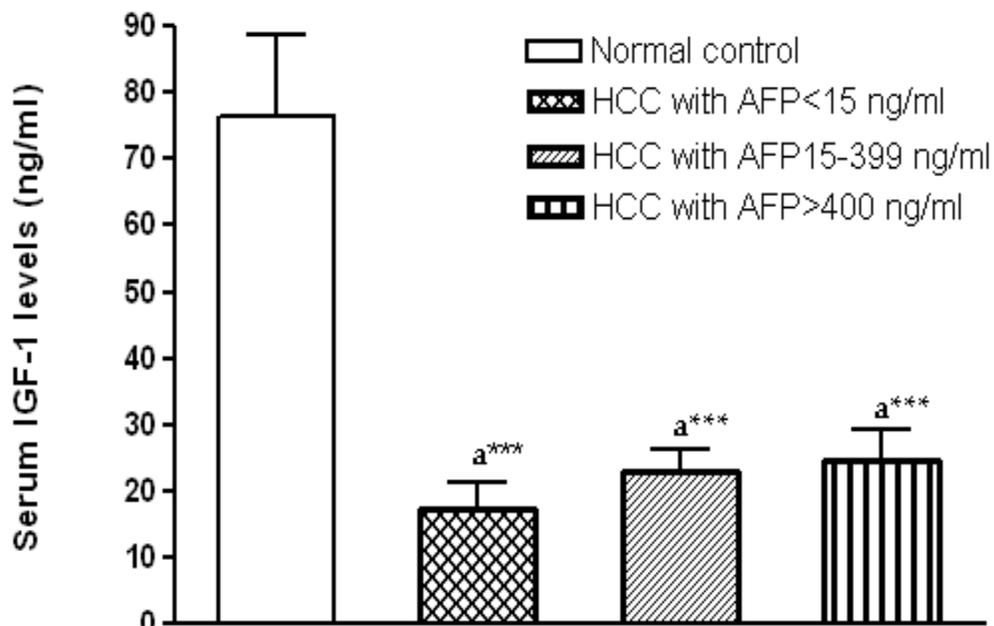
Figure 7. Serum insulin like growth factor-1 (IGF-1) level in early stage HCC patients with AFP < 15 ng/ml, those with AFP (15 to 399 ng/ml) and those with AFP > 400 ng/ml (mean ± SE). a: significantly different from control, ***$P < 0.001$.
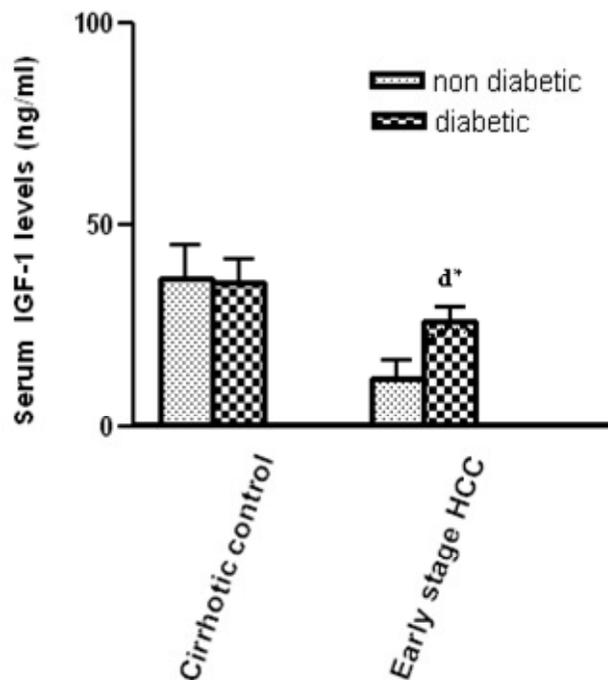
Figure 8. Serum insulin like growth factor-1 (IGF-1) levels in diabetic and non diabetic patients of cirrhotic control and early stage HCC groups (mean ± SEM). d: significantly different from non diabetic HCC patients, *P < 0. 05.
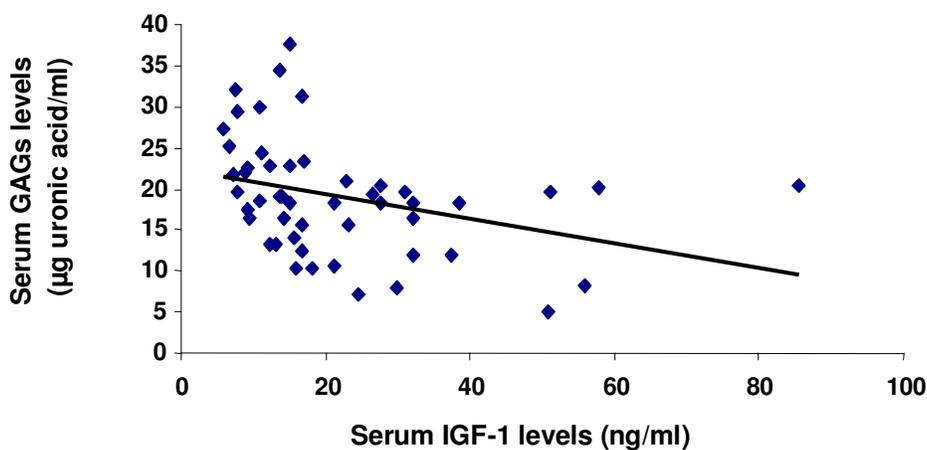
Figure 9. Significant negative correlation between serum levels of IGF-1 and GAGs in early stage HCC patients (r = -0.34, P < 0.05).

(> 400 ng/ml). In addition, both false- positive and false negative results are obtained when AFP is used as a serum marker for HCC (Giannelli and Antonaci, 2006), so

there is a real need for exploration of other marker for early diagnosis of HCC. The present study aimed to estimate whether serum levels of GAGs or IGF-1 could be
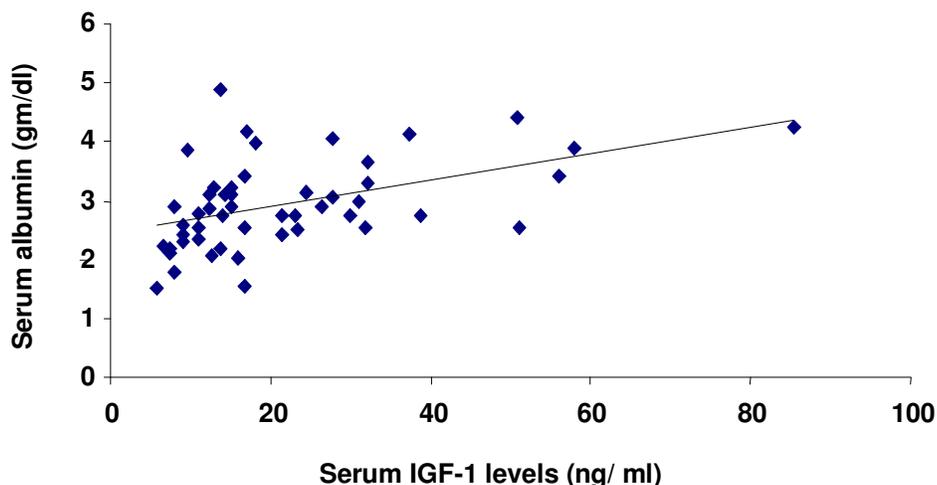
Figure 10. Significant positive correlation between serum IGF-1 and serum albumin concentrations in control cirrhotic patients (r = 0.49, *P* < 0.01).
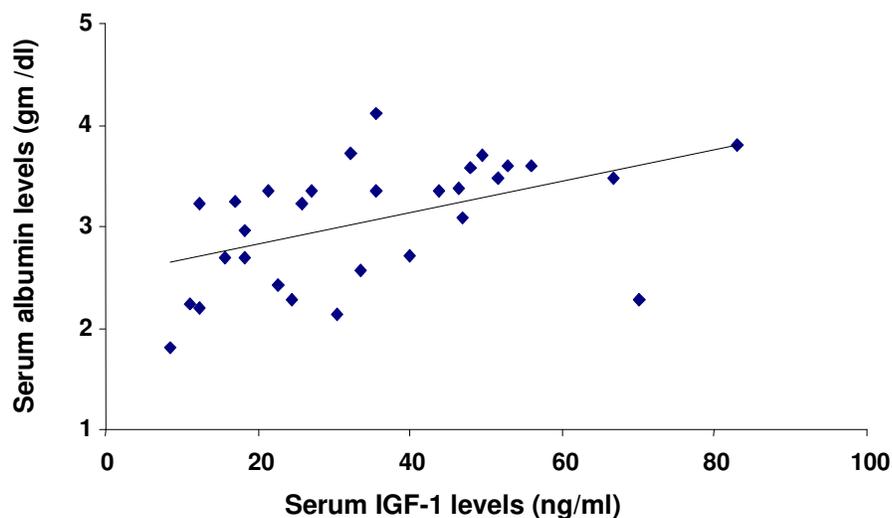
Figure 11. Significant positive correlation between serum IGF-1 and serum albumin concentrations in early stage HCC patients (r = 0.45, *P* < 0.001).

helpful as markers for early diagnosis of HCC.

In the current study, serum GAGs level was increased significantly in both cirrhotic control and HCC groups compared to normal control. Serum GAGs levels increase when production is markedly enhanced. This may be due to synovial inflammation, as seen in some rheumatological conditions, or more importantly, when liver function (SEC receptor removal mechanism) is impaired (Parés et al., 1996). The high levels of hyaluronic acid, a glycosaminoglycan of the liver extracellular matrix, which is synthesized and degraded in the liver sinusoidal cells, have been related with a decreased function of the endothelial sinusoidal cells(Testa et al., 2006; Parés et al., 1996). Recently, high production of GAGs in liver disease was documented (Jia et al., 2012; Lv et al., 2007). The finding of Jia et al. (2012) demonstrated elevated GAGs content in HCC tissues compared to that in the normal liver tissues. Such increase was found to be due to increased expression of chondroitin sulfate and dermatan sulfate. A progressive

increase in the content of chondroitin sulfate, low molecular size GAGs, and non-sulfated and di-sulfated chondroitin sulfate disaccharide units, together with a gradual decrease in heparan sulfate and significant increase in hyaluronic acid have also been found in human primary hepatic carcinoma (Lv et al., 2007).

The elevation of GAGs level may be due to the stimulation of fibroblast by fibroblast-derived growth factor and vascular endothelial growth factor; both are increased in cirrhotic and HCC patients (Chow et al., 1997; Li et al., 2003). The level of GAGs expression is regulated by a number of cytokines, among those that play significant roles are; TGF beta, TNF alpha and TGF alpha (Gressner et al., 1994). Liver cirrhosis resulted in a significant increase of plasma concentration of TNF alpha and TGF-beta (Flisiak et al., 2000). Fat-storing cells (perisinusoidal lipocytes, Ito cells) are the major connective tissue-producing cell type in liver (Gressner, 1991a). Transformed fat storing cells that is, myofibroblast-like cells are the major source of proteoglycans in injured liver (Gressner, 1991b). In areas of necro-inflammation, the cells proliferate and transform into desmin and smooth muscle alpha-actin-positive myofibroblast-like cells which synthesize a broad spectrum of significant amounts of proteoglycans, collagen and matrix glycoproteins (Gressner, 1991a). It is concluded that modified Ito (fat storing) cells will synthesize proteoglycans and play an important role in the formation of connective tissue fibers in liver fibrosis (Szende et al., 1992).

The serum level of GAGs estimated in early stage HCC patients was significantly decreased when compared with corresponding values in cirrhotic control group. This finding may result from decreased IGF-1 level in HCC. IGF-1 has a sulfation activity (incorporation of sulfate and leucine into glycosaminglycans) and stimulation of fibroblast multiplication (Corti et al., 1992). Our result proposed that the development of carcinoma in cirrhotic patients may be accompanied by a gradual significant decrease in GAGs levels as compared to before carcinogenesis. We suggest that monitoring GAGs levels in cirrhotic patients may help in predicting development of HCC. Further studies are necessary to determine a cut-off value that is diagnostic for HCC. Serum level of GAGs may increase in other diseases including rheumatoid arthritis (Friman et al., 1977), systemic lupus erythmatosus (Friman et al., 1987), chronic myeloid leukemia (Craig et al., 1988), and chronic lymphocytic leukemia, and in essential thrombocythaemia (Calabrò et al., 1998), limiting its specificity to liver diseases. Therefore, the combined measurement of serum level of GAGs and IGF-1 level may provide a more specific marker for liver diseases.

Diabetic patients with either cirrhosis or HCC in this study exhibited a significant increase in serum GAGs level when compared to the corresponding non diabetic patients. Significant positive correlations were found between serum GAGs concentration and glucose level in both cirrhotic and HCC groups (Figures 4 and 5), respectively indicating that the elevated levels of serum GAGs in diabetic patients are induced by hyperglycemia. In agreement with our results, the percentage of total GAGs in high glucose-treated medium was significantly increased compared to glucose-free medium (Han et al., 2009). One explanation of increased GAGs is presented by Takeda et al. (2001) who reported that, high glucose stimulates GAGs production through activation of protein kinase C and TGF-beta cascade. Hyperglycemia may also lead to the release of GAGs from cell proteoglycan core proteins because cultured endothelial cells treated with high glucose showed a reduction in total GAGs, while medium GAGs was increased (Han et al., 2009). We can conclude that alteration of GAGs synthesized by cells is an important pathological mechanism, which can be correlated with cell injury by hyperglycemia.

IGF-1 levels reduced significantly in both control cirrhotic and early stage HCC groups compared to normal control (Figure 6). The result concerning HCC was opposite to those in other malignancies as prostate carcinoma (Mucci et al., 2010), breast carcinoma (Baglietto et al., 2007) and colorectal carcinoma (Ma et al., 2001), reflecting the possible specificity of IGF-1 for HCC. Our results are in agreement with other reports demonstrating low IGF-1 levels in patients with cirrhosis (Conchillo et al., 2005; Donaghy et al., 2002; Guo et al., 2001; Wu et al., 2004) and HCC (Mazziotti et al., 2002; Ranke et al., 2003; Stuver et al., 2000). An important finding of the current study is that HCC patients who had normal AFP (< 15 ng/ml) showed a significantly decreased IGF-1, as compared to normal control subjects (17.4 versus 76.3 ng/ml) (Figure 7). This study suggests that the measurement of serum IGF-1 may be an important early marker for the diagnosis of early stage HCC, particularly in patients with low level of serum AFP. Reduced IGF-1 in cirrhosis and HCC could be explained by increased oxidative damage in cirrhosis and HCC, leading to increased damage of parenchymal liver cell and decrease in IGF-1 synthesis (Cantürk et al., 2003; García-Fernández et al., 2005). Mattera et al. (2003) proposed that IGF-1 was low in HCC patients because of reduced ability of GH to stimulate IGF-1 synthesis due to either a reduction of GH receptors number in the diseased liver or a post receptor defect (Donaghy et al., 2002). Low circulating IGF-1 levels in HCC may be derived also from an inhibitory effect by some tumor cytokines, like TGF beta and platelet-derived growth factor (Clemmons, 2001). Indeed, these cytokines are over-expressed in patients with HCC in relation to the degree of fibrogenic activity (Pinzani et al., 1996; Song et al., 2002).

The fibrogenic activity was observed in this study by measuring serum level of GAGs, which are the major component of extracellular matrix and was found to be increased significantly when compared to normal control subjects. In this view, the modifications of serum IGF-1 level may reflect indirectly the progression of liver fibrosis in relation to the development of HCC (Mazziotti et al., 2002). This explanation is supported by the significant negative correlation observed in our study between IGF-1 and GAGs levels in HCC patients (Figure 9). In addition, patients with cirrhosis are characterized by a variety of metabolic disturbances, including nutritional and metabolic complications such as insulin resistance, malnutrition, osteopenia and hypogonadism, all related to IGF-I deficiency (Bonefeld and Møller , 2011). It is most likely that malnutrition contributes to the reduced IGF-1 in liver disease.

IGF-1 level decreased significantly in early stage HCC group when compared to control cirrhotic group. This observation agreed with Mazziotti et al. (2002), suggesting that IGF-1 levels in these patients decreased independently from cirrhosis, and indicating the involvement of physiopathological mechanisms in addition to the parenchymal damage. This result is due to increased parenchymal damage in HCC or inhibitory effects of tumor-induced cytokines (Mazziotti et al., 2002). In addition, patients with HCC have elevated plasma nitric oxide levels compared with patients with cirrhosis, which leads to more oxidative damage for liver parenchymal cells in HCC group and further decrease in IGF-1 level (Moussa et al., 2000). Our results suggest that periodic measurement of serum IGF-1 level using Enzyme-linked immunosorbent assay (ELISA) method may be useful for HCC screening and predicting the development of HCC in patients with cirrhosis. A significant positive correlation was found between levels of IGF-1 and albumin in both cirrhosis and HCC groups (Figures 10 and 11). This finding may indicate that the decrease in IGF-1 level in cirrhotic and HCC patients may play a role in the reduction of albumin synthesis in these groups. The finding that IGF-1 replacement therapy increases albumin concentration in liver cirrhosis could confirm our suggestion (Conchillo et al., 2005). As IGF-1 is an anabolic hormone synthesized in the liver and its level decreased sharply in liver cirrhosis and HCC, the synthetic capacity of liver can be monitored by following IGF-1 level.

Our data demonstrated that diabetic HCC patients showed a significantly decreased level in serum IGF-1 level when compared to non diabetic HCC subgroup, while no significant difference was detected in diabetic cirrhotic patients as compared to non diabetic ones. This indicates that diabetes may be an important factor that decreases IGF-1 level in HCC. Liver tissue IGF-1 gene expression is greatly affected in diabetes, contributing to

reduction of serum IGF-1 level (Li et al., 2004). In addition, diabetic HCC patients have more insulin resistance and hyperinsulinaemia. Insulin is known to be an important hepatotrophic factor that stimulates the proliferation of hepatoma cells (Sasaki et al., 1993). Increased insulin level stimulates the propagation of tumor through IGF-1 receptors via phosphorylation of insulin receptor substrate 1 and activation of mitogen activated protein kinases (Rose et al., 1994). So, hyperinsulinaemia is associated with accelerated HCC growth and gives further destruction of liver cells and further reduction in IGF-1 level. Therefore, diabetic HCC patients are at high risk of propagation of tumor than non-diabetic ones.

Conclusion

GAGs and IGF-1 serum levels are disturbed in liver cirrhosis and represent a good marker of hepatic function. The development of HCC is accompanied by a significant increase in GAGs together with a significant reduction in serum IGF-1 level. Therefore, concomitant determination and monitoring of serum GAGs and IGF-1 could be used as a simple, low cost and non-invasive marker for development of HCC in cirrhotic patients, especially in patients with normal level of AFP.

Hyperglycemia in diabetic cirrhotic and HCC patients is associated with higher levels of serum GAGs compared to non diabetic patients. Such alteration in GAGs could be a pathological mechanism of liver injury. Diabetic HCC, but not cirrhotic patients had further reduction of serum IGF-1 levels as compared to non diabetics, and such reduction may be associated with HCC growth in diabetes.

ABBREVIATIONS

A/G, Albumin/globulin; **AFP,** alpha fetoprotein; **ALT,** alanine aminotransferase; **AST,** aspartate aminotransferase; **GAGs,** glycosaminoglycans; **GH,** growth hormone; **HCC,** hepatocellular carcinoma; **IGF-1,** insulin like growth factor-1; **SEC,** sinusoidal endothelial cells; **TGF-beta,** transforming growth factor beta; **TNF alpha,** tumor necrosis factor alpha.

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