Assessment of Connective Tissue Growth Factor and Hyalunoric Acid in the Diagnosis of Hepatic Fibrosis in Chronic Hepatitis B

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Chronic Hepatitis B virus is responsible for 76.3% cases of chronic hepatitis and 61.15% cases of cirrhosis in Bangladesh. Hepatic fibrosis is primary complication of chronic hepatitis B (CHB). Connective tissue growth factor (CTGF) and serum Hyaluronic acid (HA) both are involved in hepatic fibrosis. The present study was an attempt to compare between serum CTGF and HA in the diagnosis of hepatic fibrosis in CHB. This cross sectional study was done in Department of Laboratory medicine in collaboration with Hepatology and Pathology, BSMMU, Dhaka during the period of From July 2015 to June 2016.Forty patients who fulfilled the inclusion criteria of CHB were conveniently included in this study. Serum CTGF and Serum HA were measured by using of a sandwich immunoassay technique. Liver biopsy material was stained with haematoxyline & eosin and Masson's trichrome stains. The sensitivity, specificity, positive predictive value and negative predictive value, accuracy CTGF was 77.8%, 69.2%, 84.0%, 60.0%, 75% and serum HA in the diagnosis of hepatic fibrosis was 70.4%, 69.2%, 82.6%, 52.9% and 70% respectively. Serum CTGF can be used as more reliable diagnostic tool than HA for the diagnosis of hepatic fibrosis in patients with CHB. Further large scale study can be instituted to get more precise result.

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Key words: Connective tissue growth factor, hyaluronic acid, Hepatic fibrosis, Chronic Hepatitis B

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Introduction

Hepatitis B virus (HBV) infection is a serious global health problem. About 400 million people throughout the world are chronically infected with hepatitis B virus infection.[1] Chronic Hepatitis B (CHB) may be defined as chronic necroinflammatory disease of the liver caused by persistent infection with hepatitis B virus. Approximately15%-40% of chronic hepatitis B (CHB) patients will develop cirrhosis, liver failure, hepatocellular carcinoma.[2] In Bangladesh prevalence of HBV infection in healthy population is 5.4%.[3] HBV is responsible for 76.3% of cases of chronic hepatitis and 61.15% of cases of cirrhosis in Bangladesh. It poses huge burden on the health of our patients.[4]

Liver fibrosis is primary complication of chronic hepatitis which is characterized by the loss of hepatocytes, destruction of hepatic micro architecture, proliferation of hepatic fibroblasts and excessive deposition of extracellular matrix component.[5] Transforming growth factor beta (TGFβ1) is a major cytokine associated with activation of hepatic stellate cell and extracellular matrix deposition. CTGF as a downstream effector of TGFβ1-induced extracellular matrix production and fibroblast proliferation.[6]

The extra cellular matrix comprises collageneous and non collageneous proteins and hyalunoric acid. Both synthesis and deposition of these components increases during fibrogenesis. A fraction of these components is released into the systemic circulation leading to an increase in their serum concentration and these have been investigated as potential markers of the fibrotic process. The level of serum hyaluronic acid is a sensitive marker of hepatic fibrosis, reflects the severity of liver damage in patients with chronic hepatitis or cirrohosis.[7]

Liver biopsy is the gold standard to evaluate the histological stages of hepatic fibrosis and an integral part of management of chronic hepatitis B; but the procedure is invasive, blind and costly. Histological evaluation is dependent on experienced histopathologist[8]. Additionally, fibrosis is not equally distributed in the liver of some patients with liver disease. Fibrosis is missed on a single liver biopsy in 10%-30% of cases[9]. Recently several clinical studies have been attempted to identify serum markers like connective tissue growth factor (CTGF), Transforming growth factor beta (TGFβ1), hyalunoric acid, laminine, procollagen III, collagen IV, matrix metalloproteinases, and imaging technique like fibroscan that correlate with the degree of fibrosis. These non invasive markers could be used in conjunction with liver biopsy.[10] Among these non invasive markers, CTGF shows more diagnostic sensitivity and specificity.[6, 11]

Fibrogenesis is difficult to heal when it reaches the middle or later stages. Progression of fibrosis can be delayed by specific antiviral therapy. Therefore a simple, sensitive and non invasive method is needed to assess the prognosis and stages of hepatic fibrosis. It reduces the need for repeated liver biopsies. Therefore, in this study measurement of serum CTGF and serum HA was done as a noninvasive marker in the diagnosis of hepatic fibrosis in chronic hepatitis B.

Method

This cross-sectional study was conducted at the Department of Laboratory medicine in collaboration with Department of Hepatology and Department of Pathology, BSMMU,

Dhaka from July 2015 to June 2016. Forty patients who fulfilled the inclusion criteria of CHB attended in the Department of Hepatology, BSMMU, were conveniently included in this study. Patients having any condition like decompensated cirrhosis of liver, co-infected with hepatitis C virus infection, antiviral therapy, nonalcoholic fatty liver disease and hepatocellular carcinoma were excluded from the study. After taking informed consent, a careful history and the details information were recorded by the investigator in a preformed data sheet. With all aseptic precaution, 3 ml venous blood was taken before liver biopsy, allow to clot and separate serum by centrifugation at room temperature. The serum was stored at -20ºc until analysis. Serum CTGF and HA were measured in the Department of Laboratory Medicine by using Enzyme Linked Immuno Sorbent Assay (ELISA) based on sandwich principle. Cut-off value of serum CTGF is ≤ 56.6 ng/mL (DRG CTGF ELISA EIA-5195, 2011). Cut-off value of serum hyalunoric acid is ≤75ng/mL (DRG CTGF ELISA EIA-5195, 2009)

The kits were capable of detecting full length CTGF and HA. The value > cut off value is positive and the value < cut off value is negative. Needle liver biopsy was done in the Department of Hepatology by Hepatologist through right 8th or 9th intercostals space with 14Fr, 15cm Tru-cut biopsy needle. Biopsy material was fixed in 10% formalin. The specimen was sent to the Department of Pathology, BSMMU for complete histopathological examination. Haematoxyline & Eosin and Masson's trichrome stains were done to see the different stages of hepatic fibrosis by Metavir scoring system. Stages of fibrosis: The fibrosis score is also assigned a number from 0-4.

0= no fibrosis, F_1 =portal fibrosis without septa, F_2=portal fibrosis with a few septa F_3=numerous septa without cirrhosis, F_4= cirrhosis

Results

This cross-sectional study was carried out in the Department of Laboratory medicine, BSMMU, Dhaka. The cases were included in the study from the Department of Hepatology, in BSMMU. We investigated 40 chronic hepatitis B (CHB) patients without having any condition like decompensated cirrhosis of liver, co-infected with hepatitis C virus infection, antiviral drug therapy, nonalcoholic fatty liver disease and hepatocellular carcinoma. Histopathology was gold standard to identify the stages of hepatic fibrosis. According to fibrosis the patients were grouped into four, F_0=13, F_1 =6, F_2=17, F_3=4 patients were found in each group.

Table I: Distribution of the study population by age among hepatic fibrosis group (n=40)

Age (in year)	Stages of Fibrosis								p value*
	F0		F1		F2		F3		
	No.	%	No.	%	No.	%	No.	%	
≤20	0	.0	0	.0	3	17.6	2	50	
20-29	7	53.8	4	66.7	8	47.1	0	0	
30-39	4	30.8	2	33.3	6	35.3	1	25	
≥40	2	15.4	0	.0	0	0	1	25	
Mean ± SD	29.92 ± 6.17		29.00 ± 7.35		27.53 ± 5.79		30.50 ± 14.48		0.787^{ns}

*ANOVA test was done to measure the level of significance.
ns = not significant

A total of 40 patients with chronic hepatitis B were included in this study. Maximum patient's age were belonged to 20-29 years in four groups. The mean age was found 29.92 ± 6.17 years with range from 19 to 52 years in F0 group, 29.00 ± 7.35 years with range from 18 to 52 years in F1 group, 27.53 ± 5.79 years with range from 19 to 50 years in F2 group and 30.50 ± 14.48 years with range from 18 to 50 years in F3 group. The mean age difference was not statistically significant (P>0.05) in four groups. Table II: Distribution of the study population by sex among hepatic fibrosis group (n=40)

Table II: stages of fibrosis

Sex	Stages of Fibrosis								p value*
	F0		F1		F2		F3		
	No.	%	No.	%	No.	%	No.	%	
Male	9	69.2	5	83.3	15	88.2	3	75.0	0.622^{ns}
Female	4	30.8	1	16.7	2	11.8	1	25.0	

*Chi square test was done to measure the level of significance.
ns = not significant

Regarding the gender distribution of the study patients, male were predominant in four groups, which was 9 (69.2%) in F0 group, 5 (83.3%) in F1 group, 15 in F2 group (88.2%) and 3 (75.0 %) in F3 group. The difference was not statistically significant (P>0.05) between four groups.

Table III: Distribution of the study population by CTGF among hepatic fibrosis group (n=40)

CTGF	Stages of Fibrosis								p value*
	F0		F1		F2		F3		
	No.	%	No.	%	No.	%	No.	%	
Positive (≥56.6 ng/mL)	4	30.8	4	66.7	13	76.5	4	100	
Negative (≤56.6 ng/mL))	9	69.2	2	33.3	4	23.5	0	0	
Mean ± SD	53.55 ± 9.53		67.28 ± 16.10		74.95 ± 15.92		142.77 ± 27.30		0.001^{s}

*ANOVA test was done to measure the level of significance.
s = significant

Table III shows, Positive (≥56.6 ng/mL) CTGF was found 4 (30.8%) in F_0 group, 4 (66.7) in F_1 group, 13 in F_2 (76.5%) group and 4 in F_3 (100%) group of hepatic fibrosis patients and Negative (≤56.6 ng/mL)) CTGF was found 9 (69.2%) in F_0 group, 2 (33.3%) in F_1 group, 4 in F_2 (23.5%) group and 0 (100%) in F_3 group of hepatic fibrosis patients.

Table IV: Distribution of the study population by HA among hepatic fibrosis group (n=40)

HA	Stages of Fibrosis								p value*
	F0		F1		F2		F3		
	No.	%	No.	%	No.	%	No.	%	
Positive (≥75 ng/mL)	4	30.8	1	16.7	14	82.4	4	100	
Negative (≤75 ng/mL)	9	69.2	5	83.3	3	17.6	0	0	
Mean ± SD	74.28 ± 4.09		73.92 ± 6.81		85.76 ± 7.37		92.35 ± 12.29		0.001[s]

*ANOVA test was done to measure the level of significance.
s = significant

Table IV shows, Positive (≥75 ng/mL) HA was found 4 (30.8%) in F_0 group, 1 (16.7%) in F_1 group, 14 (82.4%) F_2 group and 4 (100%) in F_3 group of hepatic fibrosis patients and Negative (≤75 ng/mL) HA was found 9 (69.2%) in F_0 group, 5 (83.3%) in F_1 group, 3 (17.6%) in F_2 group and 0 (100.0%) in F_3 group of hepatic fibrosis patients.

Table V: Distribution of CTGF by Fibrosis

CTGF	Fibrosis		Total
	Positive	Negative	
Positive (≥56.6 ng/mL)	21 (77.8)	4 (30.8)	25 (62.5)
Negative (≤56.6ng/mL))	6 (22.2)	9 (69.2)	15 (37.5)
Total	27 (100)	13 (100)	40 (100)

Table V shows, Positive (≥56.6 ng/mL) CTGF was found 21 (77.8%) in positive fibrosis patients and 4 (30.8%) in negative fibrosis patients. Negative (≤56.6ng/mL)) CTGF was found 6 (22.2%) in positive fibrosis patients and 9 (69.2%) in negative fibrosis patients.

Table VI: Distribution of HA by Fibrosis

HA	Fibrosis		Total
	Positive	Negative	
Positive (≥75 ng/mL)	19 (70.4)	4 (30.8)	23 (57.5)
Negative (≤75 ng/mL	8 (29.6)	9 (69.2)	17 (42.5)
Total	27 (100)	13 (100)	40 (100)

Table VI shows, Positive (≥75 ng/mL) HA was found 19 (70.4%) in positive fibrosis patients and 4 (30.8%) in negative fibrosis patients. Negative (≤75 ng/mL) HA was found 8 (29.6%) in positive fibrosis patients and 9 (69.2%) in negative fibrosis patients.

Table VII: Sensitivity, specificity, positive and negative predictive values of the CTGF and HA evaluation for hepatic fibrosis

Validity test	CTGF	HA
Sensitivity	77.8	70.4
Specificity	69.2	69.2
PPV	84	82.2
NPV	60	52.9
Accuracy	75	70

PPN = Positive Predictive Value, NPV = Negative Predictive Value

The validity of CTGF and HA evaluation for fibrosis were correlated by calculating sensitivity, specificity, accuracy, positive and negative predictive values. The sensitivity, specificity, positive predictive value and negative predictive value, accuracy CTGF was 77.8%, 69.2%, 84.0%, 60.0%, 75% and serum HA in the diagnosis of hepatic fibrosis was 70.4%, 69.2%, 82.6%, 52.9% and 70% respectively.

Discussion

This cross-sectional study was carried in Department of Laboratory Medicine. We measured the concentration of CTGF and serum HA in patients with chronic hepatitis B (CHB) for the diagnosis of hepatic fibrosis in CHB.

In this study mean age was found 29.92±6.17 years in Fo group, 29.00 ± 7.35 years in F_1group, 27.53 ± 5.79 in F_2 group and 30.50 ± 14.48 years in F_3 group. In this study, the highest incidence of CHB patients was found at 20-29 age groups. Alam et al., (2008) found that chronic hepatitis B affects the younger population (age group 21-30) of Bangladesh. This finding was similar with our study.[12]

Analysis of gender distribution showed out of 40 patients 32 were male and 8 patients were female. Male female ratio is 4:1. Male were found 9(69.2%) in F_0 group, 5(83.3%) in F_1 group 15(88.2%) in F_2 group and 3(75%) in F_3 group. In this current study, male were predominant in four groups. Rahman (2011) observed that males were predominant in chronic hepatitis B patients in Bangladesh which was consistent with our study.[3]

In our study, mean CTGF/CCN_2 was found 53.55 ± 9.53 ng/mL in F_o group (n=13), 67.28 ± 16.10 ng/mL in F_1 group (n=6) and74.95 ± 15.92 ng/mL in F_2 group (n=17), 142.77 ± 27.30 in F_3 group (n=4). P value is 0.001 which is carried out in ANOVA test. The cutoff value of CTGF is ≤56.6ng/mL. In our study we see the serum CTGF concentration is increased in relation to the stages of hepatic fibrosis. Die et al., (2010)[6] determined the stages of hepatic fibrosis according to METAVIR scoring system. They measured serum CTGF by utilizing a commercially available ELISA method. They showed serum CTGF concentrations were 6.1ng/mL in F_0 stage, 7.1ng/mL in F_1 stage, 10.1 ng/mL in F_2 stage, 16.1ng/mL in F_3 stage, 25.7ng/mL in F_4 stage. There is significant difference found in serum CTGF levels in all stages of hepatic fibrosis[6]. Qiu et al., (2010)[11] detected serum CTGF by sandwich ELISA which is more convenient than western blot or immunohistochemistry. They demonstrated that CTGF is highly expressed in serum of

chronic hepatitis B patients than in controls, 161.50±108.27ng/mL versus 51.96± 5.81 ng/mL respectively (P value <0.001). They divided the serum sample in chronic hepatitis B in five groups according to the stages of fibrosis and CTGF level were increased along with the severity of fibrosis. Concurrently, Piao et al., (2012)[14] found serum CTGF concentration were 4 or 4.9 fold higher in patients with chronic hepatitis B as compared to healthy control and its concentration were increased in proportion to the stages of fibrosis. Gressner et al., (2006)[12] assessed the concentration of serum CTGF in chronic hepatitis patients. Their data confirmed that serum CTGF concentration was highest (5.2 fold) in the fibrosis group. So, our results were in accordance with above published studies.[6,11,13,14]

In our study, mean serum HA was found 74.28±4.09ng/mL in F_o group (n=13), 73.92 ± 6.81ng/mL in F_1 group (n=6) and85.76 ± 7.37 ng/mL in F_2 group (n=17), 92.35 ± 12.29 in F_3 group (n=4) . P value is 0.001 which is carried out in ANOVA test. Cut-off value of HA ≤75ng/mL. Mortada et al., (2012)[15] measured serum HA and determined their level with the stages of hepatic fibrosis. They showed serum level of HA was significantly higher ($p<0.05$) in fibrosis of liver. Their results were compatible with our study.

The present study has also defined the specificity of serum CTGF for diagnosis of hepatic fibrosis in CHB. The specificity of CTGF was 62.5% which was nearly consistent with the finding recorded by Qiu et al., (2010)[11].They found specificity was 71.6%. But Gressener et al., (2006)[12] and Die et al., (2010)[6] showed specificity 85% and 82.4% respectively. Their results were incompatible with our studies which may be due to sample size. The positive predictive value (PPV) of serum CTGF in current study was 88.5% which was similar to the study of Die et al., (2010)[6] and Kovalenko et al., (2009)[16]. They found specificity 82.45% and 78% respectively. On the other hand, negative predictive value (NPV) was 35.7% in our study. The NPV is diverse from the study of Die et al., (2010)[6] and Kovalenko et al., (2009)[16]. They found NPV was 72% and 77.9% respectively. Our negative predictive value is less may be due to sample size variation particularly F_4 stage.

According to our study serum CTGF and HA are able to diagnose hepatic fibrosis in chronic hepatitis B and correlates with the stages of hepatic fibrosis. They are simple, sensitive, less expensive and non invasive methods. It would also be helpful for poor, unwilling and contraindicated cases. Our results indicate that serum CTGF exhibited good diagnostic performance and capable of providing guideline for presenting hepatic fibrosis. Liver biopsy is an invasive procedure for the diagnosis and follow up purpose of the patients. Therefore, this study can be helpful for early detection and monitoring of hepatic fibrosis in hepatitis B virus infected patients and would be beneficial for our population.

Conclusion

Liver biopsy is invasive, blind and costly. It carries risk of complications. Repeated biopsy is difficult and is also contraindicated in some conditions. Fibrosis is not equally distributed in the liver and can be missed on a single liver biopsy. Histological evaluation is dependent on experienced histopathologist. Connective tissue growth factor (CTGF) are direct biomarkers of hepatic fibrosis which are simple, quick, less expensive and non-invasive method that can be carried out easily in peripheral hospital. It can be helpful in early detection and monitoring prognosis of hepatic fibrosis in CHB patients.

In this study, we found that the sensitivity, specificity, positive predictive value and accuracy of CTGF higher than HA. So, we

concluded that serum CTGF can be used as more reliable diagnostic tool than HA for the assessment of liver fibrosis in patients with chronic hepatitis B.

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