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Serum Transforming Growth Factor–B1 and Connective Tissue Growth Factor in the Diagnosis of Hepatic Fibrosis in Chronic Hepatitis B: A Cross Sectional Comparative Study

RUBAIYAT-E-MORTAZ
Research Assistant, Department of Clinical Pathology
Bangabandhu Sheikh Mujib Medical University
Md. SAIFUL ISLAM
Assistant Professor, Department of Clinical Pathology
Bangabandhu Sheikh Mujib Medical University
SHEULY FERDOUSHI
Md. SAIFUL ISLAM
Medical Officer, Department of Clinical Pathology
Bangabandhu Sheikh Mujib Medical University
TUHIN SULTANA
Associate Professor, Department of Clinical Pathology
Bangabandhu Sheikh Mujib Medical University
SALIMUR RAHMAN
Professor, Department of Hepatology
Bangabandhu Sheikh Mujib Medical University
Md. QUDDUSUR RAHMAN
Professor, Department of Clinical Pathology
Bangabandhu Sheikh Mujib Medical University
A.N. NASHIMUDDIN AHMED
Chairman & Professor, Department of Clinical Pathology Bangabandhu Sheikh Mujib Medical University
MESBAH UDDIN AHMED[1]
MS in Microbiology
Bangladesh University of Health Sciences

Abstract:

Background: *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). Transforming growth factor beta1 (TGF-β1) and*

[1] Corresponding author: mesbah.lab@gmail.com

Connective tissue growth factor (CTGF) are major profibrogenic cytokines as well as both are involved in hepatic fibrosis. ***Objective:*** *The present study was an attempt to compare between serum TGF-β1 and CTGF in the diagnosis of hepatic fibrosis in CHB.* ***Methodology:*** *This cross sectional observational study was done in Department of Clinical Pathology in collaboration with Hepatology and Pathology, Bangabandhu Sheikh Mujib Medical University, Dhaka during the period of March' 2013 to February' 2014. Forty patients who fulfilled the inclusion criteria of CHB were conveniently included in this study. Serum TGF-β1 and CTGF were measured by using of a sandwich immunoassay technique and liver biopsy material was stained with haematoxyline & eosin and masson's trichrome stains.* ***Result:*** *The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of TGF-β1 in the diagnosis of hepatic fibrosis was 69.7%, 85.7%, 72.5%, 95.8% and 37.5% respectively whereas sensitivity of CTGF was 72.7%, specificity 57.1%, accuracy 70.0%, PPV of 88.9% and NPV of 30.8%. The area under receiver-operating characteristic (ROC) curve (AUC) of TGF-β1 and CTGF were 0.842 and 0.840 respectively for identification of hepatic fibrosis.* ***Conclusion:*** *Serum TGF-β1 can be used as more reliable diagnostic tool than CTGF for the diagnosis of hepatic fibrosis in patients with CHB. Further large scale study can be instituted to get more precise result.*

Key words: Transforming growth factor beta1, Connective tissue growth factor, Hepatic fibrosis, Chronic Hepatitis B

Introduction

Chronic hepatitis is a major global health problem causing approximately 800,000 deaths per year worldwide[1]. Hepatitis B virus (HBV) infection is a clinical problem because of its worldwide distribution and potential adverse sequelae. About 400 million people throughout the world are chronically infected with HBV infection[2]. In Bangladesh, a study was done in Savar, Dhaka and found prevalence of HBV infection is 5.4%

in our population[3]. Chronic hepatitis B (CHB) affects the young and middle aged population of Bangladesh[4] and may lead to development of necroinflamation, fibrosis, cirrhosis and ultimately hepatocellular carcinoma[5]. HBV is responsible for 76.3% of cases of chronic hepatitis and 61.15% of cases of cirrhosis in Bangladesh. It possess huge burden on the health of our patients[6]. Hepatic fibrosis is primary complication of chronic hepatitis characterized by loss of hepatocytes, destruction of hepatic architecture, proliferation of hepatic fibroblasts and excessive deposition of extracellular matrix component. Cytokines play an important role in the development and progression of hepatic fibrosis among which transforming growth factor beta1 (TGF-ß1) and connective tissue growth factor (CTGF) contributes significantly. TGF-ß1 is a major cytokine associated with activation of hepatic stellate cell and extracellular matrix deposition[7]. There is a close relation between TGF-ß1 and CTGF. Both are involved in fibrogenesis. CTGF as a downstream effector of TGF-ß induced extracellular matrix production and fibroblast proliferation[8]. Hepatic fibrosis can be estimated by both invasive and non-invasive methods[9]. Liver biopsy, which is an invasive method, considered as gold standard but has three major limitations which are risk of adverse events, sampling error and intra and inter observer variability[10]. It is only about 80% accurate in fibrosis staging. 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. Histological evaluation is also dependent on experienced histopathologist[11]. TGF-ß1 and CTGF in serum are class I direct biomarker of fibrogenesis[7]. TGF-ß1 and CTGF are simple, quick, less expensive method and can be carried out in peripheral hospital with less chance of sampling error. Serum TGF-ß1 and CTGF levels correlated with the stages of hepatic fibrosis and become a valuable non invasive marker of hepatic fibrosis[12-13].

Therefore a simple, sensitive and non invasive method for early detection, assess the prognosis and different stages of hepatic fibrosis is needed. It can reduce the need for repeated liver biopsies. In this study, measurement of serum TGF-ß1 and CTGF were done as non-invasive markers to compare between serum TGF-ß1 and CTGF in the diagnosis of hepatic fibrosis in CHB.

Materials and method

This cross sectional observational study was conducted at the Department of Clinical Pathology in collaboration with Department of Hapatology and Department of Pathology, Bangabandhu Sheikh Mujib Medical University, Dhaka from March' 2013 to February' 2014. Forty patients who fulfilled the inclusion criteria of CHB[14] 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, 2 ml venous blood was taken before liver biopsy, allow to clot and separate serum by centrifugation at room temperature. The serum was stored at -20^{o}c until analysis. Serum TGF-ß1 and CTGF were measured in the Department of Clinical Pathology by using Enzyme Linked Immuno Sorbent Assay (ELISA) based on sandwich principle. Cut-off value of serum TGF-ß1 < 6000 pg/mL (DRG TGF-ß1 ELISA EIA-1864, 2012) and CTGF ≤ 56.6 ng/mL (DRG CTGF ELISA EIA-5195, 2011). The kits were capable of detecting full length TGF-ß1 and CTGF. 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 Knodell scoring system. Stages of fibrosis were as follows F0- No fibrosis, F1- Fibrous portal expansion, F3- Bridging fibrosis and F4- Cirrhosis[15]. All data was recorded systematically in a preformed data collection sheet. Sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) were calculated from the test results. Other statistical analyses of the results were obtained by ANOVA test, Chi square test, Fisher exact test. Area under the receiver-operator characteristic (ROC) curve (AUC) of serum TGFß-1 and CTGF level were also done for prediction of hepatic fibrosis. All statistical computations were performed by using statistical package of social science SPSS (17.0). Prior to the commencement of this study, the research protocol was approved by the Ethical Institutional Review Board of BSMMU, Dhaka.

Result

A total of 40 patients with HBV infection who fulfilled the criteria of CHB[14] were included. The patients were divided into four groups based on stages of fibrosis as follows: F0- No fibrosis, where 7 patients were included, F1- Fibrous portal expansion, where 25 patients were included, F3- Bridging fibrosis, where 8 patients were included and F4- Cirrhosis, no patients. No patient in F2 stage because histopathological examination was done in Knodell scoring system in which F2 stage is absent[15].

Mean±SD age of respondents in F0, F1 and F3 group was 31.71±10.97, 31.04±7.16 and 29.5 ± 6.78 years respectively and no statistical significance difference was found **(Table I).** Male and female was 82.5% and 17.5% and association between sex and different stages of fibrosis was statistically insignificant **(Table II).**

Mean serum TGF-β1 was found 6178 ± 5655 pg/ml in F0 group, 18835 ± 10146 pg/ml in F1 group and 16578 ± 11132 pg/ml in F3 group **(Table III).** Similarly mean serum CTGF was found 36.11 ± 20.51 ng/ml in F0, 63.36 ± 26.14 ng/ml in F1 and 68.09 ± 35.14 ng/ml in F3 group **(Table IV).** Mean serum TGF-β1 and CTGF difference was statistically significant (P= < 0.05) among the three groups. Positive TGF-β1 (> 6000 pg/ml) was found in 23 (69.7%) with hepatic fibrosis and negative TGF-β1 (≤6000 pg/ml) was found in 10 (30.3%) with hepatic fibrosis. Positive TGF-β1 (> 6000 pg/ml) was found in 1 (14.3%) with no fibrosis and negative TGF-β1 (≤ 6000 pg/ml) was found in 6 (85.7%) with no fibrosis **(Table V).**

The difference was statistically significant (P < 0.05) between the two groups. Positive CTGF (≥ 56.6 ng/ml) was found in 24 (72.3%) with hepatic fibrosis and negative CTGF (< 56.6 ng/ml) was found in 9 (27.3%) with hepatic fibrosis. Positive CTGF (≥ 56.6 ng/ml) was found in 2 (28.6%) with no fibrosis and negative CTGF was found in 5 (71.4%) with no fibrosis **(Table VI).** The difference was statistically significant (P < 0.05) between the two groups.

Sensitivity of TGF-β1 and CTGF were 69.7% and 72.7%, specificity 85.7% and 57.1%, accuracy 72.5% and 70.0%, PPV were 95.8% and 88.9%, NPV were 37.5% and 30.8% respectively **(Fig 1).**

The AUC for the hepatic fibrosis predictors were depicted in Table VII. ROC was constructed by using serum TGF-β1 and CTGF level of the patients with CHB. It showed serum TGF-β1 level cut off value of (> 6000 pg/ml) as the value

with a best combination of sensitivity 69.7%, specificity 85.7% and AUC for TGF-β1 0.842. Serum CTGF level cut off value of (≥ 56.6 ng/ml) as the value with a best combination of sensitivity 72.2%, specificity 58.0% and AUC for CTGF 0.840 for identification of hepatic fibrosis **(Fig 2).**

Discussion

In CHB, the pathogenesis of hepatic fibrosis has been associated with cytokines, particularly TGF-β1[16]. TGF-β1 is the main profibrogenic cytokine. A fraction of TGF-β1 or their spilt product is released into the systemic circulation leading to an increase in their serum concentration and investigated as potential markers of the fibrotic process[7]. In normal liver, hepatic stellate cells express very little TGF-β1. When injury strikes due to chronic liver diseases, inflammatory cells are drawn to the site of injury, release cytokines, hepatic stellate cells undergo activation and become fibrogenic [12].

This cross sectional study was carried out in the Department of Clinical Pathology. The concentration of serum TGF-β1 and CTGF in patients with CHB were measured and compared. 40 CHB patients attended in the Department of Hepatology, BSMMU who fulfilled the criteria were included. There is no known study done to compare between serum TGF-β1 and CTGF in the diagnosis of hepatic fibrosis in CHB in Bangladesh. It is our little endeavor to measure and compare the serum TGF-β1 and CTGF concentration for the diagnosis of hepatic fibrosis in CHB.

The age distribution of the study patients showed majority (40.0%) belonged to 20-29 years. The mean age of overall patients were found 30.85 ± 7.67 years with range from 18 to 52 years. The mean age was found 31.71 ± 10.97 years in F_0 group, 31.04 ± 7.16 years in F_1 group and 29.5 ± 6.78 years in F_3 group. Alam et al., (2008) found that CHB affects the

younger population (age group 21-30 years) of Bangladesh. This finding was similar with our study[4].

Regarding the gender distribution, 33 were male and 7 were female out of 40 patients. Male female ratio was 4:1. Male were found 71.4% in F_0 group, 84.0% in F_1 group and 87.5% in F_3 group. The female were 28.6% in F_0, 16.0% in F_1 and 12.5% in F_3 group. In this study, males were predominant among the three groups. Rahman et al., (2011) observed that males were predominant in CHB patients in Bangladesh which was consistent with our study [3].

In our study, mean TGF-ß1 were found 6178 ± 5655 pg/mL in F_0 group (n = 7), 18835 ± 10146 pg/mL in F_1 group (n = 25) and 16578 ± 11132 pg/mL in F_3 group (n = 8). The cut off value of TGF-ß1 ≤ 6000 pg/mL. Mean serum TGF-ß1 difference was statistically significant ($P < 0.05$). In this study, we observed the serum TGF-ß1 concentration was increased in relation to the stages of hepatic fibrosis. Nassef et al., (2013) measured serum TGF-ß1 and determined their level with the stages of hepatic fibrosis. They showed serum level of TGF-ß1 was significantly higher ($P < 0.05$) in fibrosis of liver [17]. Khorramdelazad et al, (2012) determined serum level of TGF-ß1 and their results showed that serum levels of TGF-ß1 was significantly ($P < 0.001$) increased in CHB patients in compared to control [18]. Elghany et al., (2008) assessed TGF-ß1 in hepatic tissues of 25 CHB patients and measured serum level of TGF-ß1[5]. A positive correlation was detected between hepatic expression of TGF-ß1 and stages of fibrosis. So, our results were in accordance with above published studies [5,17,18].

In our study, mean CTGF were found 36.11±20.51 ng/mL in F_0 group (n=7), 63.36±26.14 ng/mL in F_1 group (n=25) and 68.09±35.14 ng/mL in F_3 group (n=8). The cut off value of CTGF ≤ 56.6 ng/mL. Mean serum CTGF difference was statistically significant ($P < 0.05$). In this study, we observed the serum CTGF concentration was also increased in relation to

the stages of hepatic fibrosis. Qiu et al., (2010) detected serum CTGF and demonstrated that CTGF was highly expressed in serum of CHB patients than in controls ($P < 0.001$)[19]. Concurrently, Piao et al., (2012) found serum CTGF concentration were 4 or 4.9 fold higher in patients with CHB as compared to healthy control and its concentration were increased in proportion to the stages of fibrosis[20]. So, our results were also in accordance with these published studies[19,20].

In our study, we found positive TGF-ß1 (> 6000 pg/mL) in 23 patients with hepatic fibrosis, 1 patient with no fibrosis. Negative TGF-ß1 (≤ 6000 pg/mL) was found in 10 patients with hepatic fibrosis and 6 patients with no fibrosis. Our study showed sensitivity of 69.7% and specificity of TGF-ß1 85.7% which was nearly similar to the study of Nawar et al., (2011), the sensitivity and specificity of their studies were 65% and 94% respectively[12]. Weng et al., (2009) also found TGF-ß1 predominance in HBV-related liver fibrogenesis[21]. The PPV of serum TGF-ß1 in this study was 95.8% and Nawar et al., (2011) found PPV of 75.5%. Our result was nearly consistent with their studies[12,21]. But NPV of their study was 90.4% and in our study, NPV was 37.5%[12]. Our NPV was less may be due to sample size variation particularly F_0 stage.

This study showed positive serum CTGF (>56.6 ng/mL) in 24 patients with hepatic fibrosis, 2 patients with no fibrosis. Negative CTGF (≤56.6 ng/mL) was found in 9 patients with hepatic fibrosis and 5 patients with no fibrosis. Our study showed sensitivity of 72.7% which was nearly similar to the study of Dia et al., (2010) which was 83%[13]. The specificity of serum CTGF for diagnosis of hepatic fibrosis in CHB was 57.1% which was nearly consistent with the finding recorded by Qiu et al., (2010). Their specificity was 71.6%[19]. The PPV of serum CTGF in this study was 88.9% which was similar to the study of Dia et al., (2010) and Kovalenko et al., (2009) which were 82.45% and 78% respectively[13,22]. Our result was nearly

consistent with their studies[13,19,22]. On the other hand, NPV was 30.8% in our study and it is diverse from the study of Dia et al., (2010) in which NPV was 72%[13]. This difference is also due to sample size variation particularly F_0 stage.

The AUC for the hepatic fibrosis were depicted in our study. The AUC for TGF-ß1 and CTGF were 0.842 and 0.840 respectively. Nawar et al., (2011) showed AUC for TGF-ß1 was 0.812 and Qiu et al., (2010) showed AUC for CTGF was 0.681[12,19]. Both AUS's for TGF-ß1 and CTGF were less than our study. So, our observation in this study was within international norms[12,19].

According to our study, serum TGF-ß1 and CTGF are able to diagnose hepatic fibrosis in CHB and correlates with the stages of hepatic fibrosis. These tests are simple, reliable, less expensive and non invasive. It would also be helpful for poor, unwilling and contraindicated cases of liver biopsy. Our results indicate that serum TGF-ß1 and CTGF showed good diagnostic performance and capable of providing guideline for presenting different stages of hepatic fibrosis. Liver biopsy is an invasive procedure for the diagnosis and follow up purpose of the patients. Therefore, this study can be of help for early diagnosis and monitoring of hepatic fibrosis in chronic hepatitis B virus infected patients and would be beneficial for our population.

Conclusion

Our study revealed that TGF-ß1 and CTGF are simple, quick, less expensive and non invasive direct biomarkers. In this study, we found that specificity, PPV, accuracy and AUC of TGF-ß1 is higher than CTGF. So, we concluded that serum TGF-ß1 can be used as more reliable diagnostic tool than CTGF for the assessment of hepatic fibrosis in patients with CHB. It can be used for early detection, reduce progression, assess the prognosis in different stages of hepatic fibrosis and can also be

used as alternative of liver biopsy for the diagnosis of hepatic fibrosis in CHB where biopsy and histopathological examination are not possible.

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Table I: Age distribution among different stages of hepatic fibrosis (n=40)

Age in years	Stages of fibrosis						*P* Value
	F_0 (n=7)		F_1 (n=25)		F_3 (n=8)		
	N	%	N	%	N	%	
18-20	1	14.3	1	4.0	1	12.5	
20-29	3	42.8	11	44.0	2	25.0	
30-39	1	14.3	9	36.0	5	62.5	
40-52	2	28.6	4	16.0	0	0.0	
Total	**7**		**25**		**8**		
Mean±SD	31.71	±10.97	31.04	±7.16	29.5	±6.78	0.845^{ns}
Range (min-max)	(19	-52)	(18	-44)	(19	-37)	

ANOVA test was done; p=0.845<0.05

Table II: Sex distribution among different stages of hepatic fibrosis (n=40)

Sex	F_0 (n=7)		F_1 (n=25)		F_3 (n=8)		*P* value
	N	%	N	%	N	%	
Male (82.5%)	5	71.4	21	84.0	7	87.5	0.679^{ns}
Female (17.5%)	2	28.6	4	16.0	1	12.5	
Total	**7**		**25**		**8**		

Chi square test was done; p=0.679<0.05

Table III: Serum TGF-β1 level in different stages of hepatic fibrosis (n=40)

Stages of hepatic fibrosis	TGF-β1 (pg/ml)				P value
	N	Mean	±SD	(Min -max)	
F_0	7	6178	±5655	(2765 -18700)	
F_1	25	18835	±10146	(3445 -30000)	0.016^{s}
F_3	8	16578	±11132	(3235 -30000)	
Total	40				

ANOVA test was done; p=0.016<0.05

Table IV: Serum CTGF level in different stages of hepatic fibrosis (n=40)

Stages of hepatic fibrosis		CTGF (ng/ml)				P value
	N	Mean	±SD	(Min	-max)	
F_0	7	36.11	±20.51	(11.5	-58)	
F_1	25	63.36	±26.14	(4.7	-125)	0.031[s]
F_3	8	68.09	±35.14	(3	-118.3)	
Total	**40**					

ANOVA test was done; p=0.031<0.05

Table V: Distribution of study patients according to hepatic fibrosis with TGF-β1

TGF-β1	Fibrosis				P value
	Positive (n=33)		Negative (n=7)		
	N	%	N	%	
Positive (>6000 pg/ml)	23	69.7	1	14.3	0.010[s]
Negative (≤6000 pg/ml)	10	30.3	6	85.7	

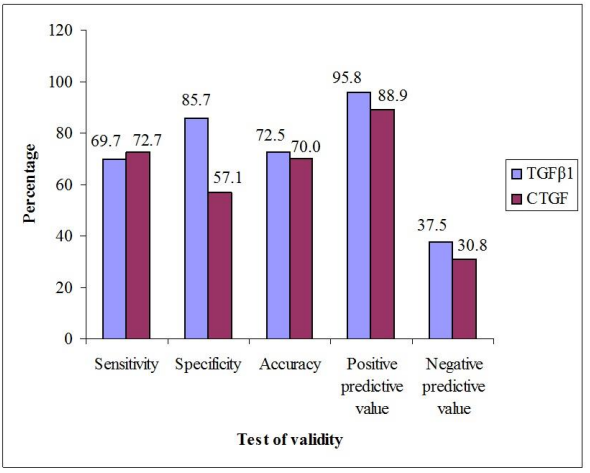
Fisher exact test was done; p=0.010<0.05

Table VI: Distribution of the study patients according to hepatic fibrosis with CTGF (n=40)

CTGF (Serum connective tissue growth factor)	Fibrosis				P value
	Positive (n=33)		Negative (n=7)		
	N	%	N	%	
Positive (≥56.6 ng/ml)	24	72.3	2	28.6	0.039[s]
Negative (<56.6 ng/ml)	9	27.3	5	71.4	

Fisher exact test was done; p=0.039<0.05

Fig: 1 Validity of TGF-β1 and CTGF in diagnosis of hepatic fibrosis:



Receiver-operator characteristic (ROC) curve of serum TGF-β1 and CTGF level for prediction of hepatic fibrosis

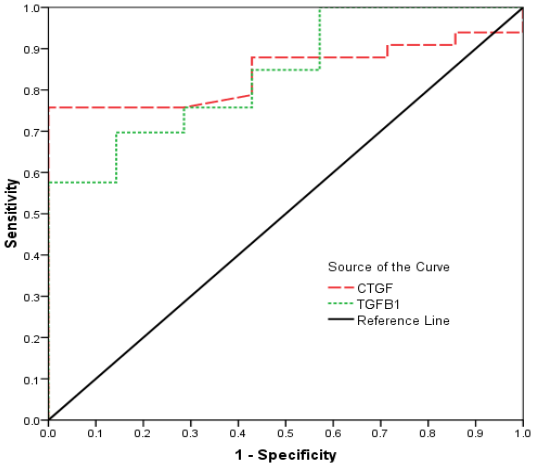


Fig 2: Receiver-operator characteristic curves of serum TGF-β1 and CTGF level.

Table VII: Receiver-operator characteristic (ROC) curve of serum TGF-β1 and CTGF level for prediction of hepatic fibrosis

	Cut of value	Sensitivity	Specificity	Area under the ROC curve (AUC)	95% Confidence interval (CI)	
					Lower bound	Upper bound
TGF-ß1	> 6000 pg/ml	69.7	85.7	0.842	0.722	0.964
CTGF	≥56.6 ng/ml	74.2	58.0	0.840	0.698	0.982