Original Article

Relation of Apoptosis Marker CK-18 fragment M30 with hepatic Necroinflammation in patients with HBV related Chronic Liver Disease

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ABSTRACT:

Background and Objective: Caspase*-cleaved cytokeratin 18 (CK18 M30) is a potential clinically useful biomarker in liver disease as it is released from hepatocytes during apoptosis. Chronic hepatitis B virus (HBV) infection affects more than 400 million people worldwide. For treatment of chronic hepatitis B, it is very essential to know the necro inflammatory status of liver. Cytokeratin (CK) 18 is an intermediary filament protein, expressed in hepatocytes, which is proteolytically cleaved during liver damage. In this study, we aimed to investigate whether serum CK-18 fragment M30 level significantly related with the hepatic necroinflammatory activity in patients with HBV related compensated chronic liver disease (CLD)..**Methods:** This was a prospective observational study. All patients who met the inclusion and exclusion criteria were assessed for liver biopsy. The total sample was 40 patients. This study was conducted in Department of Hepatology, Bangabandhu Sheikh Mujib Medical University. Per cutaneous transthoracic liver biopsy was done. Specimens were sent to department of pathology, BSMMU for METAVIR scoring. **Result:** Among 40 CHB patients, the highest frequency was found at 21-30 age groups, male 31(77.5%) and female 9(22.5%), the mean HBV DNA PCR was found 5.3±1.7 (IU/ml). The mean AST and ALT were found 40.2±20.2 (U/L) and 66.4±68.2 (U/L) respectively. 16 patients were HBeAg positive and 24 patients were HBeAg negative. CK-18 M30 level in both HBeAg negative CHB patients and HBeAg positive patients were almost similar(128.8±32.91 and 123.9±28.1), Activity METAVIR score of hepatic necro inflammation reveals, A0 was 0 (0.0%), A1 was 7(17.5%), A2 was 21(52.5%), A3 was 12(30.0%),Correlation between factors and activity METAVIR score on spearman correlation test reveals, Correlation co-efficient (r value)-age was 0.204, GGT was 0.287 , ALT was 0.333, AST was 0.360, Serum CK-*

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18 fragment M30 level was negatively correlated with activity score of hepatic necro inflammation (r= -0.073; p=0.357). The correlation between ALT and AST with activity score were statistically significant (p<0.05). The area under the receiver-operator characteristic (ROC) curves for prediction of serum CK-18 fragment M30 level reveals - area under curve(AUC) 0.307, which gave a cut off value of 100 U/L with 78.8% sensitivity and 14.3% specificity for prediction of significant necro inflammation (A2 and A3). ***Conclusion:*** *This study indicates there was no correlation between serum CK-18 fragment M30 level and hepatic necroinflammatory activity in patients with HBV related compensated chronic liver disease (CLD). As a result, CK 18 M30 cannot be used as an accurate non-invasive predictor of significant inflammatory activities in patients with CHB.*

Key words*: Chronic Hepatitis B, Chronic Liver Disease, Necro-inflammatory status, Cytokeratin (CK)-18 fragment M30, Hepatic Fibrosis*

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INTRODUCTION:

There are an estimated two billion people with serological markers of present or past HepatitisB virus (HBV) infection globally; 257 million of these are chronically infected.[1] The outcomesof acute HBV infection range from complete recovery to fulminant liver disease. A failureto clear HBV after acute infection may lead to either inactive or active chronic infection,which can induce hepatic insufficiency, end-stage liver disease including liver cirrhosis (LC)and hepatocellular carcinoma (HCC).[2,3]Bangladesh is within the intermediate zone of prevalence (5.4%) of HBV infection and HBeAg negative variant is predominant cause of chronic hepatitis in incidentally detected HBsAg positive patient.[6] Transmission of HBV occurs either perinatally or horizontally. Chronic hepatitis affects the younger and middle aged population of Bangladesh and may lead to development of cirrhosis and hepatocellular carcinoma. This favours horizontal transmission in early childhood contrary to vertical transmission.[7,8]Chronic hepatitis B diagnosis is based on clinical examination and laboratory tests. The assessment of the severity of the liver disease includes LFT, HBV DNA, hepatic ultrasound and liver Biopsy.[5]

Patients with significant hepatic inflammation and fibrosis are at the highest risk of complications like cirrhosis of liver and hepatocellular carcinoma.[9] Histological examination of liver biopsy is the current gold standard for the detection of liver damage. This procedure provides important information regarding the severity of necroinflammatory activity and fibrosis, features potentially useful for predicting treatment response and prognosis.[9]Liver biopsy has a number of limitations. Liver biopsy is invasive, costly, and limited by sampling error and poor intra and inter observer variability.[10-12]Many recent studies clearly indicate that liver biopsy is prone to sampling errors and may underestimate the extent of liver injury.[13]Considering these limitations and patient reluctance to undergo liver biopsy, noninvasive predictors of histological severity are desperately needed.

Cytokeratin is the major intermediate filament protein in cells. These are subdivided into type I (acidic) and type II (basic) keratins. Cytokeratin-18 is type I keratin. Keratin expression is tissue specific with different pairs. Adult hepatocytes express keratin 18 (K18; type I) and keratin 8 (K8; type II) exclusively. It forms cytoplasmic network. CK-18 maintains normal cellular and mitochondrial structure. It is also involved in apoptosis.

There is increasing evidence that liver cell damage in chronic HBV infection is mediated by the induction of apoptosis. In apoptosis hyperphosphorylation of keratin filament occurs. Phosphorylated CK8/CK18 pair is the substrate forpro-caspase 3 and 9. These enzymes caspases become catalytically active and the effector caspases cleave CK-18. This keratin pairs in turn have been broken down and collapse the cytoplasmic and nuclear cytoskeleton. It leads to the condensation of chromatin, which is the hallmark of apoptosis. Caspase-cleaved CK18 fragment is released into the extracellular compartment. A monoclonal antibody, M30, specifically recognizes a fragment of CK18 cleaved at Asp396 (M30-antigen). An M30-based sandwich ELISA assay determines the circulating levels of M30-antigen and may serve as surrogate serum biomarker of hepatocyte apoptosis.[12]

METHODS AND MATERIALS:

Patients who were HBsAg +ve for more than 06 months with age- 18 to 65 years and HBV DNA value >2000 IU/ml were included in the study. Exclusion criteria were patient with HBsAg +ve for <6 months, co- infection with HCV, HIV etc. patient with history of anti-viral treatment, alcohol consumption >30gm/day for male and>20gm/day for female, Non-alcoholic fatty liver disease, patient with decompensated cirrhosis of liver, patient with co-morbid condition (COPD, CKD, CCF etc.), patient who fails to give consent for biopsy. Total 40 patients were included after matching criteria.

Serum levels of M30-antigen were determined by commercially available immunoassays (M30-Apoptosense ELISA kit, Peviva AB, Bromma, Sweden) according to the manufacturer's instructions in the department of microbiology and immunology, BSMMU.

Per cutaneous transthoracic liver biopsy performed using a true-cut biopsy needle, G14, 15cm in length (sample length 1.5cm) with available resuscitation facilities. All biopsies were fixed with 10% formalin solution and stained with hematoxylin-eosin and Masson Trichrome stain. Experienced single pathologist, not aware about the clinical and biochemical parameters of any patient using the METAVIR scoring system evaluated biopsies. Patient were observed for 48 hours and then discharged from hospital.

Statistical Analysis

The demographic information, relevant history, examination findings and investigation reports of all the study subjects was recorded in previously prepared data collection sheet. After compilation, the data were presented in the form of tables, figures and graphs, as

necessary. Statistical analysis of the results as done by using computer based software, SPSS version.20 (SPSS Inc, Chicago, IL, USA).All values were presented as mean ± standard deviations (SDs) for continuous data and as percentages for categorical data. Qualitative data were analyzed by Chi-square test and quantitative data were analyzed by student's t-test and anova test. Biochemical scoring was calculated using available formula. The AUROC, sensitivity, specificity and cut off values for biochemical indices were measured. A probability 'P' value of 0.05 or less was considered as significant. The association between serum biochemical indices with Fibrosis staging and the histological severity of CHB was evaluated by Spearman's correlation test.

RESULTS:

Table 1: Baseline characteristics of the study population (n=40)

Investigation	Range (min, max)	Mean±SD
Age (in years)	18, 50	26.5±7.7
Sex (male/female)	-	31/9
Hb%	11.5, 16.2	13.7±1.3
TC	5, 12	7.4±1.7
Neutrophil (%)	41, 77	60.1±9.4
Lymphocyte (%)	18, 46	31.2±7.8
Monocyte (%)	1, 8	4.5±1.9
Eosinophil (%)	0, 15	4.2±3.0
Basophil (%)	0, 2	0.10±0.37
Platelet count ($\times 10^9$/L)	100, 400	234.3±70.1
*HBV DNA PCR (IU/ml)	2.2, 8.8	5.3±1.7
AST (UL)	19, 118	40.2±20.2
ALT (U/L)	22, 397	66.4±68.2
Prothrombin Time (sec)	10.5, 15.2	12.4±0.7
INR	0.88, 107	1.1±0.2
GGT	12, 110	30.2±17.9

All values are expressed as mean±SD or number (%).

Among the 40 patients, 16 patients were HBeAgpositive and 24 patients were HBeAg negative.

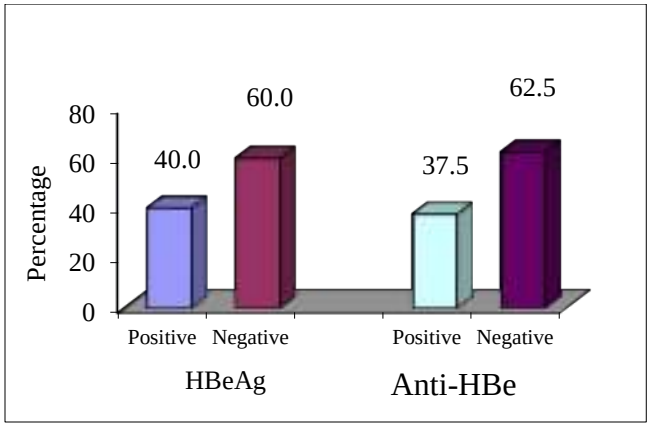

Fig. 1: Bar diagram shows HBeAg status of the study patients.

Table 2: Comparison of Demographic details and Laboratory findings between HBeAg positive and HBeAg negative CHB patients (n = 40).

HBeAg status	All patients (n = 40)	HBeAg Positive CHB (n = 16)	HBeAg Negative CHB (n = 24)
*Age	26.5±7.7	24.9±9.2	27.6±6.7
**Sex (male/female)	31/9	13/3	17/7

Hb%	13.7±1.3	13.7±1.5	13.7±1.3
TC	7.4±1.7	7.1±1.7	7.7±1.7
Platelet count (*10^9/L)	234.3±70.1	218.5±45.1	244.8±81.9
*HBV DNA PCR (IU/ml)	5.3±1.7	7.0±0.9	4.2±1.0
AST (UL)	40.2±20.2	42.3±17.8	38.8±21.9
ALT (U/L)	66.4±68.2	62.1±41.5	69.3±82.0
Prothrombin Time (sec)	12.4±0.7	12.3±0.7	12.4±0.8
INR	1.1±0.2	1.1±0.6	1.2±0.2
GGT	30.2±17.9	32.6±23.6	28.6±13.2
Activity score			
A0	0 (0.0%)	0(0.0%)	0 (0.0%)
A1	7 (17.5%)	4 (25.0%)	3 (12.5%)
A2	21 (52.5%)	8 (50.0%)	13 (54.2%)
A3	12 (30.0%)	4 (25.0%)	8 (33.3%)
Fibrosis score			
F0	1 (2.5%)	0 (0.0%)	1 (4.2%)
F1	5 (12.5%)	2 (12.5%)	3 (12.5%)
F2	27 (67.5%)	10 (62.5%)	17 (70.8%)
F3	5 (12.5%)	3 (18.8%)	2 (8.3%)
F4	2 (5.0%)	1 (6.3%)	1 (4.2%)
Serum CK -18 fragment M30 level (Unit/L)	125.4±30.2	128.8±32.9	123.9±28.1

Table 03: Multiple comparison of variables in study patients according to Activity METAVIR score of hepatic necro inflammation (n=40)

	Activity score						**P value**
	A1 (n=7)		**A2 (n=21)**		**A3 (n=12)**		
	n	**%**	**n**	**%**	**n**	**%**	
Age (in years)	21.3	±4.6	27.7	±7.1	27.4	±9.3	0.145ns
Sex (male/female)	5/2		15/6		10/2		0.728ns
Prothombin time (sec)	12.3	±0.7	12.4	±0.7	12.3	±0.8	0.911ns
Serum CK -18 fragment M30 level	140.6±27.5		121.8±25.1		124.4±37.6		0.350ns
AST	28.0	±8.4	38.5	±14.6	50.2	±28.5	0.055ns
ALT	42.9	±14.0	53.1	±29.6	103.3±112.2		0.072ns
GGT	31.0	±35.3	29.4	±13.3	31.2	±11.7	0.956ns
Platelet count	258.0±61.5		237.9±68.3		214.2±77.6		0.407ns
HBV DNA PCR	6.0	±2.1	5.1	±1.5	5.4	±1.6	0.457ns

All values are expressed as mean±SD or number (%)

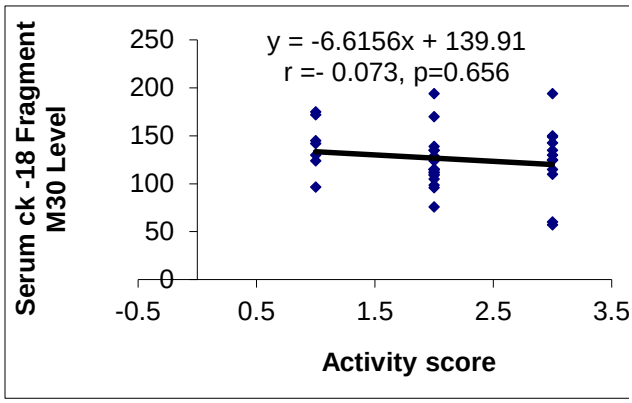

Fig. 2: Scatter diagram showing negative correlation (r= -0.073; p=0.357) between activity score and serum CK -18 fragment M30 level.

Table 4: Correlation between factors and activity score of necro inflammation on spearman correlation test.

Factors	Correlation co-efficient (r value)	p-value
Age	0.204	0.206[ns]
GGT	0.287	0.087[ns]
ALT	0.333	0.036[s]
AST	0.360	0.016[s]
Prothombin time	0.150	0.357[ns]
Serum CK -18 fragment M30 level	-0.073	0.656[ns]
HBV DNA PCR	-0.098	0.549[ns]
Platelet count	-0.289	0.070[ns]

DISCUSSION:

Increased CK-18 M30 level significantly correlates with the activity of hepatic necroinflammation and reflects as the non-invasive marker for significant inflammation (CB, Bae et al. 2013). So, measurement of serum CK-18 M 30 level can be useful for the predication of significant inflammation in CHB patients. In this study, most of the patients were young with 30 patients (75%) below the age of 30 years. Mean age of the patients found 26.5 ± 7.7 with the highest frequency at 21-30 years' age group (Table-1).

Among 40 CHB patients a male predominance was observed, male 31(77.5%) and female 9(22.5%). Among the 40 patients, 16 patients were HBeAg positive and 24 patients were HBeAg negative. Predominance of HBeAg negative patients was also similar in previous study done in Bangladeshi people.[14]In this study, comparison between HBeAg negative CHB patients and HBeAg positive patients reveals that HBeAg negative CHB patients are older (27.6 ±6.7 versus 24.9 ±9.2 years),AST and HBV DNA levels are lower (38.8 versus 42.3 U/L and 4.2±1.0 Vs 7.0±0.9 IU/ml respectively).They have significant fibrosis (82.2% Vs 75%) and significant inflammation (87.5% Vs 75%)(Table 2).These results are almost similar with another study conducted in Dept of

hepatology, BSMMU.[15] In this study, CK-18 M30 level in both HBeAg negative CHB patients and HBeAg positive patients are almost similar (128.8±32.91 and 123.9±28.1) **(**Table 3). In this study, 17.5% patients have not significant necroinflammation (A0, A1) and 85.5% patients have significant necroinflammation (A2, A3). Serum CK-18 M30 level are not significantly increased in a stepwise fashion from A0 to A3. But AST and ALT levels are increased in a stepwise fashion from A0 to A3. Sherbiny WA et al., 2015 conducted a study in patients with chronic hepatitis C. Results showed that serum CK-18 M30 concentrations were significantly increased in a stepwise fashion from A0 to A3. Our study does not support this finding.

Correlation between factors and activity METAVIR score on spearman correlation test reveals, Serum CK-18 fragment M30 level was negatively correlated with activity score of hepatic necro inflammation (r= -0.073; p=0.357). The correlation between ALT and AST with activity score were statistically significant (p<0.05). Our study shows negative correlation with activity of necroinflammation.

The area under the receiver-operator characteristic (ROC) curves for prediction of serum CK -18 fragment M30 level reveals - area under curve(AUC) 0.307, which gave a cut off value of 100 U/L with 78.8% sensitivity and 14.3% specificity for prediction of significant necroinflammation (A2 and A3). Study conducted by Bae CB *et al*., 2013 showed combined measurements of serum M30-antigen level (>344 U/L) and AST (>78 IU/L) provided the most accurate identification of significant inflammation, showing 38.2% sensitivity, 96.1% specificity. Our study differs from that result.

CONCLUSION:

This study indicates there was no correlation between serum CK-18 fragment M30 level and hepatic necroinflammatory activity in patients with HBV related compensated chronic liver disease (CLD). As a result, CK 18 M30 cannot be used as an accurate non-invasive predictor of significant inflammatory activities in patients with CHB.

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