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INCREASED SERUM APOPTOTIC CYTOKERATIN-18 FRAGMENTS IN CHRONIC HEPATITIS C (CHC) PATIENTS WITH NORMAL ALANINE AMINOTRANSFERASE (ALT).

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Abstract

For 40 adult patients with CHC Gr. I; 21 with normal ALT and Gr. II; of 19 with elevated ALT levels togetherwith 20 controls Gr. III. Results showed that Gr.I and Gr. II serum CK-18 fragments levels were significantly correlated with age and was higher than that of Gr. III ($p<0.05$) but lower than in Gr. II ($p<0.05$).There was a statistically significant positive correlation ($p<0.05$) between serum CK-18 and ALT and AST levels ($p < 0.05$ and < 0.01 ,respectively) in Gr.I & Gr.II and also highly significant positive correlation with HCV-RNA- load ($p<0.001$).Liver biopsies in Gr.I showed METAVIR (A1) activity in 17 out of 21 patients and fibrosis (F2) was significantly higher than in Gr.II($p<0.05$). while those with (F3) showed no such difference.A statistically positive correlation ($P<0.05$) was observed between CK-18 and METAVIR necroinflamatory grade (A) and fibrosis stage (F).

In conclusion, in CHC patients with persistently normal ALT, serum CK-18 fragments level is increased and correlates with the necroinflammatory changes in liver tissue as well as the viral load.

Introduction

Egypt suffers from the morbidity and mortality of HCV with its resulting cirrhosis and HCC (Miller and Abu-Raddad, 2010). In all forms of hepatitis, the inflammatory process causes hepatocyte death through different mechanisms, including necrosis and apoptosis (Fischer *et al.,* 2007 and Eren *et al.,* 2010). Apoptosis is a process of cell deletion that plays a fundamental role in the maintenance of tissue homeostasis in humans (Najimi *et al.,* 2009). There is increasing evidence suggesting that liver cell damage in chronic HCV infection is mediated by the induction of apoptosis whose importance has originally been proposed in view of patho-morphological features, (Bantel and Schulze-Osthoff, 2003). These includes cell shrinkage and fragmentation of the nucleus particularly in areas of piecemeal necrosis, the presence of acidophilic bodies, and focal cell dropouts in the liver lobule, which are characteristic features of individually infected hepatocytes in CHC Cytokeratins (CK) are the major intermediate filament proteins in the liver and any cellular damage that alters

the hepatocyte membrane integrity would result in the release of CK (or their fragments) into the circulation (Bateman and Hübscher, 2010).

Aminotransferases are considered an indirect parameter of hepatocellular damage. However, they do not allow to distinguish the specific mode of cell damage or cell death with either necrosis or apoptosis and their serum levels do not accurately reflect the extent of liver injury. The upper limit of normal (ULN) for ALT fails to identify many patients with hepatic injury and aminotransferases may also be released via increased membrane permeability without cell destruction (Zeuzem *et al.*, 1996; Prati *et al.*, 2002 and Kronenberger *et al.*, 2005). The discordance between serum aminotransferase levels and histologically assessed liver damage has been well documented in HCV-infected patients with persistently normal ALT levels who can show substantial liver inflammation and fibrosis on liver biopsy (Bantel *et al.*, 2001 and Puoti *et al.*, 2003).

During apoptosis, cells die without membrane injury, possibly leading to a reduced release of aminotransferases. Therefore, apoptosis could explain the discordance between histological activity and serum aminotransferase levels (Kronenberger *et al.*, 2005).

Generally hepatitis C virus (HCV)- infected patients with persistently normal alanine aminotransferase (ALT) levels can show hepatic inflammation and fibrosis in liver biopsy. Cytokeratins (CK) are the major intermediate filament protein in the liver representing substrates for caspases (intracellular cysteine proteases) during hepatocyte apoptosis. Altered hepatocyte membrane integrity with cellular damage may release CKs (or their fragments) into the circulation. For all these considerations, the measurement of CKs and their fragments in the serum, which reflects the apoptotic process, would be valuable for the noninvasive detection of the necroinflammatory activity during the course of hepatitis (Linder, 2007 and Linder *et al.*, 2010).

The aim of this study, therefore, was to determine the serum level of CK-18 fragments (the M3-epitope on CK-18 fragments) in CHC patients with persistently normal ALT levels, compared to those with elevated ALT and healthy controls, and to assess its histopathological correlation in liver biopsy.

Subjects and Methods

This is an observational, case – control, study that was conducted according to the guide lines of the Research Ethics Committee (REC) of Benha Faculty of Medicine and its University Hospitals during the period from January to December 2011. It was carried out on 60 subjects attending the Department of Hepatology, Gastroenterology and Infectious Diseases (in collaboration with the Department of Clinical Pathology), Benha University Hospitals. The Cases were 40 adult patients with CHC, they were 29 males (72.5%) and (11) females (27.5%) who were subdivided into 2 groups: Group-I: comprised 21 patients with persistently normal AlT levels (defined as 3 ALT values below the upper limit of normal (ULN) taken at least 4 weeks apart within a period of at least 6 months). Group-II: comprised 19 patients with elevated ALT levels. Twenty healthy subjects {14 males (70%) and 6

females (30%)} with negative HCV-Ab and HBsAg and having repeatedly normal ALT levels were taken as the control group (Group-III).

All cases were immunocompetent adults (between 18 - 60 years old), positive for HCV-Ab using the enzyme linked immunosorbeny assay *(ELISA) kit and **HCV-RNA-PCR. Patients with the following criteria were excluded from the study: age under 18 or above 60ys, co-morbid liver conditions (*e.g.* HBV infection, alcoholics, *etc.*), pregnancy, patients receiving any medications which may affect ALT level within the 6 months preceding the study, patients with established cirrhosis (METAVIR - F4) and/or HCC and those refusing the medical consent.

All the studied cases were subjected to thorough history taking, clinical examination and laboratory investigations including CBC, liver profile tests, serum creatinine, hepatitis viral markers, HCV-RNA-PCR and serum α fetoprotein. Serum level of CK-18 fragments was assessed in both cases and control groups (table-1). Abdominal ultrasonography and ultrasound-guided liver biopsies were done, for all cases, under local anaesthesia using 16 gauge Trucut needles with biopsy gun (US biopty) and the METAVIR scoring system (Bedossa and Poynard, 1996) was applied for histopathological grading and staging.

Quantitative measurement of serum caspase- generated CK- 18 fragments (M3-epitope on CK-18 fragments) by TPS-ELISA:

The *TPS-ELISA is a one step enzyme linked sandwich immunoassay for the quantitative measurement of the M3-epitope of soluble fragments of CK-18 in serum. Patient samples, standards and controls react during incubation with wells coated with solid phase monoclonal catcher antibodies directed against CK proteins, simultanously with a HRP (Horseradish Peroxidase) conjugated monoclonal antibody (M3) used as a detector. Excess unbound HRP-conjugate is removed by a washing step. Tetra-methylbenzidine substrate is added and after incubation, the reaction is finished. The developed colour is directly proportional to the concentration of CK in the test sample. The absorbance is measured in a Microplate Reader at 450 nm. Quantitation is achieved by the construction of a standard curve using known concentration of CK protein. By comparing the absorbance obtained from a sample containing an unknown amount of CK protein with that obtained from the standards, the concentration of CK protein in the test sample can be determined.

Statistical analysis: collected data were organized, tabulated and statistically analyzed using SPSS (Statistical package for social sciences) version 16. For quantitative data; the range, mean and standard deviation were calculated. The difference between 2 means was analyzed using Student's (t) test and for comparison between > 2 means, the F value of analysis of variance (ANOVA) was calculated. Pearson's correlation coefficient (r) was calculated to test the association

*Abott Diagnostics, Wiesbaden, Germany.
**The Amplicor HCV monitor test of Roche Diagnostics, Mannheim, Germany.

*IDL Biotech AB Company, Bromma, Sweden.

between 2 variables. Levels of significance: P-value >0.05 indicates that results are not significant, P<0.05 indicates significant results and P<0.001 indicates highly significant results. Receiver operating characteristics (ROC) curve that plots the sensitivity versus 1-specificity for all possible cut off values was used to assess the diagnostic performance. The accuracy index is the area under the ROC curve (AUROC) which is 0.5 for a random model and 1 for a perfect model. Values above 0.7 indicate useful testing while values above 0.8 indicate high diagnostic accuracy.

Results

The main demographic data and laboratory findings of the studied groups are illustrated in table-1 and the histopathological findings of the studied cases in table-2. Cases with persistently normal ALT levels (group -I) had significantly higher serum CK-18 fragments level than the control group ($p<0.05$) and lower levels (but not statistically significant) than cases of group-II. A statistically significant positive correlation ($p<0.05$) between CK-18 fragments level and age of the studied 40 cases was observed. Cases group (groups I and II) showed a significantly higher serum CK -18 levels ($p<0.05$) than the control group (group-III). There was a statistically significant positive correlation ($p<0.05$) between serum CK-18 and ALT as well as AST levels ($p<0.05$ and <0.01, respectively) in the studied cases group. There was a statistically highly significant positive correlation between CK-18 levels in whole cases and HCV-RNA- load ($p<0.001$).

Table-1: Data of the studied groups.

General characteristics (Mean±SD)	Cases		Controls	P-value *Cases control*
	Group I Normal ALT (No. 21)	Group-II Elevated ALT (No. 19)	Group-III (No. 20)	
Age (years)	38.5±10.3	38.5±11.3	35.25±5.63	0.450
Male / Female	14/7	15/4	14/6	0.676
Hb (gm/dl)	13.9±1.7	14.2±1.5	14.13±0.67	0.665
WBCs (x 10^3/mm^3)	6.3±2.3	6.4±1.6	6.33±1.32	0.968
Platelets (x 10^3/mm^3)	215.4±45.2	204.0±41.6	214.4±21.26	0.614
INR	1.1±0.1	1.1±0.3	1.02±0.04	*0.003
ALT (U/L)	30.0±6.4	74.5±18.0	17.90±5.82	**0.000
AST (U/L)	32.3±12.2	58.4±27.2	15.25±4.78	**0.000
Alkaline Phosphatase (U/L)	111.7±73.7	128.4±57.9	66.40±22.9	**0.005
S.Albumin (gm/dl)	4.2±0.4	4.3±0.3	3.91±0.42	**0.006
Total Bilirubin (mg/dl)	0.66±0.2	0.9±0.3	0.63±0.13	**0.001
S.Cytokeratin-18 (U/L)	66.0±59.7	105.4±87.7	17.66±22.61	**0.000

Table-2: Histopathological findings in the studied cases groups applying the METAVIR scoring system.

METAVIR	Cases				χ^2	P-value
	Group - I		Group - II			
	No.	%	No.	%		
Necroinflammatory Grade (A)						
A0	1	4.8	1	5.3	9.36	*0.025
A1	17	81.0	7	36.8		
A2	3	14.3	8	42.1		
A3	0	0.0	3	15.8		
Fibrosis stage (F)						
F0	1	4.8	1	5.3	0.352	0.950
F1	1	4.8	1	5.3		
F2	14	66.7	11	57.9		
F3	5	23.8	6	31.6		

Considering the histopathology, the number of cases with METAVIR mild activity (A1) in group-I was significantly higher than those in group-II (17 vs 7 with $p<0.05$) while the number of cases with moderate activity (A2) was significantly higher in group-II than group-I (8 vs 3 with $p<0.05$) and none of the cases in group-I had severe activity (A3) in his liver biopsy compared to 3 cases in group-II. On the other hand and despite normal enzymes, the number of patients with significant fibrosis (F2) was significantly ($p<0.05$) higher in group-I than in group-II. Moreover, the number of patients with advanced fibrosis (F3) showed no statistically significant difference between both groups. A statistically significant positive correlation ($P<0.05$) was observed between CK-18 and METAVIR necroinflamatory grade (A) and fibrosis stage (F) among the studied cases.

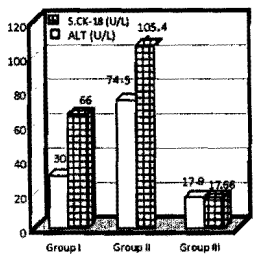

Fig.1: Cytokeratin-18 and ALT levels among the studied groups.

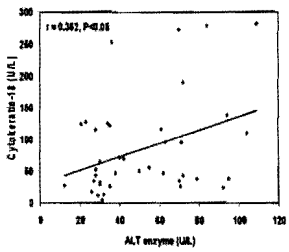

Fig.2: Correlation between CK -18 and ALT in the studied cases.