The Role of Bcl-2 and Bax as Markers of Disease Progression in Hepatitis C Virus Infected Patients

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Many apoptotic markers have been linked to hepatic cell injury in HCV-related liver diseases, and hence could be used as potential markers for early detection of the disease. The present study aimed to assess the role of apoptotic markers Bcl-2 and Bax in the pathogenesis of chronic HCV-related liver diseases. A total of 85 participants were enrolled into the study; 70 chronic HCV patients (35 non-cirrhotic and 35 cirrhotic), and 15 healthy controls. The serum levels of Bcl-2 and Bax were assayed in all participants by ELISA. Bcl-2 and Bcl-2/Bax ratio were significantly higher in non-cirrhotic patients than the cirrhotic and controls (*P* < 0.001). Bax was significantly higher in cirrhotic patients compared to the other groups (P < 0.001). Positive and negative correlations were found between serum Bcl-2, Bax, Bcl-2/Bax ratio and HCV viral load in non-cirrhotic and cirrhotic patients respectively. These findings provide an evidence that apoptosis is dysregulated in patients with chronic HCV.

Hepatitis C virus (HCV) infection is a devastating health problem and a leading cause of chronic liver disease worldwide [1]. Hepatitis C infection causes acute symptoms which are generally mild and non-specific in only 15% of patients [2]. About 80% of those exposed to the virus develop a chronic infection with its consequences of liver cirrhosis and hepatocellular carcinoma [3]. It has been reported that about 700,000 persons die annually as a consequence of HCV-related complications; all of which could be avoided if proper antiviral treatment is provided at an early stage of the disease [4]. The pathogenesis of chronic HCV infection has not been clearly elucidated. Hepatitis C virus is RNA virus without apparent cytopathic effects, therefore in chronic infection the hepatocellular damage is generally believed to be immune-mediated [5].

Apoptosis or programmed cell death plays an important role in the maintenance of cellular homeostasis through removal of aged, damaged, and hyperproliferative cells [6]. Apoptosis can be induced via two major pathways. The first pathway is the death receptor pathway (extrinsic pathway), which is triggered by binding of FAS ligand (FASLG) to FAS (CD95). This leads to activation of caspase 8 (CASP8), which subsequently activates effecter caspases 3, 6, and 7 (CASP3, CASP6, and CASP7) [7]. The second pathway; intrinsic pathway or Mitochondrion-dependent apoptosis is mediated by the mitochondria via the B-cell Leukemia/Lymphoma 2 (Bcl-2) family in response to DNA damage, oxidative stress, and viral proteins [8].

Bcl-2 family has emerged as a dominant regulator of apoptosis through the regulation of the mitochondrial-mediated pathway. Bcl-

2 family includes anti-apoptotic (Bcl-2, Bcl-xl, Mcl-1) and pro-apoptotic proteins (Bax, Bad, and Bak). Bcl-2 is classified as an important anti-apoptotic protein, thereby it is considered as an oncogene. It regulates programmed cell death by providing a survival advantage to rapidly proliferating cells. Bax protein promotes apoptosis by enhancing cell susceptibility to apoptotic stimuli. Moreover, Bcl-2 inhibits apoptosis by interacting and forming inactivating heterodimers with Bax [9].

Apoptosis has been implicated not only in the pathogenesis of HCV infection, but also in determining the individual susceptibility to the infection [10-11]. Additionally, it has been shown that apoptosis is the cause of HCV-induced cell death. Bax, which is a pro-apoptotic member of the Bcl-2 family that induces apoptosis of the host cell through a Bax-triggered, mitochondria-mediated mechanism, is activated by HCV infection. Also, it has been suggested that the cell susceptibility to apoptosis is determined by the Bcl-2: Bax ratio [12]. Considering the high prevalence of HCV infection in Egypt, the current study was conducted to assess the potential role of Bcl-2 and Bax in the pathogenesis of chronic hepatitis c virus related liver diseases.

Patients and Methods

Study design and patients

This study was carried out at the central research laboratory, faculty of medicine, Sohag University, during the period from July 2015 to April 2016. The study included 70 chronic HCV patients who were recruited from the outpatient clinics and the inpatient wards of Internal Medicine department of Sohag university hospital. The study population was divided into 2 groups; group 1 (n=35) chronic HCV patients with no evidence of cirrhosis and group 2 (n=35) chronic HCV patients with cirrhosis; Child-Pugh class B/C (13). Exclusion criteria were co-infection with HBV or HIV, diabetes mellitus, hypertension, other systemic diseases, hepatocellular carcinoma or other malignancy. Fifteen age and sex matched healthy controls (HCV and HBV seronegative) were included in the study (group 3). The study was approved by the Ethics Committee of Sohag faculty of medicine, Sohag University and written informed consents were obtained from all participants.

All the patients were subjected to the following:

- Through history taking and complete clinical examination with stress on jaundice, ascites, lower limb oedema, hepatomegaly, splenomegaly and encephalopathy.

- Abdominal ultrasound. To assess the echo pattern and size of the liver and the presence of periportal fibrosis, the presence of ascites, the size of spleen or any other abnormalities.

Laboratory investigations

a- Liver function tests (total protein, albumin, total bilirubin, direct bilirubin, SGOPT and SGPT) were measured by Cobas c311 Chemistry Analyzer System (Roche Diagnostics, Germany).

b- Prothrombin concentration was done by Thrombol –S Kit using Fibrintimer (Dade Behring-Germany).

c- Complete blood cell count (CBC) was done by CELL– DYN 3700 (Abbott Diagnostics, USA).

d- Hepatitis markers; HCV antibodies and HBs Ag were detected by Architect i1000SR system (Abbott Diagnostics, USA).

e- Detection of HCV-RNA and viral load was done by StepOne real-time PCR system (Applied Biosystem, USA) as follow:

- RNA Extraction

HCV RNA was extracted from serum samples using QIAamp Viral RNA Mini kit (Qiagen, Germany) following the manufacturer's instructions.

- Real time quantitative PCR

HCV viral load was quantified using specific TaqMan probe-based technology in a StepOne Real Time-PCR system (Applied Biosystems, USA). The reaction mixture was used in a total volume of 25 µ including 8.5 µ of the sample extract and 16.5 µ of the master mix which prepared by: 12.5 µ 2X RT-

PCR Buffer, 0.5 µ of HCV Forward primer, 0.5 µ HCV Revere primer, 0.5 µ HCV Probe, 0.5 µ IPC Forward Prime, 0.5 µ IPC Reverse, 0.5 µ Primer IPC Probe and 1 µ of 25X RT-PCR. Thermal profile was adjusted as follow: incubation at 45°C for 10 min to transcribe viral RNA to cDNA by reverse transcriptase (RT). This was followed by AmpliTaq gold activation at 95° C for 10 min, followed by 45 cycles of three PCR-steps amplification; denaturation at 95° C for 15 sec, f and annealing and extension at 60° C for 45 sec. The concentration of RNA copies in the sample was calculated automatically by the software provided by the manufacturer and was interpreted as IU/ml using a standard curve obtained from quantification of serial dilution of the included standards.

Assessment of serum level of Bcl-2 and Bax

Blood samples were withdrawn from the patients and controls for detection of serum level of Bcl-2 and Bax by Enzyme Linked Immunosorbent Assay using human Bcl-2 and Bax ELISA kit (Invitrogen™, USA). Specimens were allowed to clot for 2 hours at room temperature then centrifuged for 15 minutes at 1000×g. The supernatants were collected, divided into aliquots and stored at -80˚C (≤ 6 months) to avoid loss of bioactivity and contamination. This assay employs the quantitative sandwich enzyme immunoassay technique. The assay was done according to the manufacturer's protocol and results were read using the Stat fax 2600 microplate reader (Awareness Technologies, USA).

Statistical Analysis

The collected data were processed and analyzed using the Statistical Package for Social Sciences (SPSS) computer program version 16.0. Quantitative data were expressed as means and standard deviation or median and range. The non-parametric Mann–Whitney test was used for comparing two quantitative variables. Kruskal–Wallis test was used for comparison between more than two quantitative variables. Spearman's correlation coefficient was used for measuring correlation between the serum level of Bcl-2, Bax and Bcl-2/Bax ratio and the other laboratory parameters in all chronic HCV patients. A 5% level was chosen as a level of significance in all statistical tests used in the study. P- Values less than 0.05 were considered significant.

Results

The study included 70 chronic HCV patients (47 males and 23 females); their ages ranged from 40-66 years. A total of 15 healthy volunteers were included in the study as a control group. The clinical and the laboratory data of the three studied groups are shown in table 1 and 2.

Table 1. Clinical features of the HCV infected patients and controls

Parameters	Chronic HCV non-cirrhotic (group1) (n=35)	Chronic HCV cirrhotic (group2) (n=35)	Controls (group3) (n=15)
Age (Year)			
Mean ± S.D. Range	50.31±5.27 (40-58)	53.77±6.15 (45-66)	36.6±5.98 (28-49)
Gender			
Female Male	10 (28.5 %) 25 (71.5 %)	13 (37.1 %) 22 (62.9 %)	7 (46.7 %) 8 (53.3 %)
Jaundice	0	15 (42.9 %)	0
Ascites	0	32 (91.4 %)	0
Hepatomegaly	0	33 (94.3 %)	0
Encephalopathy	0	13 (37.1 %)	0
Vascular manifestations	0	18 (51.4 %)	0

Table 2. Comparison of the laboratory parameters between Chronic HCV patients and controls

Parameters	Chronic HCV non-cirrhotic (group1) (n=35)	Chronic HCV cirrhotic (group2) (n=35)	Controls (group3) (n=15)	*P*-value	Sig. group
Hb (g/dl)	13.65 ± 1.87	7.91 ± 3.11	14.38 ± 1.63	0.001	2 vs. 1,3
WBCs (/mm³)	5300 ± 1700	4903 ± 1140	5620 ± 1571	NS	NS
Lymphocyte count (/mm³)	1581 ± 453	1390 ± 420	1492 ± 413	NS	NS
Platelets (10^3/mm^3)	211 ± 43.00	119 ± 53.26	236 ± 53.01	0.007	2 vs. 1,3
P.T (s)	12.5 ± 0.5	13.1 ± 0.7	12 ± 0.9	<0.001*	2 vs. 1,3
Total bilirubin (mg/dl)	0.98±0.46	1.9±2.8	0.46±0.11	<0.001*	2 vs. 1,3
Direct Bilirubin (mg/dl)	0.26 ±0.18	1.04 ±2.47	0.13±0.35	0.022*	1,2 vs.3
Total protein (g/dl)	6.68±0.63	6.5±0.75	7.6±0.38	<0.001*	3 vs.1,2
Serum albumin (g/dl)	4.11±0.37	2.40±0.63	4.18±0.19	<0.001*	1,3 vs. 2
ALT (IU/L)	36.8 ±16.06	50.89±32.36	23±6.65	<0.001*	1,2 vs. 3
AST (IU/L)	56.69±25.57	51.74±26.77	23±5.23	<0.001*	1,2 vs. 3
HCV viral load (IU/ml)	1388257.2 ± 1734312.1	2289192.5 ± 2086144.6		0.004*	2 VS. 1

Hb: hemoglobin; WBCs: White blood cells; P.T: prothrombin time; ALT& AST, alanine & aspartate aminotransferase; NS, not significant. Data were expressed as mean±SD. * Statistically significant difference ($P < 0.05$)

Bcl-2 serum level showed a highly significant increase in chronic HCV non-cirrhotic patients while it decreased significantly in cirrhotic patients as compared to controls ($P<0.001$). On the contrary, there was a highly significant elevation of Bax serum level in cirrhotic patients and a significant decrease in non-cirrhotic group ($P<0.001$). Although, Bcl-2/Bax ratio showed a highly significant elevation in non-cirrhotic group, it decreased significantly in cirrhotic patients in comparison to controls ($P<0.001$). The comparison of serum Bcl-2, Bax and Bcl-2/Bax ratio between the three studied groups are shown in table (3) and Figures (1-4).

Table 3. Comparison of serum Bcl-2, Bax and Bcl-2/Bax between chronic HCV patients and controls.

Parameter	Chronic HCV non-cirrhotic (group1) (N=35)	Chronic HCV cirrhotic (group2) (N= 35)	Controls (group3) (N= 15)	*P*-value	*P1* (1 vs 2)	*P2* (1 vs 3)	*P3* (2 vs 3)
Bcl-2 (ng/ml)	13.2±7.71	3.7±2.4	5.9±4.31	<0.001*	<0.001*	0.001*	0.001*
Bax (ng/ml)	2.7±1.03	5.9±1.8	3.8±1.4	<0.001*	<0.001*	0.023*	<0.001*
Bcl-2/Bax ratio	7.1±6.13	0.82±0.65	1.46±0.87	<0.001*	<0.001*	0.001*	0.014*

Data were expressed as mean±SD. *$P< 0.05$ is significant

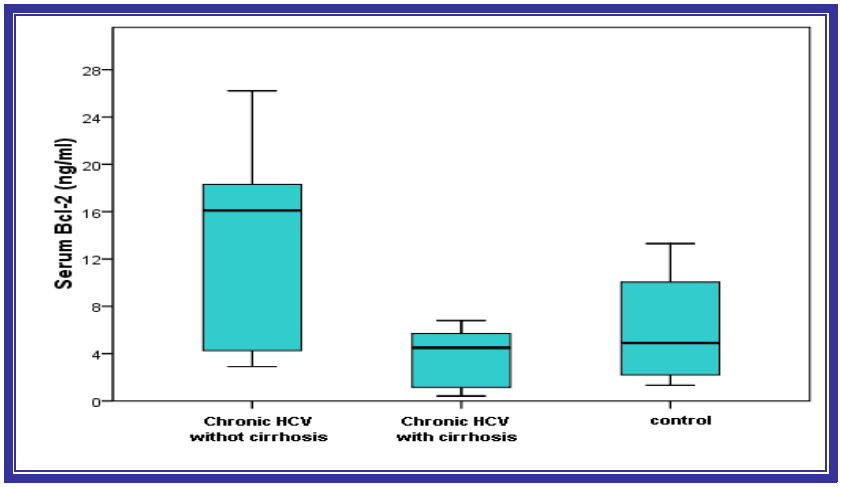

Figure 1. Box plot for comparison of the median of serum Bcl-2 among chronic HCV patients and controls.

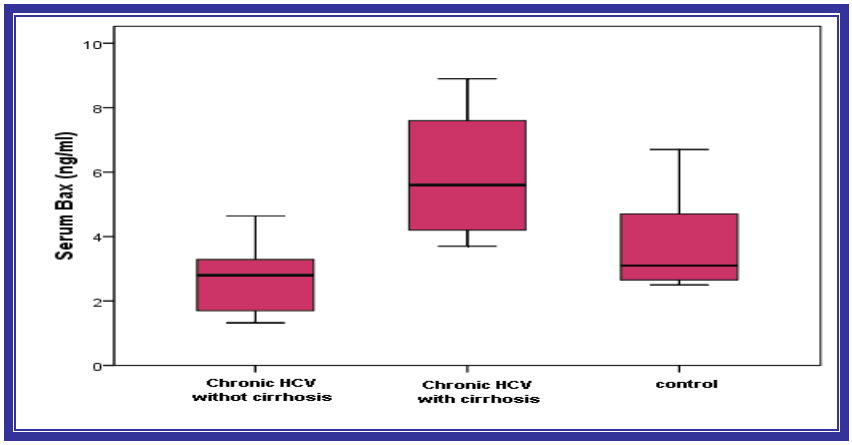

Figure 2. Box plot for comparison of the median of serum Bax between chronic HCV patients and controls.

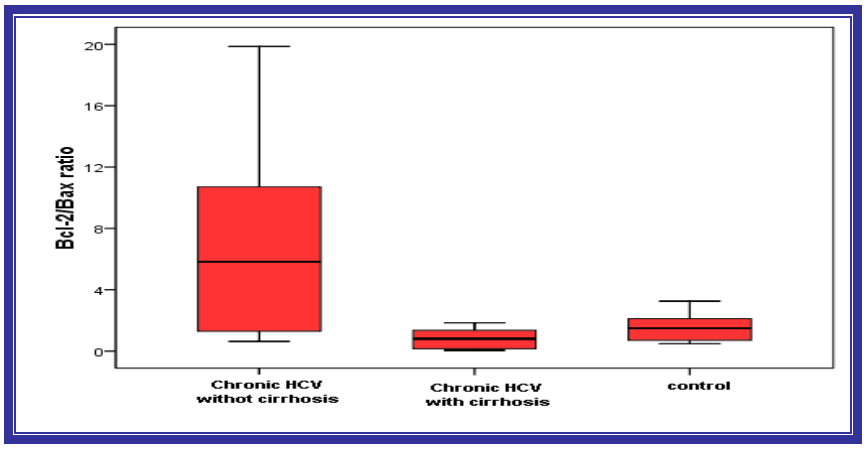

Figure 3. Box plot for comparison of serum Bcl-2/Bax ratio between chronic HCV patients and controls.

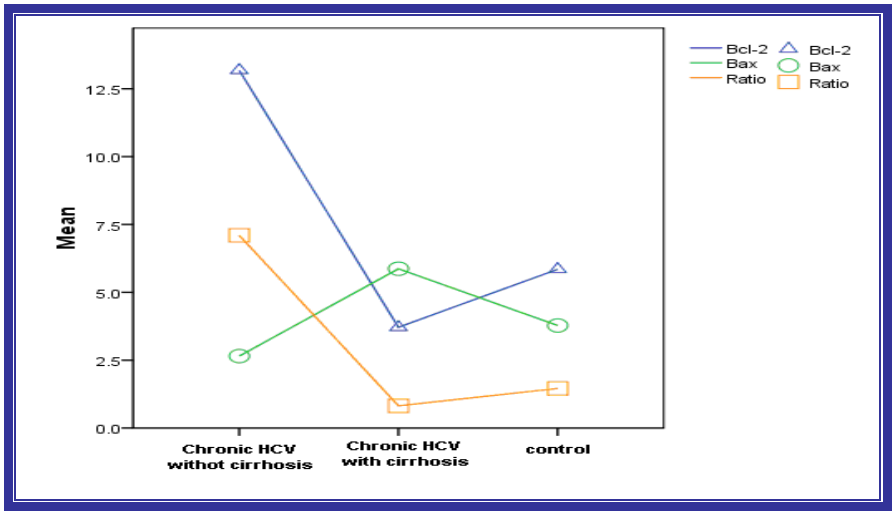

Figure 4. Mean plot for the mean values of serum Bcl-2, Bax and Bcl-2/Bax ratio in the three groups.

Serum apoptotic markers (Bcl-2, Bax and Bcl-2/Bax ratio) showed statistically significant positive correlations (P<0.05) with total protein, albumin, ALT, AST and HCV viral load of all the studied HCV patients while significant negative correlations were found with total and direct bilirubin (table 4). Moreover, positive correlations were noticed between serum Bcl-2, Bax, Bcl-2/Bax ratio and HCV viral load of non-cirrhotic group but negative correlations were reported in cirrhotic patients {table (5) and Figures (5-7)}.

Table 4. Correlation between serum Bcl-2, Bax, Bcl-2/Bax ratio and other parameters in all chronic HCV patients (n= 70).

Parameter	Bcl-2(ng/ml)		Bax (ng/ml)		Bcl-2/Bax ratio	
	r	*P*-value	r	*P*-value	r	*P*-value
Total bilirubin(mg/dl)	- 0.067	NS	0.071	NS	- 0.068	NS
Direct bilirubin(mg/dl)	- 0.192	NS	0.204	NS	- 0.207	NS
Total protein (g/dl)	0.517	< 0.001*	- 0.483	< 0.001*	0.536	< 0.001*
Albumin (g/dl)	0.882	< 0.001*	- 0.986	< 0.001*	0.933	< 0.001*
ALT(IU/L)	- 0.716	< 0.001*	0.662	< 0.001*	- 0.723	< 0.001*
AST(IU/L)	- 0.704	< 0.001*	0.472	< 0.001*	- 0.628	< 0.001*
HCV viral load (IU/ml)	- 0.416	< 0.001*	0.463	< 0.001*	- 0.462	< 0.001*
Bcl-2 (ng/ml)	--	--	- 0.922	< 0.001*	0.985	< 0.001*
Bax (ng/ml)	- 0.922	< 0.001*	--	--	- 0.969	< 0.001*

r: Spearman's correlation coefficient. **P*< 0.05 is significant.

Table 5. Correlation between serum Bcl-2, Bax, Bcl-2/Bax ratio and HCV viral load (IU/ml) in non-cirrhotic and cirrhotic patients (n= 70)

Parameter	Bcl-2 (ng/ml)		Bax (ng/ml)		Bcl-2/Bax ratio	
	r	*P*-value	r	*P*-value	r	*P*-value
HCV viral load (IU/ml) in non-cirrhotic group	-0.517	0.001*	0.513	0.002*	-0.513	0.002*
HCV viral load (IU/ml) in cirrhotic group	-0.009	NS	0.009	NS	-0.009	NS

r: Spearman's correlation coefficient. **P*< 0.05 is significant.

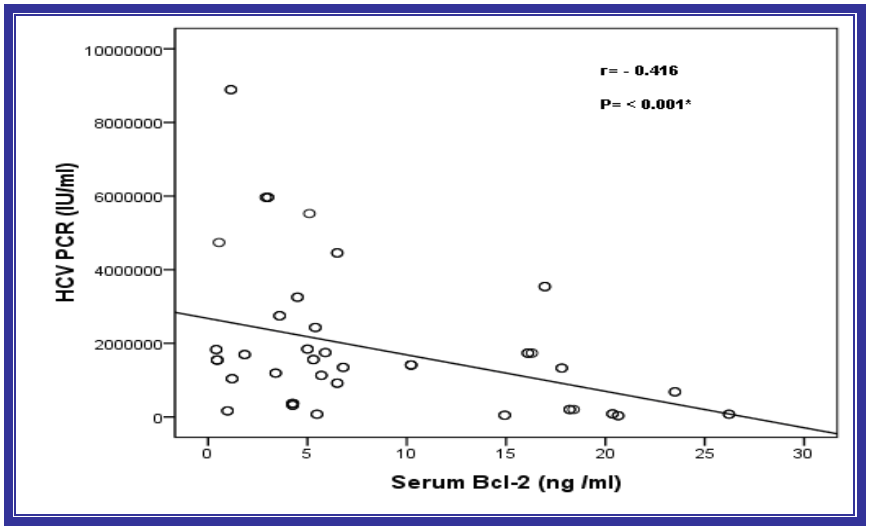

Figure 5. Scatter diagram for correlation between serum Bcl-2 and HCV viral load (IU/ml) in all chronic HCV patients (n= 70)

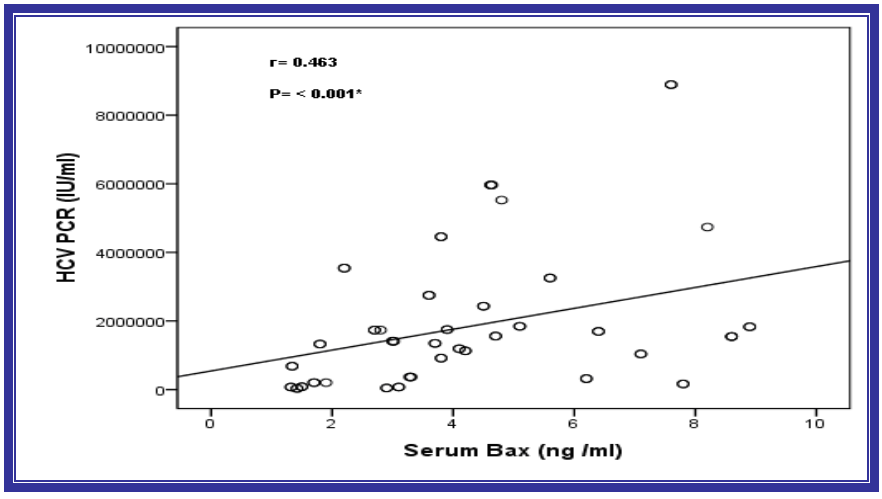

Figure 6. Scatter diagram for correlation between serum Bax and HCV viral load (IU/ml) in all chronic HCV patients (n= 70)

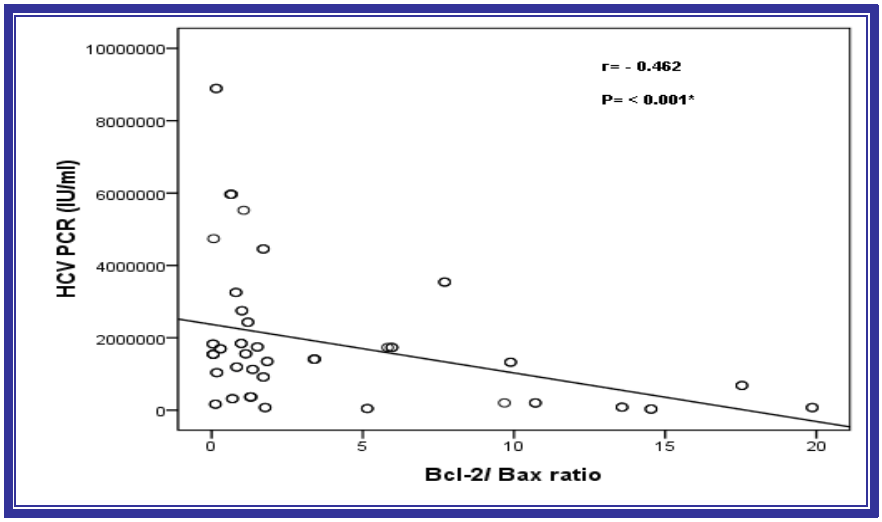

Figure 7. Scatter diagram for correlation between serum Bcl-2/ Bax ratio and HCV viral load (IU/ml) in all chronic HCV patients (n= 70).

Discussion

The present work showed a linear decrease of both Bcl-2 level & Bcl-2: Bax ratio and linear increase of Bax level in correlation with the degree of chronicity of the hepatic viral infection. These findings were in alignment with the results reported by Shehata *et al.* [14]. These authors reported a statistically significant down regulation of Bcl-2 percentage expression in peripheral blood lymphocytes together with a statistically significant increased expression of Bax and a significantly decreased Bcl-2/Bax ratio was observed in patients with cirrhosis and ascites.

The incriminating role of immunity and apoptosis in the pathogenesis of HCV infection was further reinforced by the study conducted by Nakamoto *et al.* [15]. They demonstrated that viral persistence and progression of liver disease in chronic hepatitis C is attributed to apoptosis of peripheral blood mononuclear cells (PBMC) subsets as a result of the down-regulation of Bcl-2expression, As Bcl-2 can block or delay the apoptotic death of a virus-infected cell, viral Bcl-2 homolog can contribute to viral latency or result in the establishment of persistent viral infection in the absence of cell lysis.

Tsamandas *et al.* [16] assessed the expression of Bcl-2 and Bax in liver biopsies from patients with chronic hepatitis C (HCV) by pathologic examination. The degree of liver impairment was negatively correlated with Bcl-2 protein expression and positively correlated with Bax expression. These data provided another evidence about the possible role of apoptosis role in the development and progression of liver disease. On estimation of the degree of inflammation and fibrosis there is a dose relation between the apoptotic process and the evolution and progression of human liver disease. They reported that apoptosis seems to play a significant role during the early stages of chronic liver disease and fibrosis, whereas the expression of anti-apoptotic

gene Bcl-2 mRNA, from hepatocytes, in the late fibrotic stages implies that this oncogene may be related to the presence of end-stage liver disease. These data coincided with the results of many previously published studies [17-18].

Alenzi *et al.* [19] concluded that in HCV infected patients, the proliferation activity is altered and apoptosis is apparently dysregulated. The estimation of apoptotic proteins may have a prognostic and/or diagnostic potential in these patients. They found that the serum Bcl-2 assayed by ELISA was elevated in 82 % in HCV infected patients HCC free versus 100% HCC patients, with no significant difference between the two groups ($P < 0.05$). Moreover, cytoplasmic staining of Bcl-2 as detected by immunohistochemistry was found in 16% of chronic HCV patients without HCC versus 8 % in HCC patients.

The results of the present study are not in congruent with that reported by El-Bendarya *et al.* [20] who found that there is lack of difference in Bcl-2 expression in peripheral blood T cells between HCV-infected patients, either compensated or decompensated, and normal controls. The finding that the expression of Bcl-2 is not increased in the peripheral T cells of patients with chronic HCV infection signifies that Bcl-2 does not exert an anti-apoptotic effect in HCV-infected patients.

Up till now, the precise ways in which viral proteins affect the cellular pathways of apoptosis and the ways that apoptosis contributes to the immune clearance or persistence of viral hepatitis remain unclear. One plausible mechanism is that the Apoptotic bodies containing intact virions may be phagocytosed by adjacent cells, thus promoting the spread of the virus without inducing an inflammatory response or invoking the immune response. Nevertheless, this postulated mechanism needs to be addressed in further studies [21].

In conclusion, apoptotic markers may be a useful non-invasive surrogate marker of disease activity in HCV infection; increased serum Bax level with decreased serum Bcl-2 level and Bcl-2/Bax ratio were detected with the progression of the disease that provide prognostic and/or diagnostic potential for chronic HCV infected patients.

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