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Correlation Between Serum Apoptotic Markers and Hepatitis C viral load In Chronic Hepatitis C Virus-Related Liver Disease

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Abstract

Hepatitis C virus (HCV) is considered the most common etiology of chronic liver disease in Egypt. Developing non invasive tests that can predict liver injury represents a growing medical need. Considering that hepatocyte apoptosis plays a role in chronic hepatitis C pathogenesis; evaluation of the presence of different apoptotic markers that correlate with liver injury in HCV- related liver diseases could be a way for this achievement. The aim of the present study was to identify the correlation between serum apoptotic markers **Bcl-2** and Bax with hepatitis C viral load in the chronic hepatitis C virus related liver diseases. The study included three groups ; group **1: 35** compensated chronic HCV patients without any suspected evidence of liver cirrhosis and with Child - Pugh score of **5-6** (class A), Group **2: 35** decompensated chronic HCV patients with Child - Pugh score of **10-15** (class C) and group **3: 15** healthy volunteers as a control group. The results collectively suggest that statistically significant correlations were noticed between serum Bcl-**2**, Bax, Bcl-**2**/Bax ratio and HCV PCR in chronic hepatitis C virus-related liver disease.

Keywords: Chronic HCV, hepatitis, Bcl-**2**, Bax

Introduction

Hepatitis C virus (HCV) infection is a serious health problem as it is the major cause of chronic hepatitis. Globally, there is continuous increase in the morbidity and mortality attributable to HCV infection. Approximately **700,000** persons die each year from HCV-related complications, which include cirrhosis and hepatocellular carcinoma (HCC) all of which could be avoided if proper antiviral treatment is provided **(WHO, 2016).** However, there are problems facing HCV curing such as the asymptomatic nature of the disease that contributes to the unawareness of infected persons of their infection, in addition those who are diagnosed, the access to their treatment remains low in many settings **(Esmat, 2013).**

The pathogenesis of chronic HCV infection has not been clearly defined. HCV is an RNA virus without apparent cytopathic effects, therefore in chronic infection the hepatocellular damage is generally believed to be immune-mediated **(Kar and Hazam, 2009).**

Apoptosis or programmed cell death plays a pivotal role in the maintenance of cellular homeostasis through removal of aged, damaged, and hyperproliferative cells **(Guilherme et al., 2012).** Apoptosis of hepatocytes has a significant role in the pathogenesis of HCV infection evidenced by liver inflammation and fibrosis. There are alternative sites of HCV replication, such as the PBMCs, therefore, chronic hepatitis C should be considered as a

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systemic disease rather than a local one. Moreover, the HCV ability to directly infect those cells might affect their function and down-modulate apoptotic events leading to chronic viral replication in target cells **(Garcia, 2012).**

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 specifically considered an important anti-apoptotic protein and is thus classified as an oncogene. It regulates programmed cell death by providing a survival advantage to rapidly proliferating cells while Bax protein promotes apoptosis by enhancing cell susceptibility to apoptotic stimuli. Bcl-**2** inhibits apoptosis by interacting and forming inactivating heterodimers with Bax **(Panasiuk et al., 2010)**. Considering the high prevalence of HCV infection in Egypt, the current study was conducted to assess the correlation between apoptotic markers Bcl-**2** and Bax with hepatitis C viral load In chronic hepatitis C virus-related liver disease.

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 January **2015** to June **2015**. 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 according to Child – Pugh score table (**1)** :

; 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. 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 **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 patteren and size of the liver and the presence of periportal fibrosis, the presence of ascites, the size of spleen or any other abnormalities.

obtained from all participants.

-Laboratory investigations:

a- Liver function tests (total protein, albumin, total bilirubin, direct bilirubin, SGOPT and SGPT) were measured by Cobas c**311** 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:

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***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 **2**X 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 **25**X 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.

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Results

The study included group **1:** include **35** compensated chronic HCV patients (**25** males) their ages ranged from **40 – 58** years with a mean ± S**.D of 50.31 ± 5.27** years, group **2:** include **35** decompensated chronic HCV patients (**22** males) their ages ranged from **45 – 66** years old with a mean ± **S.D of 53.77 ± 6.15** years, group **3: 15** healthy individuals as a control group (**8** males) their ages ranged from **28 – 49** years with a mean ± S.D of **36.6 ± 5.98** years.

The clinical and the laboratory parameters in the three studied groups are shown in table (**2** and **3**).

The comparison of serum Bcl-**2**, Bax and Bcl-**2**/Bax ratio between the three studied groups are shown in Figure **(1),** Bcl-**2** serum level showed a highly significant increase in group 1 while it decreased significantly in group **2** as compared to controls (P value < **0.001**). On the contrary, there was a highly significant elevation of Bax serum level in group 2 and a significant decrease in group **1** (P value **< 0.001**). Although, Bcl-**2**/Bax ratio showed a highly significant elevation in group 1, it decreased significantly in group **2** in comparison to controls **(P value < 0.001).**

Table (4) and figures **(2-7)** showed Correlation between serum Bcl-**2,** Bax expression, Bcl-**2**/Bax ratio and HCV PCR (IU/ml) in group **1** and group **2** patients. Statistically significant correlations (P. value ≤ **0.05)** were noticed between Bcl-**2,** Bax expression, Bcl-**2**/Bax ratio and HCV PCR in group **1** but negative correlations were reported in group **2.**

Table (1) : Child – Pugh score

Measure	1 point	2 points	3 points
Total billirubin (mg/dl)	**< 2**	**2 - 3**	**> 3**
Serum albumin (g/dl)	**> 3.5**	**2.8 – 3.5**	**< 2.8**
Prothrombin time (s)	**< 4**	**4 - 6**	**> 6**
Ascities	**None**	**Mild or suppressed with medication**	**Moderate to sever or refractory**
Hepatic encephalopathy	**None**	**Grade I - II**	**Grade III - IV**

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Table (2): Clinical features of the HCV infected patients and controls

Parameters	**Group 1 (n=35)**	**Group 2 (n=35)**	**Group 3 (Controls) (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**

Group I: chronic HCV patients with no evidence of cirrhosis (asymptomatic group); group **2:**chronic HCV patients with cirrhosis (decompensated group); group **3:** healthy controls (control group)

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Table (3): Comparison of the laboratory parameters between Chronic HCV patients and controls

Parameters	Group 1 (n=35)	Group 2 (n=35)	Group 3 (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**	**0.106**	***NS***
Lymphocyte count (/mm³)	**1581 ± 453**	**1390 ± 420**	**1492 ± 413**	**0.098**	***NS***
Platelets (10 ^{9}/L)	**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 (g/dl)	**36.8 ±16.06**	**50.89±32.36**	**23±6.65**	**<0.001***	***1,2 vs. 3***
AST (g/dl)	**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***

Group I: chronic HCV patients with no evidence of cirrhosis (asymptomatic group); group **2**:chronic HCV patients with cirrhosis (decompensated group); group **3:** healthy controls (control group) 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**)

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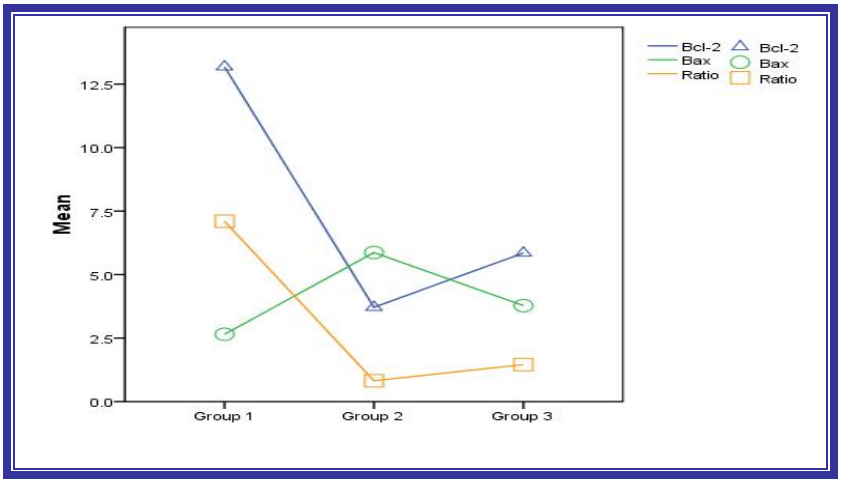

Figure (1): Mean plot for the mean values of serum Bcl-2, Bax and Bcl-2/Bax ratio in the three groups

Table (4): Correlation between serum Bcl-2, Bax, Bcl-2/Bax ratio and HCV viral load (IU/ml) in group 1 and group 2 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 group 1	-0.517	0.001*	0.513	0.002*	-0.513	0.002*
HCV viral load (IU/ml) in group 2	-0.009	0.957	0.009	0.957	-0.009	0.957

r: Spearman's correlation coefficient
p< 0.05 is significant

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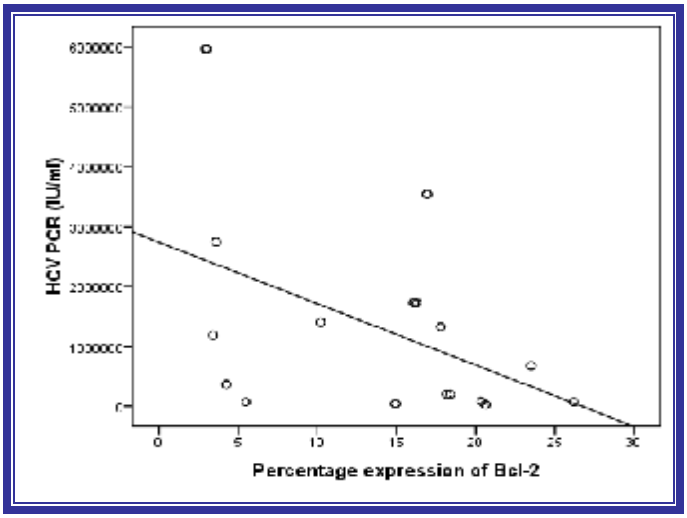

Figure (2): Scatter diagram for correlation between **Bcl-2** percentage expression and HCV PCR (IU/ml) in group **1 (n= 35)**

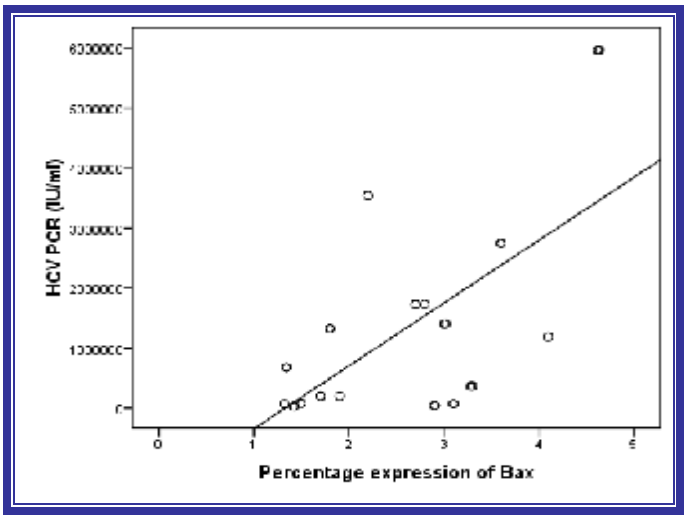

Figure (3): Scatter diagram for correlation between Bax percentage expression and HCV PCR (IU/ml) in group1 **(n= 35)**

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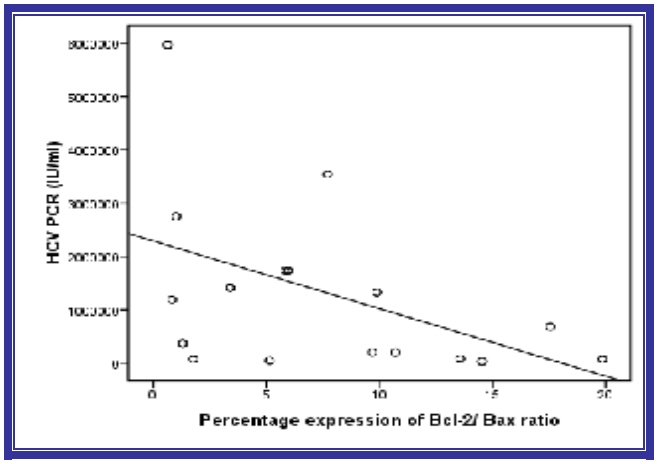

Figure (4): Scatter diagram for correlation between Bcl-**2/** Bax ratio percentage expression and HCV PCR (IU/ml) in group 1 (n = **35)**

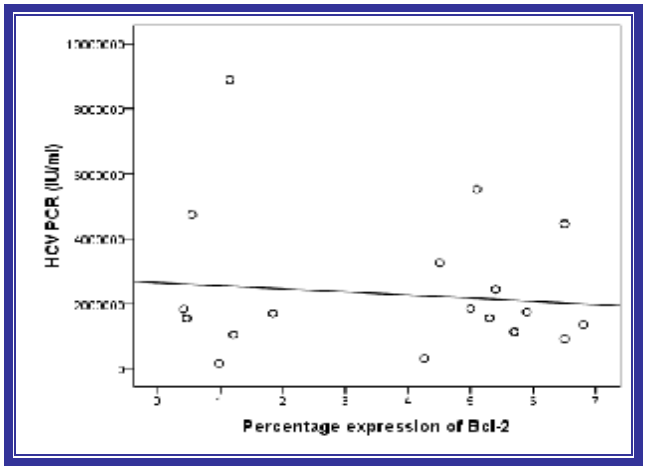

Figure (5): Scatter diagram for correlation between Bcl-**2** percentage expression and HCV PCR (IU/ml) in group **2 (n= 35)**

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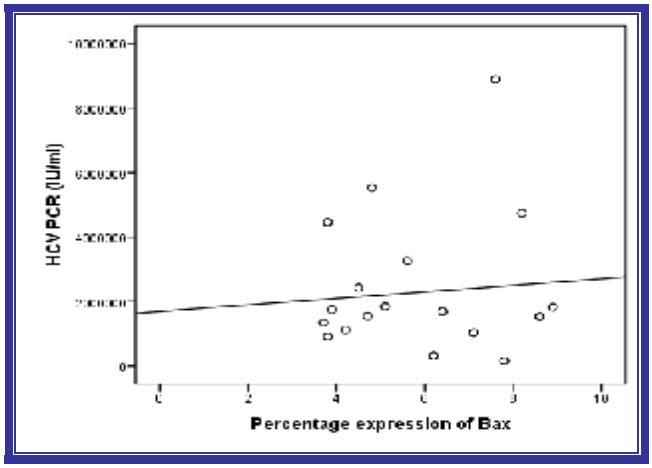

Figure (6): Scatter diagram for correlation between Bax percentage expression and HCV PCR (IU/ml) in group **2 (n= 35)**

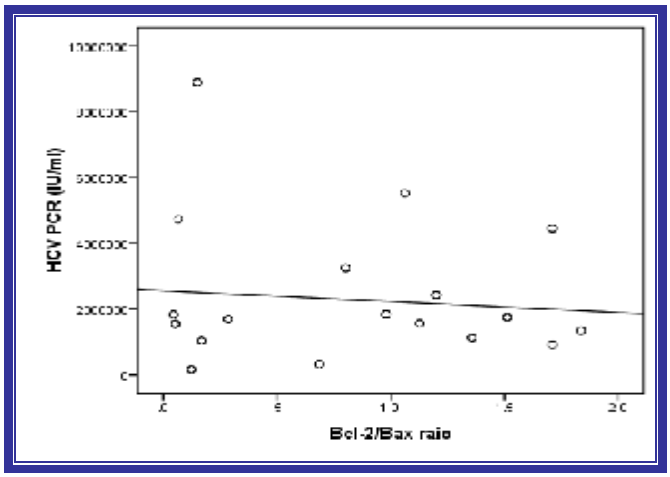

Figure (7): Scatter diagram for correlation between Bcl-**2**/Bax percentage expression and HCV PCR (IU/ml) in group **2 (n= 35)**

Discussion

This study showed the correlation between serum Bcl-**2**, Bax expression, Bcl-**2**/Bax ratio and HCV PCR (IU/ml) in group **1** and group **2** patients. It showed Statistically positive significant correlations (P. value ≤ **0.05**) between Bcl-**2,** Bax expression, Bcl-**2**/Bax ratio and HCV PCR in group **1** but negative correlations were reported in group **2**, also the study found increase in serum Bax, decrease of serum Bcl-**2** and decrease Serum Bcl-**2**/Bax ratio with chronic HCV infection. These results are in agreement with a study done by **Chen et al., 2000** found that the expression level of Bax subfamily in the decompensated chronic hepatitis was significantly higher than that of Bc1-subfamily and suggested that Bcl-family is involved in the regulation of apoptosis in the chronic liver diseases. Also **Papakyriakou et al., 2002** provided evidence of increased apoptosis in severe chronic viral liver disease without

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over expression of the apoptosis inhibiting Bcl-**2** protein.

Alenzi et al., 2010 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.

Nakamoto et al., 2002 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-**2**expression, 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.

On the other hand, **El-Bendarya et al., 2014** 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.

Conclusion

There is correlation between apoptotic markers and HCV PCR (IU/ml) in chronic hepatitis C virus-related liver disease so that apoptotic markers may provide prognostic potential for chronic HCV infected patients.

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