A Study of BCL-2 Oncoprotein among Egyptian Patients with Chronic Hepatitis C and Patients with Hepatocellular Carcinoma

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ABSTRACT

Objective: To investigate the presence of bcl-2 protein as an apoptotic marker in serum of Hepatitis C positive patients with and without hepatocellular carcinoma and correlate them with liver function tests and AFP.

Methods: Three group of patients were included in this study, their age ranged from 20 to 70 years: Group I (healthy control) No = 20. Group II (Non-HCC) No = 20. Group III (HCC) No = 20.

HCV was diagnosed based on HCV-RNA positivity using polymerase chain reaction (PCR). HBsAg and anti-HCV antibodies were detected enzyme-linked immunosorbent assay (ELISA) technique. Intracellular BCL2 oncoprotein was detected by flow cytometer.

Results: Results of the study showed that it shows increase in the level of AST and ALT among patients infected with HCV with or without HCC than normal subjects. AFP is significantly higher among group II and group III that group I (P = 0.001). BCL2 was higher among group II and group III in comparison to group I. With a statistically significant difference (P = 0.001, 0.0 respectively), regarding BCL-2, it was significantly higher in group III in comparison with group II (P = 0.0).

Conclusion: Apoptotic markers like FAS and BCL-2 play important role in the pathogenesis and outcome of liver diseases especially caused by hepatitis viruses.

KEY WORDS

Hepatitis c, carcinoma, BCL-2, flow cytometer, FAS

INTRODUCTION

Hepatitis C virus (HCV) is an important cause of persistent infection of the liver and can progress to hepatic cirrhosis and leads to hepatocellular carcinoma (HCC)¹. Multiple factors are responsible for disease progression. Host viral interaction is influenced by several factors that result in a unique individual disease pattern²).

Hepatocellular carcinoma (HCC) caused by hepatitis c virus is a major health problem especially among Egyptian patients³. Other etiologic factors associated with HCC such as hepatitis B (HBV) viruses, excess alcohol consumption and exposure to fungal aflatoxin also exits⁴. It is important to understand the mechanism that orchestrate HCV-mediate oncogenesis⁵. HCV encodes a single polyprotein, which is processed to yield at least 10 proteins (structural and non-structural)⁶).

HCV infection particularly its proteins are responsible for regulating transcriptionally cellular genomes especially apoptotic genes as reported by many studies⁷).

Apoptosis is cell death directed by specific genes regulating. This process is characterized by cytoplasmic fragmentation and nuclear condensation⁸,⁹. Apoptosis process is one of the main mechanisms explain the pathogenesis of chronic viral hepatitis patients⁴,⁵,¹⁰. The mechanism of apoptosis is initiated first by death receptor activation of the mitochondria; this process cause’s release of specific proteins called apoptotic proteins. These proteins are mainly responsible for most of changes observed during apoptosis process as biological and morphological changes¹¹).

Regarding apoptosis, there are two pathways, one is the extrinsic pathway which is activated by ligand engagement of cell surface death receptors and the other is the intrinsic (mitochondrial) pathway. BCL-2 family of proteins that regulate activation of the intrinsic apoptotic pathway in response to cellular stresses such as DNA damage, γ-irradiation¹²).

Regarding the types of BCL2 there are several types. These types
are divided mainly into the pro and anti-apoptotic types. BCL-2, and other types like BCLXL, BCL-w, Mcl-1, Brag-I, and AI inhibit the apoptosis process, whereas Bax, Bid, Bak, and Bad promote apoptosis. There must be a balance as excess apoptosis leads to severe damage to the liver also failure of apoptosis is an important determinant for the development of hepatocellular carcinoma.

In studying Bcl-2 there is an antigen and a protein, antigen is a protein that cause inhibition of cell death by apoptosis mechanism, and Bcl-2 protein prevent apoptosis of the normal liver cells and also enhances the antioxidant capacity of the cells so prolong the liver cell survival.

In case of chronic hepatitis caused by hepatitis c, bcl-2 expression is increased to accept the publication of their results.

Assessment of Bcl-2 expression in cancer tissues can be done by immunohistochemistry. However, its detection requires the availability of cancer tissues from biopsy specimen and if take long time in addition to the time needed for preparation of specimen and interpretation of the results.

Several methods can be used to detect BCL-2 as immunohistochemistry flow cytometer and Western blot. The advantage of the Flow cytometer over other methods, as this method is rapid, sensitive and simple. In addition, it can be used to detect more than one marker at the same site.

Our study aimed to investigate the presence of bcl-2 protein as an apoptotic marker in serum of Hepatitis C positive patients with and without hepatocellular carcinoma and correlate, them with liver function tests and AFP.

Subjects and Methods

Patients in the present study were collected from Gastroenterology and hepatology unite, specialized medical hospital, Mansoura University during the period between May 2017 to December 2018.

Three group of patients were included in this study, their age ranged from 20 to 70 years:

**Group I (healthy control) No = 20**: (10 males and 10 females), ranged from 20 to 70 years. The Mean ± SD is 53.62 ± 5.030.

**Group II (Non-HCC) No = 20**: (13 males and 7 females). Mean ± SD of 54.350 ± 5.875.

**Group III (HCC) No = 20**: (8 males and 7 females) with a mean ± SD of 52.24 ± 6.09.

Ethical guidelines of Research Ethics committee for clinical studies from Gastroenterology and hepatology unite, specialized medical hospital, Mansoura University was approved before the start of this research.

Patients included in the study fulfilled these inclusion criteria: age 20-70 years, ALT and AST is high within 6 months prior to the study, positive HCV antibodies, HCV-RNA, type 4 genotype of HCV and histological proven chronic hepatitis with or without HCC.

Exclusion criteria in this study is autoimmune hepatitis, any chronic diseases patient with positive Hepatitis B surface antigen.

Patients and control subjects included in the study were asked to give written informed voluntary consent to participate in the study, and to accept the publication of their results.

From patients and control, 5 ml of venous blood was collected. This sample is divided into two tubes:

i. Routine investigations: complete blood count by SYSMEX NE 1500. Liver function tests AST, ALT, Total protein, Albumin, Total bilirubin and conjugated bilirubin by the fully automated chemical auto-analyzer (Dimension-ES).

HbsAg and anti-HCV antibodies were detected by enzyme-linked immunosorbent assay (ELISA) technique (Biopkit, S.A, Barcelona, Spain).

1. Three ml in a plastic tube and left to clot then centrifuge, serum separated is used to determine HCV-antibodies, complete liver function, alpha-fetoprotein and qualitative HCV- RNA PCR.

2. Two ml of blood were collected in EDTA tube for complete blood count (CBC) and for assessment of BCL-2 oncoprotein by flow cytometer.

Patients and controls were subjected to

ii. Virologic assessment

HCV-RNA viral load was determined using Real-Time PCR. HCV Genotype was determined by real-time PCR.

iii. Detection of intracellular BCL-2 oncoprotein.

- Prepare two-tube one test and the other is control each tube 50-µl blood samples.
- Prepare two-tube one test and the other is control each tube 50-µl blood samples.
- Vortex each tube then incubates for 15 minutes at room tempera-

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**Table 1. Characteristic data of the studied groups**

<table>
<thead>
<tr>
<th></th>
<th>Control No= 20</th>
<th>Without HCC No = 20</th>
<th>With HCC No = 15</th>
<th>P value</th>
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<td>65</td>
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<td>Above 50Yrs</td>
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<td>7</td>
<td>35</td>
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<td>Mean</td>
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<td>54.350 ± 5.875</td>
<td>52.24 ± 6.09</td>
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<td>Hb:</td>
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<td>No %</td>
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<tr>
<td>Below 8 g/dl</td>
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<td>10</td>
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<tr>
<td>Above 8 g/dl</td>
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<tr>
<td>Above 4x10⁶/µl</td>
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<td>Mean ± SD</td>
<td>7.23 ± 1.64</td>
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<td>95</td>
<td>14</td>
<td>70</td>
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<tr>
<td>Mean ± SD</td>
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<td>07.0 ± 53.0</td>
<td>112.0 ± 30.0</td>
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</table>

HCC- Hepatocellular carcinoma- *statistically significant
ture, and then add PBS and centrifugate for 5 minutes at 3000 r.p.m.
-100 μl of Dako intrastrain B (Permeabilization) is added to the pellet.
-Ten μl of FITC-conjugated monoclonal antibody is added as control. To the test tube, ten μl of FITC conjugated monoclonal mouse anti-human BCL-2 oncoprotein is added, vortex the two tubes, then incubate both tubes in dark at room temperature for 15 min., after that add 2 ml of PBS then centrifuge again for 5 min.
-Finally, resuspend the pellet in 0.5 ml PBS.
-Analyze by flow cytometer.

**BCL-2 analysis by flow cytometer:**
-Analysis of the samples were done by using (Coulter EPICS XL-MCL Argon Laser, U.S.A) flow cytometry. After warming up the argon laser (488 nm) of the flow cytometry for 30 minutes, then load the protocol of analysis.
-Analysis of the isotopic control was done before analysis of monoclonal antibody (BCL-2 oncoprotein) where the light scatter was received on both forward and side scatter detectors to show the cell population in the basic histogram and to exclude the non-specific binding.
- Sample analysis done by gating on 5000 lymphocytes.

**Statistical Analysis:**
-Data analysis in the study was done using SPSS version 11. Comparison between studied groups was done by Mann Whitney-T test.

**RESULTS**

Table 1 shows demographic data of studied groups showing HCV infected patients without HCC were common among male patients (75%) than female patients. Also, HCC is more common among male than females (53.3%). HB, RBCs count and platelet count were significantly lower among group II and III in comparison with group I (p value = 0.001, 0.01 and 0.001, respectively).

Table 2 shows biochemical data of HCV infected patients with and without HCC. It shows increase in the level of AST and ALT among patients infected with HCV with or without HCC than normal subjects.

Table 3 shows a significant difference between the three different groups regarding result of AFP (P = 0.001).

Table 5 shows that BCL2 was significantly higher among group II and group III in comparison with group I. (P = 0.001, 0.0), regarding BCL-2 results, it was higher in group III in comparison with group II with a statistically significant difference (P value = 0.0).

**DISCUSSION**

The current study was conducted in Gastroenterology and hepatology unite, specialized medical hospital, Mansoura University. It was per-
formed of three groups (group 1 includes healthy subjects matching the same age and the socioeconomic status. This group was taken as a control group. Group 2 consist of 20 patients with hepatitis C virus infection without hepatocellular carcinoma and group 3 included 15 patients with hepatitis C viral infection and hepatocellular carcinoma mean age of the studied groups was (53.62 ± 5.030, 54.350 ± 5.875 and 52.24 ± 6.09 respectively).

In this study, several factors were analyzed and are associated with prognosis of the disease. Of these factors, HB, RBCs and platelets ALT and AST, α-fetoprotein (AFP), and viral genotype. HB, platelets, RBCs were significantly lower among group II and group III in comparison with the control group. (P value = 0.01 for HB and 0.001 for RBCs and platelets respectively.

AFP is a specific antigen and it is the most common serological biomarker and is most important for serological screening and early diagnosis of HCC. However, its diagnostic accuracy is unsatisfactory as it has a wide variation in its sensitivity and specificity of AFP is considered as non-specific high biomarker especially in the early stages of HCC.

In this study, regarding the results of AFP, there was a significant difference between the three studied groups (P = 0.001). Sensitivity was 75% while specificity 65%. Similar results were detected in many studies. Another recent study by Alhadi et al. reported that AFP serum level had greatly increased in the cancer group by 154-fold as compared to the liver cirrhosis group.

A study by Daniele et al. showed that AFP sensitivity of AFP was 41-65% and the specificity was 80-90% when used to detect HCC and the cut-off for AFP was 20 ng/ml. Johnson showed that elevated levels of AFP could also be found in non-malignant chronic liver disease. For this reason, AFP cannot be used as a screen for and diagnose HCC a sole tool and we have to depend on other marker soon to complement the role of AFP and improve the outcome of these patients.

Regarding the results of AST, ALT among patients in this study. It was significantly higher among patients with chronic hepatitis and with hepatocellular carcinoma than normal subjects. These results were in agreement with several studies.

BCL-2 could be detected by several methods, like flow cytometer, Western blot and immunohistochemical. Flow cytometer has several advantages than other methods, as it is simple, easy and rapid method.

Results of BCL-2 in our study showed higher expression on patients with HCC compared with those normal subjects matched by age and sex (p = 0.001). Also, it was significantly higher in group I patients with chronic hepatitis C without HCC rather than those with HCC and the control (P = 0.000 in both groups).

These finding are also in agreement with Tsamandas et al. in his study. BCL-2 was significantly higher among patients with chronic hepatitis. Nakamoto et al., in another study demonstrated that the down-regulation process of BCL-2 expression might cause viral resistance and consequent progression of the disease among infected patients.

Our results agreed with El- Araby et al., study who reported that the level of BCL-2 is expressed highly in patients with HCV- viral infection than normal subjects. Also, it was significantly higher in HCC patients with liver cirrhosis. Another study by Placzek et al, reported an increase in the apoptosis in chronic HCV patients but no overexpression of apoptosis inhibiting BCL-2 protein. In his study that apoptosis can be because of host mechanism in order to decrease and limit viral replication and spread. Viruses have several strategic ways to delay or block the apoptotic process thus allow the virus to replicate. Some viruses also can induce apoptosis process by acting on intra-cellular mechanisms in the host cells. The apoptotic bodies that carry the intact virion can be phagocytosed by the nearby cells and this can promote spread of virus without any inflammatory reactions. The ways in which the virus and its proteins can affect the cellular pathway of apoptotic mechanism and the way of immune-clearance or persistence of the virus is still not clear.

BCL-2 can cause a delay or inhibit apoptotic death of the viral infected cells, consequently the viral BCL-2 homology can cause viral latency or cause establishment of viral infection in the absence of cell lysis.

Cell death is not significantly caused by reduction in BCL-2 oncprotein but the cell will be it more liable to apoptotic process, expression of BCL-2 also is a not a protector of apoptotic process. Thus, BCL-2 does not universally present apoptosis and independent mechanisms are present for regulation of apoptosis process among infected patients.

CONCLUSION

Apoptosis is important in the pathophysiology of Human liver diseases especially in those patients infected with chronic hepatitis C virus. It is important to understand the mechanisms of apoptosis and its regulation as it will improve the methods for diagnosis and therapeutic modulation.

Induction of apoptosis may be useful for treatment of some disorders which apoptosis is suppressed and on the other hand, in some disorders that are characterized by excessive apoptosis, its inhibition of this process is useful in treatment of such conditions.

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REFERENCES

12) Muller M, Strand S, Hug H, Heinemann E, Walczak H, Hofmann W, Strommel W. Drug induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild type p53. J Clin Invest 1997; 99: 403-
BCL-2 Oncoprotein among Egyptian Patients with Chronic Hepatitis C and Patients with Hepatocellular Carcinoma


34) Aghoram R, Cai P, Dickinson JA. Alpha-fetoprotein and/or liver ultrasonography for screening of hepatocellular carcinoma in patients with chronic hepatitis B. *Cochrane Database Syst Rev* 2012; Cd 002799.