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Original Article:

Investigation of Bcl-2 and PCNA in Hepatocellular Carcinoma: Relation to Chronic HCV

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ABSTRACT

Background and Objectives: Bcl-2 family members can be functionally divided into anti-apoptotic and pro-apoptotic groups. The balance between these two groups may determine the fate of tumor cells. In hepatocellular carcinoma (HCC), this balance is often tilted towards the anti-apoptotic members in tumor cells, leading to resistance to cell death and rapid proliferation.

Material and Methods: In the current study, we investigated Bcl-2 and proliferating cell nuclear antigen (PCNA) immunohistochemically, using specific monoclonal antibodies in liver tissues obtained from two patient groups. The first group included fifty patients infected with hepatitis C virus (HCV) without hepatocellular carcinoma, the other group included twenty five HCV-infected patients but with confirmed HCC. Serum Bcl-2 was assayed using enzyme immunoassay.

Results: Results showed serum Bcl-2 was elevated in 82% versus 100% in HCC-free and HCC patients, respectively. Moreover, cytoplasmic staining of Bcl-2 was found in only 16% of chronic HCV patients without HCC, versus 8% in HCC patients. On the other hand, nuclear staining of PCNA was detected in 100% of HCC patients, but in none of the HCV patients without HCC.

Conclusion: The results collectively suggest that in HCV-infected patients with and without HCC, apoptosis is dysregulated and proliferation activity perturbed. There may be prognostic and/or diagnostic potential in estimating Bcl-2 and PCNA proteins in these patient groups.

Key Words: *Bcl-2 – PCNA – Apoptosis – HCC – HCV.*

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INTRODUCTION

According to the World Health Organization (WHO), approximately 350 million people are chronically infected with HBV and 170 million are infected with HCV **[1,2]**. Egypt is overwhelmed by the highest prevalence of HCV in the world, with estimates ranging from 6 to 28% and a reported average of approximately 13.8% **[3,4]**. Many investigations in Egypt have also shown the increasing importance of HCV infection in the etiology of HCC, now estimated to account for 40-50% of cases **[5-7]**. Just as many people were infected with HBV as with HCV during the parenteral antischistosomal therapy campaign; however, HBV only caused chronic infections in 5% or less of infected individuals, whereas chronic HCV infection developed in 70% to 80% **[8,9]**. Hepatocellular carcinoma (HCC) comprises nearly 6% of all incident cancer cases worldwide, with the overwhelming majority occurring in the developing world. One of the least curable malignancies, HCC is the third most frequent cause of cancer mortality among men worldwide **[10]**. Hepatocellular carcinoma is the second most frequent cause of cancer incidence and mortality among men in Egypt **[11]**.

Apoptosis is widely accepted as a prominent tumor-suppression mechanism. Bcl-2 family has emerged as a dominant regulator of apoptosis in cancer cells. The mitochondrial-mediat-

ed pathway of apoptosis is regulated by the Bcl-2 family of anti-apoptotic (Bcl-2, Bcl-xl, Mcl-1) and pro-apoptotic proteins (Bax, Bad, and Bak). Bcl-2 inhibits apoptosis by interacting and forming inactivating heterodimers with Bax/Bak **[12]**. Defects in apoptosis signaling contribute to tumorgenesis and chemotherapy resistance of HCC cells. In HCC, there is an imbalance between the pro- and anti-apoptotic effect(s) of Bcl-2 family members. The expression of anti-apoptotic Bcl-xL and Mcl-1 is increased in HCC, whereas the expression of pro-apoptotic bid and Bak proteins is decreased (reviewed in 13).

Proliferating cell nuclear antigen (PCNA), an essential regulator of the cell cycle, is a 36kDa molecule which is highly conserved among species. It has been shown that PCNA serves as a co-factor for DNA polymerase delta in S-phase and is involved in DNA repair during DNA synthesis **[14,15]**. The temporal pattern of PCNA expression makes it a useful tool to study cell proliferation. It starts to accumulate in the G1 phase of the cell cycle, reaches the highest level during the S phase and decreases during the G2/M phase **[16]**. PCNA was found valuable in studying the proliferative activity in different tumors including HCC **[17,18]**. The obtained data give a clear evidence that the proliferation activity and the programmed cell death are altered in HCC patients with HCV differently from those without HCV.

In the current study, we investigated the Bcl-2 and PCNA proteins expression immunohistochemically in liver tissues, and also measured serum Bcl-2 using enzyme immunoassay, in order to study the prognostic and/or diagnostic potential of these two markers in HCV infected patients with and without HCC.

MATERIAL AND METHODS

Study subjects:

The present study was conducted on 75 HCV infected patients (50 patients without focal lesions and the other 25 patients with focal lesions diagnosed as hepatocellular carcinoma-HCC) from the endoscopy unit and the surgery department of National Liver Institute (NLI), Minufiya University, Minufiya, Egypt during the time period from January 2007 to May 2008.

The patients were classified into two groups, the first group was chronic HCV patients without HCC, and the second were HCV infected patients with HCC.

All patients gave a thorough medical history, and received a complete clinical examination, abdominal ultrasound and laboratory investigations including; total bilirubin (TB), direct bilirubin (DB), total protein (TP), serum albumin (S.Alb), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and serum creatinine. Hepatitis B surface antigen (HBsAg), anti-HCV antibodies were detected by ELISA (Diasorium Kit; Diasorium SR, Italy) and RT-PCR for HCV RNA (Amplicor PCR; Roche Molecular Systems, Inc., Pleasanton, Calif, USA). All patients were strictly positive for anti-HCV and HCV RNA and negative for HBV. The study protocol respected the most recent Declaration of Helsinki (WMA, 2008), written informed consent and Research Ethics Committee approval were obtained from all cases.

Immunohistochemical staining of Bcl-2 and PCNA:

All chemicals and reagents were purchased from Zymed Laboratories (Invitrogen) unless mentioned otherwise. Bcl-2 and PCNA proteins were detected immunohistochemically by using commercial monoclonal antibodies, namely anti-Bcl-2 (Dako, Cambridge, UK) and anti-PCNA (Dako, Cambridge, UK) in avidin-biotin complex using diaminobenzidine tetrahydrochloride (DAB) as a chromogen. Four μm sections were cut from each of the paraffin blocks of the liver samples onto sialinised slides and processed eventually for primary and secondary antibodies incubation and then visualized using DAB chromogen. Presence of a brown colored end product at the site of the target antigen was indicative of positive reactivity. Isotype matched antibody instead of the primary antibody was used in the negative control tissues. B cell lymphoma cell line was used as a positive control for Bcl-2 Mab. Both positive and negative controls were run with each batch and the staining experiment was considered when the negative and positive control slides were strictly negative and positive. Only unequivocal nuclear positivity was interpreted as immunopositive for PCNA and Bcl-2. The labeling index was

determined by counting the number of labeled nuclei in 100 cells using a light microscope and subsequently expressed as a percentage. It was also accordingly converted into (–) (immunonegative); (+) (up to 25%); (++) (26-50%); (+++) (51-75%) and (++++) (>75%) as described earlier by others for PCNA **[19]** and by us for Bcl-2 **[20]**.

Determination of soluble Fas (sFas) in serum by ELISA method:

Soluble Fas in serum samples was detected with a Sandwich ELISA kit (R & D system, MN, USA), according to the manufacturer instructions without modification.

Determination of soluble Bcl-2 in serum by ELISA method:

The estimation of serum Bcl-2 was carried out using (Bender Medsystem, Viena, Austria) ELISA kit. This kit is a sandwich enzyme immunoassay and utilizes mouse Bcl-2 monoclonal antibody specific for human Bcl-2 protein. The procedures recommended by the manufacturer were followed without modification. The optical absorbance values were read on a micro-ELISA auto-reader at 490nm. All samples were tested in duplicate and assayed on two separate occasions. All samples were coded and read blinded in the assay.

Determination of apoptotic cells by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) in tested hepatocytes:

Hepatocytes resuspended in Dulbecco's MEM (Gibco) were mixed with equal volumes of 8% paraformaldehyde for 10 minutes. Cells were stained with TUNEL kit (Frag EL: Calbiochem, Nottingham, UK) according to the manufacturer instructions without modification. Viable cells stained blue whilst apoptotic cells appeared as small fragmented bodies staining bright green.

Statistical analysis:

Statistical analysis was carried out using Microsoft Excel spreadsheet and the StatView SE + graphics software. The probability of a significant difference between groups was determined by Chi-square or Wilcoxon Signed rank test as appropriate for nominal data and student *t*-test for quantitative data. *p*-values ≤0.05 were considered statistically significant. All software programs were run on a Macintosh computer.

RESULTS

Patients' criteria:

The patients' characteristics and biochemical data are shown in Tables (1,2). These data showed that males were numerically dominant in chronic HCV patients without HCC, and in HCV infected patients with HCC. Hemoglobin concentration and total leucocytic count were statistically decreased in HCC patients versus the HCC free patients ($p<0.01$ and $p<0.05$ respectively). Red blood cells and platelet counts were insignificantly decreased in HCC patients versus the HCC free patients ($p>0.05$ for both). AST was found significantly higher in HCC group ($p>0.05$) but ALT was found statistically decreased in HCC group ($p>0.001$) versus the HCC free patients. Serum total bilirubin, total protein, serum albumin were significantly lower in HCC group versus the HCC free patients ($p<0.001$, $p<0.05$ and $p<0.001$, respectively). In HCC group serum alkaline phosphatase was insignificantly increased ($p>0.05$) and alpha fetoprotein was highly increased ($p<0.001$).

Bcl-2 and PCNA:

The results showed that the serum Bcl-2 was elevated in 82% in the group without HCC versus 100% in patients with HCC (Table 3). 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 (Table 3). On the other hand, nuclear staining of PCNA was detected in 100% of HCC patients versus none of HCV patients without HCC. It is worthy to note that the Bcl-2 staining was found positive in five cases from non-tumorous areas of the HCC patients (20%). On the other hand, these non-tumorous areas of the HCC patients were found all stained positive for PCNA (100%). Moreover, the Bcl-2 labeling index was decreased from chronic active hepatitis to cirrhosis to HCC (29.8%, 26.8% and 17.4% respectively). In HCC patients, the PCNA labeling index was increased from 16.9% to 30.6% to 46.8% from grade-1 to grade-2 to grade-3, respectively.

Table (1): Characteristics of HCV infected patients with and without HCC.

Variable	Non-HCC N = 50		HCC N = 25	
	No.	%	No.	%
Sex:				
Male	40	80	21	84
Female	10	20	4	16
Age:				
Below 56 Yrs	30	60	9	36
Above 56 Yrs	20	40	16	64
Hb:				
Below 8g/dl	31	62	13	52
Above 8g/dl	19	38	12	48
RBCs:				
Below 4x 10^6/µl	35	70	16	64
Above 4x 10^6/µl	15	30	9	36
Thrombocytes (Platelets):				
Below 150x 10^3/µl	26	52	16	64
Above 150x 10^3/µl	24	48	9	36

Table (2): Biochemical data of HCV infected patients with and without HCC.

Variable	Non-HCC		HCC	
	No.	%	No.	%
Aspartate aminotransferase (AST):				
Normal	11	22	5	20
Elevated	39	78	20	80
Alanine aminotransferase (ALT):				
Normal	12	24	10	40
Elevated	38	76	15	60
Serum total bilirubin:				
Normal	15	30	10	40
Elevated	35	70	15	60
Serum proteins:				
Normal	7	14	7	28
Decreased	43	86	18	72
Serum albumin:				
Normal	8	16	8	32
Decreased	42	84	17	68
Alkaline phosphatase:				
Normal	49	98	12	48
Elevated	1	2	13	52
Alpha fetoprotein:				
Normal	34	68	0	0
Elevated	16	32	25	100

Table (3): Immunohistochemical results of liver samples obtained from HCV infected patients with and without HCC using Bcl-2 and PCNA specific monoclonals antibodies.

Variable	Non-HCC		HCC	
	No.	%	No.	%
Bcl-2:				
Negative	20	40	23	92
Positive				
+	15	30	0	0
++	10	20	1	4
+++	5	10	1	4
PCNA:				
Negative	50	100	0	0
Positive				
+	0	0	3	13.5
++	0	0	13	50
+++	0	0	9	36.5

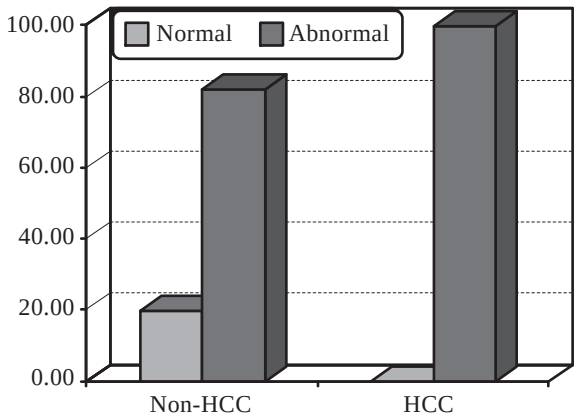

Fig. (1): Serum Bcl-2 levels as measured by ELISA. The normal ranged from 11.3 to 30U/ml. The serum Bcl-2 was found normal in nine non-HCC patients (18%) versus none of HCC patients. The serum Bcl-2 was found elevated in 41 non-HCC patients (82%) versus 25 HCC patients (100%).

DISCUSSION

The regulation of programmed cell death (PCD), or apoptosis, is an essential determinant of the cell life span. The mammalian Bcl-2 family of apoptosis-associated proteins consists of members that inhibit apoptosis (Bcl-2, Bcl-xl, Mcl-1, A1, etc) and others that induce apoptosis (Bax, Bak, Bad, Bcl-xs, Bik, etc), and the balance between pro-apoptotic and anti-apoptotic members determines the fate of the cells in many systems **[21,22]**. The study aimed to investigate whether the Bcl-2 and PCNA can be used as diagnostic and prognostic markers in patients infected with viral hepatitis C with or without HCC.

In the current study, we found that the serum Bcl-2 assayed by ELISA was elevated in 82% versus 100% in HCC free and HCC patients, respectively, with no significant difference between the two groups ($p<0.05$). These results are in agreement with Lacronique et al. **[23]** who stated that Bcl-2 expression is elevated in liver of cirrhotic patients and this increase may correlate with the development of HCC. Moreover, they explained their results suggesting that Bcl-2 is playing a role in tumorgenesis by inhibiting apoptotic death rather than promoting cell proliferation. In normal liver, Bcl-2 family members have a number of essential roles in liver homeostasis. Although Bcl-2 is not generally expressed in human hepatocytes **[24]**, ectopic Bcl-2 expression delays hepatocyte cell cycle progression **[25]**. Over-expression of either Bcl-2 or Bcl-xL in mouse liver protects hepatocytes from Fas-induced apoptosis and liver destruction in a dose-dependent manner **[26]**. We are aware of the difference in measuring the Bcl-2 (serum Vs cytoplasm) using different methods (ELISA Vs Immunohistology). The discrepancy was claimed to be due to the sensitivity of the two techniques.

In the present study, cytoplasmic staining of Bcl-2 was observed in 60% of chronic HCV patients without HCC versus 8% in HCC patients. In HCC, Bcl-2 is usually absent while Bcl-xL is predominately expressed. Using Northern blotting, researchers reported that Bcl-2 and Bcl-xL may play important roles in regulating the apoptosis of normal liver and HCC **[27]**. Some reports showed that increased level of Bcl-2 RNA was frequently present in HCC **[28]**. A high dramatic difference was found in the expression of Bcl-2 between HCC group and non-HCC group. It is clear that the Bcl-2 related apoptosis played an important role in hepatocarcinogenesis.

However, its protein product is either absent **[29]** or present only in a very small proportion of tumor cells in HCC tissues **[30]**. The findings suggest a post-translational mechanism of Bcl-2 protein degradation, indicating that Bcl-2 may not play an important role in hepatocarcinogenesis **[31]**. In contrast, other studies have shown an increased level of Bcl-2 protein in HCC **[32,33]** and it may be involved in the development of HCC. This concept is supported by a study showing that the over-expression of Smad3, a major TGF-ß signaling transducer, reduces the susceptibility to hepatocarcinogenesis in a mouse model by reducing the level of Bcl-2 to sensitize hepatocytes to apoptosis **[34]**. This observation is in agreement with data demonstrating that excess Bcl-2 expression in TGFα/Bcl-2 double transgenic mice delays the development of liver tumors induced by the growth factor **[25]** and that Bcl-2 inhibits c-myc-induced liver carcinogenesis **[35]**.

Furthermore, in vivo electrophoretic transfer of Bcl-2 antisense oligonucleotide (ASO) into liver can inhibit the development of HCC in rats **[36]**. The differential expression of Bcl-2 may be related to the status of p53 since Bcl-2 is remarkably up-regulated in p53-positive HCC tissues, but down-regulated in p53-negative tissues **[37]**. Alterations of both p53 and Bcl-2 proteins have been observed during hepatocarcinogenesis **[32]**, and the over-expression of p53 correlates with a high level of proliferation of cell nuclear antigen (PCNA), HCC dedifferentiation and advanced HCC stages **[38]**.

Collectively, in agreement with our results the serum Bcl-2 is elevated in chronic hepatitis and HCC patients **[39]**. On the other hand, in normal liver tissue the Bcl-2 is not expressed, but in chronic hepatitis and HCC it is barely detected in liver tissue. The pattern of serum and tissue Bcl-2 is seemingly altered in chronic hepatitis and HCC to initiate the cell survival and antiapoptotic effect. Overexpression of bcl-2 gene could lead to apoptosis inhibition and enable cell survival **[40]**. Thus, it seems clear that EIA is more sensitive than immunohistochemistry in the detection of Bcl-2.

PCNA, also known as cyclin, is a cell cycle related nuclear protein which accumulates in the late G1 and S phases of proliferating cells. It was used to evaluate cell proliferation as a progression marker in various tumors including HCC **[39]**. In the present study, cases without HCC were all negative for PCNA and all HCC cases were apparently positive for PCNA, and this may indicate a powerful proliferative activity of liver cells in HCC. It was considered in many studies that PCNA expression is indicative for an increased risk of developing HCC in HCV patients **[40,41]**. Since, we compared serum levels of Bcl-2 between patients with HCC and those without it, it was most likely clear that

non-apoptotic hepatocytes are the major producers of Bcl-2 (data not shown).

In the present study, non-tumorous areas in all cases of HCC were positive for PCNA while all HCV cases without HCC were negative. Comparable results were obtained with other investigators **[24,39]**. PCNA protein is one of the central molecules responsible for decisions of life and death of the cell. The PCNA gene is induced by p53, while PCNA protein interacts with p53-controlled proteins Gadd45, MyD118, CR6 and, most importantly, p21, in the process of deciding cell fate. If PCNA protein is present in abundance in the cell in the absence of p53, DNA replication occurs. On the other hand, if PCNA protein levels are high in the cell in the presence of p53, DNA repair takes place. If PCNA is rendered non-functional or is absent or present in low quantities in the cell, apoptosis occurs **[42]**. It was concluded that the apoptotic cells are consistently negative for PCNA and vice versa, suggesting that apoptotic cells do not show proliferative activity **[43]**. Additionally, since the analyses were done only on the expression of Bcl-2 and PCNA, it is hard to understand their implications for either apoptosis or survival of liver cancer. Therefore, we have examined if the degree of apoptosis in liver cancer is correlated with anti- or pro-apoptotic molecules. We found a trend correlation between sFas concentration and the level apoptosis in hepatocytes (r=0.4) (data not shown).

In conclusion, we herein report that in HCV infected patients with and without HCC, apoptosis is apparently dysregulated and the proliferation activity is altered. The estimation of these proteins may have a prognostic and/or diagnostic potential in these patients. The diagnostic and/or prognostic significance of these proteins requires further study with a large cohort of patients.

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