

Bcl-2 and p53 expression in hepatic tissues of Egyptian patients with Chronic Hepatitis C

Ahmed Allam,¹ Sami Gabr,² Jamaan Ajarem,³ Mostafa Abdel-Maksoud⁴

Abstract

Objective: To investigate B-cell-lymphoma-2 and tumour protein p53 expression in hepatic tissues of human cases of Chronic Hepatitis C.

Methods: The case-control study was conducted from December 2011 to February 2014 at the out-patient department of Gastroenterology Surgical Centre, Faculty of Medicine, Mansoura University, Mansoura, Egypt, and comprised healthy individuals and treatment-naïve chronic hepatitis C patients who had undergone liver biopsy. Liver biopsy was taken from patients prior to antiviral therapy or any other anti-fibrotic therapy. Serum marker levels were investigated on the day of biopsy or within 5 days after it. Blood platelet count was also investigated using standard methods. Formalin-fixed, paraffin-embedded sections were stained with haematoxylin and eosin and for apoptosis detection, B-cell-lymphoma-2 and tumour protein p53 expression in tissue was investigated through immunohistochemistry. Slides were labelled with patient identification numbers and then reviewed and graded blindly by a senior pathologist. SPSS 16 was used for statistical analysis.

Results: Of the 140 subjects in the study, 120(85.7%) were patients with a mean age of 39 ± 8.7 years (range: 11-64 years) and 20(14.3%) were healthy controls with a mean age of 38.6 ± 7.4 years (range: 14-66 years). The patients had higher body mass index but the difference was not significant ($p > 0.05$), while the difference in the levels of alanine transaminase, aspartate aminotransferase, alpha fetoprotein and platelet count was significant ($p < 0.05$ each). The highest expression of B-cell-lymphoma-2 was detected in chronic hepatitis C stage, while the highest expression of p53 was detected in hepatocellular carcinoma stage.

Conclusion: The expression of both B-cell-lymphoma-2 and tumour protein p53 might play diagnostic role during the different stages of the disease.

Keywords: Apoptosis, Chronic hepatitis, Hepatocellular carcinoma, Cirrhosis. (JPMA 65: 1186; 2015)

Introduction

Hepatitis C virus (HCV) is a hepatotropic virus that affects over 180 million people worldwide. It belongs to the family Flaviviridae and the genus Hepacivirus and its genome contains a linear, positive-strand ribonucleic acid (RNA) molecule of ~9,500 nucleotides.¹ HCV is the leading cause of chronic liver diseases and hepatocellular carcinoma (HCC).² HCC is amongst the top three cancer causes of death worldwide with hepatitis B and C viruses (HBV/HCV) as the main aetiological agents.³ Egypt has the highest HCV prevalence worldwide and as many as 20% of infected individuals will develop liver cirrhosis, on average 20-30 years after infection which finally develops to liver cancer.⁴ Moreover, Egypt is one of the global hot

spots of HCC⁵ as it is the second most common malignancy in Egypt.⁶ Surgical hepatic resection has been considered the first-line treatment which is most effective and radical treatment for HCC, but HCC is usually associated with poor liver function owing to chronic hepatitis or liver cirrhosis.⁷ Apoptosis is a tightly regulated process that plays a major role in carcinogenesis and the pathways governing it are complex.⁸ Apoptosis is characterized by cytoplasmic fragmentation and nuclear condensation.⁹ The pro- and anti-apoptotic permutations regulating cell viability vary according to species, cell type and also between normal and cancer cells.¹⁰ In liver, apoptosis and apoptosis-related proteins contribute to intra-hepatic bile duct development and the previous studies have demonstrated the expression of apoptosis related proteins in normal and diseased human liver, including instances of acute and of chronic hepatitis (CH).¹¹ An increasing number of genes that are involved in the control of apoptosis and consequently play a major role in cancer development have been discovered. The tumour suppressor gene, p53 (a transcription factor that is often rate-limiting for deoxyribonucleic acid [DNA]

.....
¹Beni-Suef University, Faculty of Science, Zoology Department, Beni-Suef, Egypt, ^{1,3,4}King Saud University, College of Science, Zoology Department, Riyadh, Saudia Arabia, ²Rehabilitation Research Chair, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia, Department of Anatomy, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

Correspondence: Sami Gabr. Email: nadalab2009@hotmail.com

damage-induced apoptosis) and the proto-oncogene, B-cell leukaemia/lymphoma-2 (Bcl-2) were two of the earliest identified cancer genes.¹² A major proportion of HCCs present mutation of the gene that encodes p53.¹³ Mutant p53 proteins were first discovered in transformed murine cell lines, while Bcl-2 translocations were first identified in human follicular lymphoma.¹⁴ P53 was proposed to activate cell cycle checkpoints, whereas Bcl-2 was shown to inhibit cell death.¹⁵ The last few years have witnessed the emergence of strong genetic and biochemical ties between these two proteins, and it has become increasingly evident that signalling between p53 and Bcl-2 is of fundamental importance to cancer biology.¹⁶ Early studies of p53 focussed on the ability of tumour-derived p53 mutants to promote cell growth and transformation. Subsequently, p53 was characterised as an essential mediator of cell cycle arrest in response to diverse cellular stresses.¹⁷ It now appears that the primary action of p53 in apoptosis is to directly and indirectly regulate the activity of the Bcl-2 family proteins. Members of this family are either inducers (BAX, BAD, BAK, BID, BIK, and BCL-XS) or inhibitors (Bcl-2, Bcl-XL) of apoptosis.¹⁸ It has recently been shown that Bcl-2 expression by mouse hepatocytes protects them from Fas-mediated apoptosis, suggesting the potential for alternative approaches to the prevention of hepatic failure due to viral hepatitis in man.¹⁹

Progress of chronic liver failure is associated with depletion of hepatocytes along with liver fibrosis. The transition from liver growth and hyper-cellularity to cell depletion and atrophy raises the question as to which process is responsible for cell loss. Bcl-2 presence has been reported in areas of cholangiolar proliferation and focally, in hepatocytes in patients with cirrhosis caused by HCV.²⁰ Also, Bcl-2 expression was shown in periportal hepatocytes of the bile duct-ligated rat, where it is believed to protect metaplastic hepatocytes from cholestasis-induced apoptotic death.²¹ On the other hand, emerging evidence has shown that p53 gene participates in human carcinogenesis as tumour suppressors.²² P53 was shown to inhibit the cell growth of human hepatocellular carcinoma.²³ The current study was planned to investigate Bcl-2 and p53 expression as apoptotic markers in hepatic tissues of HCV patients, and to correlate the progression of liver apoptosis as prognostic markers in the assessment of HCV pathogenicity.

Subjects and Methods

The case-control study was conducted from December 2011 to February 2014 at the out-patient department of Gastroenterology Surgical Centre, Faculty of Medicine,

Mansoura University, Mansoura, Egypt. A total of 140 adults were included. Out of these, 20 healthy individuals (15 men and 5 women, between 14 and 66 years of age) with a mean age of 38.6 ± 7.4 were selected as controls from a population undergoing standard annual physical examination and biological measurements for medical insurance and 120 treatment-naïve CHC patients who had undergone liver biopsy (100 men and 20 women, aged from 11 to 64 years of age) with a mean age of 39 ± 8.7 were included as cases. The study was conducted after obtaining clearance from the institutional ethics committee and informed consent from each participant. The sample size was calculated with an estimated difference of 5 per cent between the two groups for estimating Bcl-2 and p53 expression proteins. Accordingly, we needed 116 or more in the HCV group to have 90% power, but at the same power, we needed 19 subjects in the control group.

Only those patients were included who abstained from alcohol abuse for more than 6 months; with a proven HCV viremia, HCV RNA positivity and genotype determinations. Liver biopsy was taken from patients prior to antiviral therapy or any other anti fibrotic therapy. Serum marker levels, such as alanine transaminase (ALT), aspartate aminotransferase (AST) and alpha fetoprotein (AFP), were performed on the day of biopsy or within 5 days after liver biopsy.

Those with human immunodeficiency virus (HIV) and/or hepatitis B virus (HBV) co-infection, other causes of chronic liver diseases, hepatocellular carcinoma and prior liver transplantation were excluded. Also, subjects with iron supplementation, overweight and obesity (body mass index [BMI]: ≥ 25 and $\geq 30 \text{ kg/m}^2$), having previously received interferon therapy, and insufficient liver biopsy were excluded.

All the subjects completed a structured questionnaire with questions regarding demographic data and daily medication use. Venous blood samples from each patient were collected either before the administration of pre-operative drugs on the day of biopsy or within 5 days after biopsy. Samples were given a coded study identification number and were shipped frozen at -80°C for analysis.

Diagnosis of chronic hepatitis C (CHC) was established by elevated ALT levels in persons having HCV antibody (anti-HCV) detected by a third-generation enzyme immunoassay (EIA). Samples were subjected firstly for the detection of HCV RNA qualitatively as previously described.^{23,24} Reverse transcription polymerase chain

reaction (RT-PCR) was done for the identification of HCV RNA. RNA was extracted from 100µl patient's sera using Quigen RNA extraction kit, according to the kit protocol. Nested PCR was performed using *Thermus aquaticus* DNA (Taq DNA) polymerase enzyme (Fermentas Technologies, USA) in a volume of 20µl reaction mix. The nested PCR products were visualised on 2% agarose gel under ultraviolet (UV) light using Uvitec gel documentation system.

HCV RNA quantification was done by using Smart Cycler II Real-time PCR (Cepheid, Sunnyvale, California, USA) with HCV RNA quantification kits (Sacace Biotechnologies, Italy). The Smart Cycler II system is a PCR system by which amplification and diagnosis were accomplished at same time with TaqMan technology (Applied Biosystems, Foster City, California, USA) using fluorescent probes to investigate amplification after each replicating cycle. The lower and upper detection limits of the used assay were 250 and 5.0×10^8 iu/mL, respectively. Specimens yielding values above the upper limit were diluted 100-fold, retested and the obtained values were multiplied by this dilution factor to get the actual HCV RNA concentration in iu/mL.

HCV genotypes were identified by reverse hybridisation method using Line Probe assay (INNO-LiPA HCV II kit, Innogenetics, Swigdrecht, Belgium) according to the manufacturer's instructions.

A previously validated questionnaire²⁵ was used to collect demographic and medical information. Laboratory test results used in this study were all performed using standard methods. Serum AFP was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, USA).

Hepatic biopsies were obtained from all cases by a surgeon after computed tomography (CT) or magnetic resonance imaging (MRI) studies. A preoperative clinical diagnosis of primary liver cancer was made on the basis of an elevated serum AFP level (≥ 400 ng/ml) and characteristic features of the disease that were visible in the CT or MRI scans. The histological diagnosis of cirrhosis and HCC were based on the internationally based criteria. For histological examination, liver biopsies were obtained using an automatic 16-gauge tru-cut needle (biopsy gun) which provides adequate specimens for evaluation and fewer cases with tissue fragmentations. Liver biopsy specimens analysed were at least 15-25mm long with complete portal tracts (>10 CPTs). Formalin-fixed, paraffin-embedded sections were stained with haematoxylin and eosin (H&E) and with Masson's Trichrome. Slides were labelled with patient

identification numbers and then reviewed and graded blindly by a senior pathologist, and the mean length of liver biopsy and the number of portal tracts were assessed (including only the complete, intact portal tracts). The degree of fibrosis was scored according to the METAVIR system,²⁶ and no fibrosis was defined as F0, mild fibrosis as F1, moderate fibrosis as F2, severe fibrosis as F3, and cirrhosis as F4. Significant fibrosis was also defined as F2-F4. Hepatic inflammatory activity was also scored.

Immunohistochemical (IHC) reaction was performed using an avidin biotin complex immunoperoxidase technique on paraffin sections. p53 and Bcl-2 were detected using an anti human p53, and Bcl-2 monoclonal antibody (Dako A/S, Glostrup, Denmark), respectively. The mean percentage of p53 and Bcl2-positive liver cells was used as IHC scoring system. For the negative control, the primary antibody was omitted. Cytoplasmic staining for Bcl-2 was considered positive. However, positive p53 expression was based on nuclear staining. The Bcl-2 and p53 expression was evaluated semi quantitatively. Bcl-2 positivity was graded as negative; no staining, (+) 25% of the cells, stained; (++) 26-60% of the cells, stained; (+++) 61-100% of stained liver cells.

Statistical analysis was performed using SPSS 16. Patient baseline characteristics and results were descriptively summarised and reported as mean $\pm$ standard deviation (SD) or frequency (percentage) of patients with a condition. Comparisons between groups were made using Student's t test, one-way analysis of variance (ANOVA) and Mann-Whitney U- test for continuous variables. $P < 0.05$ was considered significant.

Results

Of the 140 subjects in the study, 120(85.7%) were patients with a mean age of 39 ± 8.7 years (range: 11-64 years) and 20(14.3%) were healthy controls with a mean age of 38.6 ± 7.4 years (range: 14-66 years). Among the cases there were 100(83.3%) men and 20(16.6%) women. Among the controls, 15(75%) were men and 5(25%) were women.

CHC patients had higher BMI (24.7 ± 3.8) than the controls (23.7 ± 3.4) ($p > 0.05$). AST (63.56 ± 46.8) and ALT (79.65 ± 58.51) levels in patients were significantly higher than those of controls who had low AST (22.3 ± 6.3) and ALT (28.3 ± 5.6) ($p < 0.05$). Also, a highly elevated level of AFP (19.6 ± 4.4) in patients was recorded compared to controls (3.4 ± 2.7) ($p < 0.05$). A significantly decreased level of plasma platelets (196 ± 56.5) in patients compared to controls (250 ± 24.4) was detected ($p < 0.05$). The mean length of liver biopsy core (LBC) was

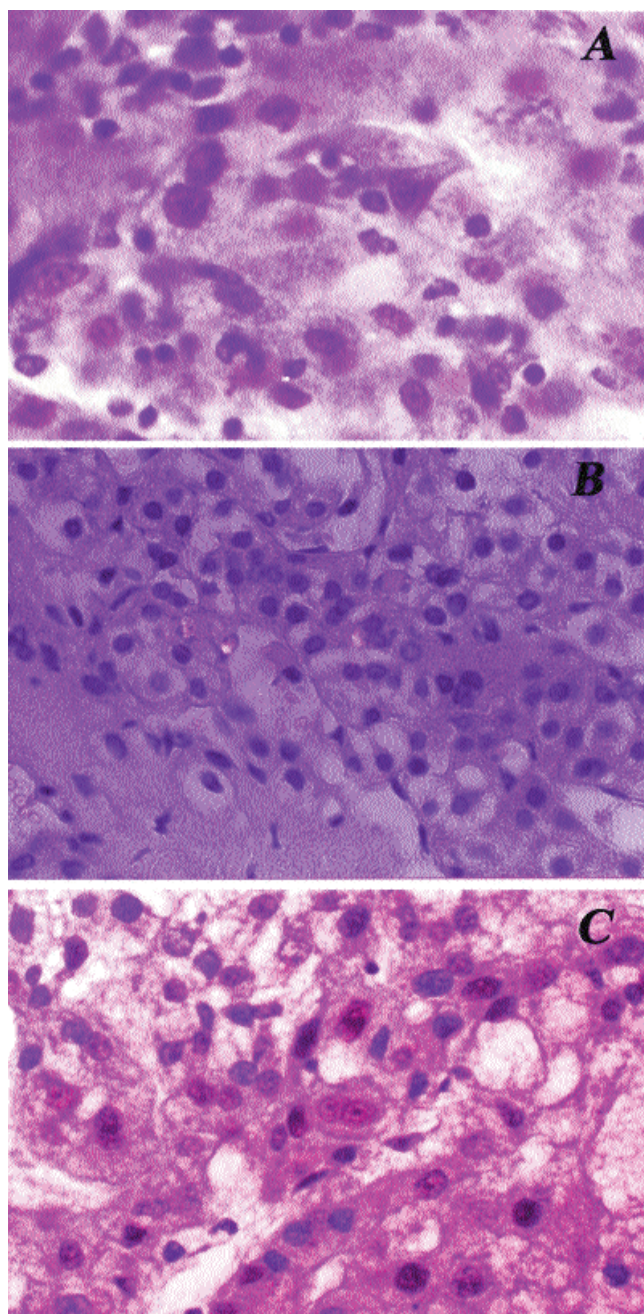


Figure-1: Photomicrograph showing different apoptotic rates in hepatocytes from Hepatitis C virus positive patients. A: Chronic hepatitis C stage; B: Cirrhosis stage. C: Hepatocellular carcinoma stage. Sections were stained with Haematoxylin and eosin (H&E) and analysed under light microscopy. Original magnification, 400x.

17.9±0.52cm and the mean number of portal tracts (NoPs) was 14±6.5. Duration of HCV per years, HCV-RNA, HCV genotypes, stage of fibrosis and necro inflammation were also recorded (Table-1).

Different apoptotic rates in hepatocytes from HCV

Table-1: Demographic, laboratory and histological characteristics of 140 patients with chronic hepatitis C and control subjects.

Characteristics	All CHC Patients N, mean±SD, (%)	Controls N, mean±SD	P*
No.	120	20	
Age (Year) ++	39±8.7	38.6±7.4	0.001**
Gender (Male/Female) ++	100/20	15/5	
BMI (kg/m2) ++	23.7±3.4	24.7 ±3.8	0.52
AST (IU/ml) +	63.56±46.8	22.3 ± 6.3	0.001**
ALT (IU/ml) +	79.65 ±58.51	28.3 ± 5.6	0.001**
AFP (ng/ml) +	19.6 ±4.4	3.4 ±2.7	0.001**
Platelets (109/L) ++	196 ± 56.5	250 ± 24.4	0.001**
Duration of HCV (years)	6.5 ± 1.7	-	-
HCV-RNA (IU/ml)	6.7 × 105	-	-
HCV Genotypes			
4	115 (96)		
2,4	5(4)		
Viral Load	25.6±8.3		
Stage of Fibrosis, (METAVIR) n (%)	114/120 (95)		
F0	6 (5)		
F1	15 (12.5)		
F2	25 (20.8)		
F3	35 (29.1)		
F4	39 (32.5)		
Population, n			
F0-F1	21 (17.5)		
F2-F4	99 (82.5)		
F0-F3	81(67.5)		
F4	39 (32.5)		
Mean length of liver biopsy core (LBC +SD)	17.9 ± 0.52 cm.		
mean number of portal tracts(NoP+SD)	14 ± 6.5		
Necro inflammation			
A0-A1	20(20)		
A2-A3	80 (80)		

*p for controls vs all HCV patients; ++Student t test; +Mann Whitney U test; * p<0.05; **p<0.01;

SD: Standard deviation

BMI: Body mass index;

ALT: Alanine amino transferase

AST: Aspartate amino transferase.

AFP: Alpha fetoprotein

HCV: Hepatitis C virus

RNA: Ribonucleic acid

positive patients were noted (Figure-1); the highest apoptotic rate was found in hepatocellular carcinoma stage (C). In cirrhosis stage (B), the apoptotic rate was higher than that in CHC stage (A). The highest expression of Bcl-2 was detected in CHC stage (Figure-2A). In cirrhosis stage (B), the expression of Bcl-2 was still higher than that in hepatocellular carcinoma stage (C). In contrast, the highest expression of p53 was detected in hepatocellular carcinoma stage (C). In cirrhosis stage (B), the expression of p53 was higher than that in chronic hepatitis C stage (A) which had the lowest expression level of p53.

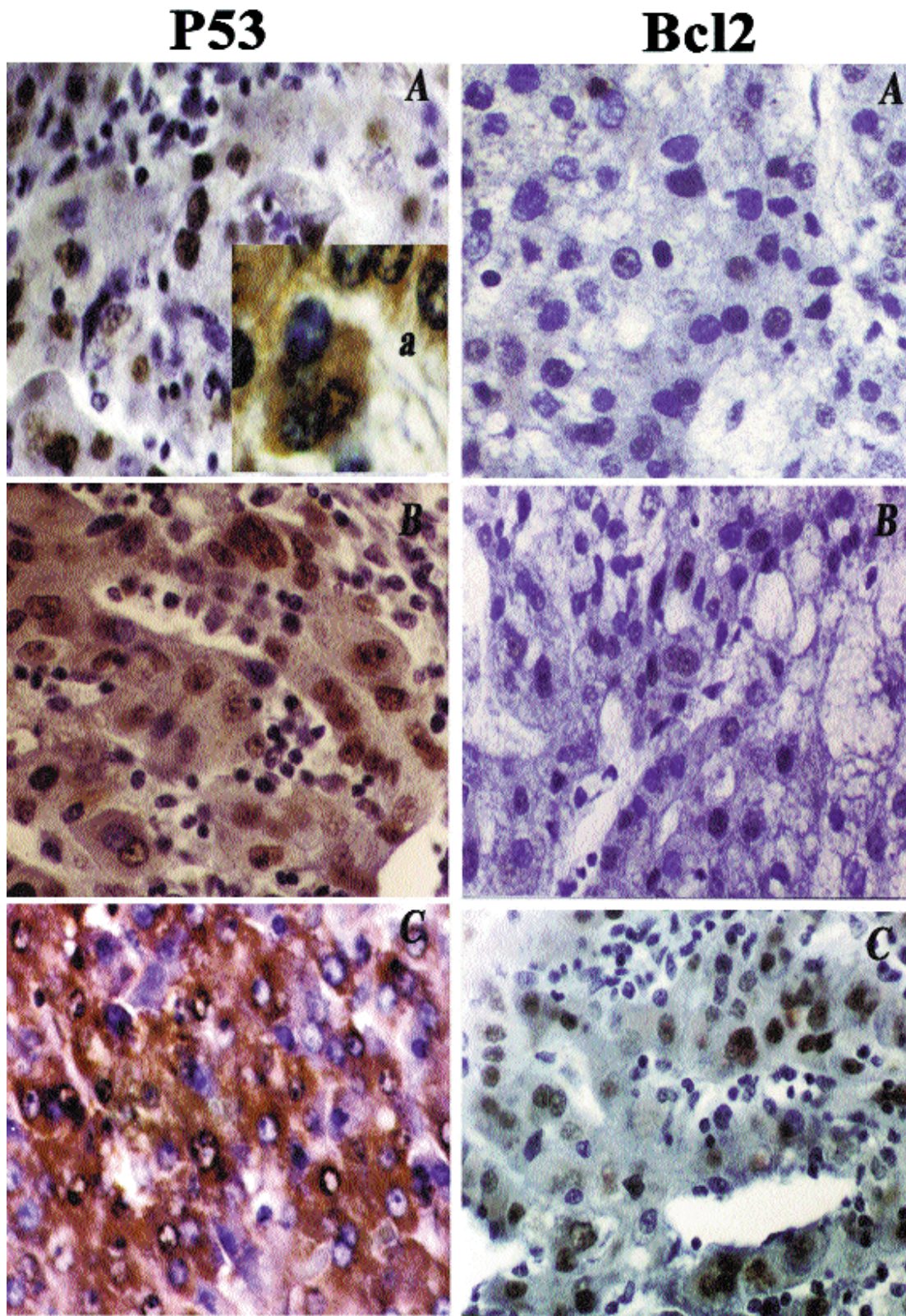


Figure-2: Photomicrograph showing tumour protein p53 (left panel) and B-Cell-Lymphoma-2 (right panel) expression in hepatocytes from Hepatitis C patients. A: Chronic hepatitis C stage; B: Cirrhosis stage. C: Hepatocellular carcinoma stage. Original magnification, 400x. The insertion (a) represents an active dividing cell.

Discussion

Recently, it has been thought that apoptosis may constitute an important mechanism in carcinogenesis.¹⁰ Apoptotic and anti-apoptotic genes produce proteins that regulate apoptosis. The dysfunction of their expression may contribute to carcinogenetic pathways.²⁷ The liver is continuously exposed to a large antigenic load that includes pathogens, toxins, tumour cells and dietary antigens. Amongst the hepatitis viruses, only HBV and HCV cause chronic hepatitis; this can progress to cirrhosis and HCC. Of the different antiviral defence systems employed by the tissue, apoptosis significantly contributes to the prevention of viral replication, dissemination and persistence.²⁸ Apoptotic cell death of hepatocytes emerges as a fundamental component of virtually all acute and chronic liver diseases. The liver tissue repair, inflammation, regeneration and fibrosis may all be triggered by apoptosis.²⁹ In Egypt, liver biopsy (LB) remains the gold standard to assess liver apoptosis in CHC. So in the current study, we took LBs from CHC patients to investigate the hepatocytes apoptosis and the histological expression of both Bcl-2 and p53 proteins during the different stages of CHC. Our results are in accordance with previous reports whereas CHC infection was associated with higher BMI. Many studies have reported the association of hepatic cirrhosis and metabolic factors such as obesity or high BMI.³⁰ As a hepatic disease, CHC is expected to be associated with liver dysfunction. We investigated the plasma level of liver enzymes as a biomarker for liver dysfunction. We recorded a higher level of both ALT and AST enzymes, which is in agreement with most published reports.³¹

Another important biomarker for CHC infection is AFP level in plasma. The elevated level of AFP in plasma of CHC patients compared to the control is in agreement with other reports.³² Clinically, our results correlate and support the previous findings as there was a significant decrease in serum platelets of the patients compared to the controls. Also, the several parameters of activity and progression of the chronic liver disease, including METAVIER fibrosis stages (F0-F4), necro inflammatory activity grades (A0-A3) and viral load (25.6 ± 8.3) were similar to that reported before.³³

Among the most important proteins that regulate apoptosis, the pro-apoptotic gene p53, and the anti-apoptotic gene Bcl-2, were considered major players in carcinogenesis¹⁴ and the dysfunction of their expression may contribute to carcinogenetic pathways. So, we investigated the expression of both p53 and Bcl-2 in hepatic tissue sections taken from HC-infected patients

during the three different stages of the disease which are CHC stage (A), cirrhosis stage (B) and HCC stage (C). The highest apoptotic rate was seen in HCC stage as indicated from H&E stained hepatic tissue sections and as confirmed by the highest expression of p53 and the lowest expression of Bcl-2. Our data supports previous studies that stated that death receptors are expressed on the surface of hepatocytes to facilitate the elimination of cells infected with hepatotropic viruses.³⁴ Taken together, our data reveals that HC infection can cause harmful effects in human patients, of which, the most important is hepatocytes apoptosis that can be confirmed by the elevated expression of p53 and the decreased expression of Bcl-2 in hepatic tissue sections of HC patients.

Conclusion

Hepatocytes apoptosis is a characteristic feature in histological sections of HCV patients. The highest apoptotic rate was seen in HCC stage that can be confirmed by the elevated expression of p53 and the decreased expression of Bcl-2 in tissue sections while the lowest apoptotic rate was seen in CHC stage.

Acknowledgement

We are grateful to the Deanship of Scientific Research at King Saud University for financial assistance.

References

1. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; 244: 359-62.
2. Abd Elrazek AE, Bilasy SE, Elbanna AE, Elsherif AE. Prior to the oral therapy, what do we know about HCV-4 in Egypt: a randomized survey of prevalence and risks using data mining computed analysis. *Medicine (Baltimore)*. 2014;93:e204.
3. Khan G, Hashim MJ. Burden of Virus-associated Liver Cancer in the Arab World, 1990-2010. *Asian Pac J Cancer Prev*. 2015; 16:265-70.
4. Jimenez AP, Mohamed MK, Eldin NS, Seif HA, El Aidi S, Sultan Y, et al. Injection drug use is a risk factor for HCV infection in urban Egypt. *PLoS One*. 2009; 4:e7193.
5. Wahab MA, Shehta A, Hamed H, El Nakeeb A, Salah T. Predictors of recurrence in hepatitis C virus related hepatocellular carcinoma after hepatic resection: a retrospective cohort study. *Eurasian J Med*. 2014; 46:36-41.
6. Omran DA, Awad AH, Mabrouk MA, Soliman AF, Aziz AO. Application of Data Mining Techniques to Explore Predictors of HCC in Egyptian Patients with HCV-related Chronic Liver Disease. *Asian Pac J Cancer Prev*. 2015; 16:381-5.
7. Abdelraouf A, Hamdy H, El Erian AM, Elsebae M, Taha S, Elshafey HE, et al. Initial experience of surgical microwave tissue pre-coagulation in liver resection for hepatocellular carcinoma in cirrhotic liver. *J Egypt Soc Parasitol*. 2014; 44:343-50.
8. Jiang M, Milner J. Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells. *Genes Dev*. 2003; 17:832-7.
9. Cory S, Adams JM. The Bcl2 family: Regulators of the cell life-or-death switch. *Nat Rev Cancer*. 2002; 2:647-56.
10. Cummings MC, Winterford CM, Walker NI. Apoptosis. *Am J Surg*

- Pathol. 1997; 21:88-101.
11. Tsamandas AC, Thomopoulos K, Gogos C, Tepetes K, Kourelis T, Ravazoula P, et al. Expression of bcl-2 oncoprotein in cases of acute and chronic viral hepatitis type B and type C. A clinicopathologic study. *Dig Dis Sci*. 2002; 47:1618-24.
12. Basu A, Haldar S. The relationship between Bcl2, Bax and P53: consequences for cell cycle progression and cell death. *Mol Hum Reprod*. 1998; 4:1099-109.
13. Brito AF, Abrantes AM, Pinto-Costa C, Gomes AR, Mamede AC, Casalta-Lopes J, et al. Hepatocellular carcinoma and chemotherapy: the role of p53. *Chemotherapy*. 2012; 58:381-6.
14. Hemann MT, Lowe SW. The p53-Bcl-2 connection. *Cell Death Differ*. 2006; 13:1256-9.
15. Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM. The t (14; 18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science*. 1985; 229:1390-3.
16. Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood*. 1992; 80:879-86.
17. Eliyahu D, Raz A, Gruss P, Givol D, Oren M. Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. *Nature*. 1984; 312:646-9.
18. Reed JC. Double identity for proteins of the Bcl-2 family. *Nature*. 1997; 387:773-6.
19. Lacronique V, Mignon A, Fabre M, Viollet B, Rouquet N, Molina T, et al. Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. *Nat Med*. 1996; 2:80-6.
20. Tsamandas AC, Thomopoulos K, Zolota V, Kourelis T, Karatzas T, Ravazoula P, et al. Potential role of bcl-2 and bax mRNA and protein expression in chronic hepatitis type B and C: a clinicopathologic study. *Mod Pathol*. 2003; 16:1273-88.
21. Kurosawa H, Que FG, Roberts LR, Fesmier PJ, Gores GJ. Hepatocytes in the bile duct-ligated rat express Bcl-2. *Am J Physiol*. 1997; 272:G1587-93.
22. Hu S, Zhao L, Yang J, Hu M. The association between polymorphism of P53 codon 72 Arg/Pro and hepatocellular carcinoma susceptibility: evidence from a meta-analysis of 15 studies with 3704 cases. *Meta Gene*. 2013; 1:126-37.
23. Zhang Q, Cao LY, Cheng SJ, Zhang AM, Jin XS, Li Y. p53-induced microRNA-1246 inhibits the cell growth of human hepatocellular carcinoma cells by targeting NFIB. *Oncol Rep*. 2015; 33:1335-41.
24. Abdel-Hamid M, El-Daly M, Molnegren V, El-Kafrawy S, Abdel-Latif S, Esmat G, et al. Genetic diversity in hepatitis C virus in Egypt and possible association with hepatocellular carcinoma. *J Gen Virol*. 2007; 88:1526-31.
25. Kazi AM, Khalid W. Questionnaire designing and validation. *J Pak Med Assoc*. 2012; 62:514-6.
26. Bedossa P. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. *Hepatology*. 1994; 20:15-20.
27. Portt L, Norman G, Clapp C, Greenwood M, Greenwood MT. Anti-apoptosis and cell survival: a review. *Biochim Biophys Acta*. 2011; 1813:238-59.
28. Ghavami S, Hashemi M, Kadhoda K, Alavian SM, Bay GH, Los M. Apoptosis in liver diseases--detection and therapeutic applications. *Med Sci Monit*. 2005; 11:RA337-45.
29. Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. *Toxicol Sci*. 2002; 65:166-76.
30. Thomopoulos KC, Arvaniti V, Tsamantas AC, Dimitropoulou D, Gogos CA, Siagris D, et al. Prevalence of liver steatosis in patients with chronic hepatitis B: a study of associated factors and of relationship with fibrosis. *Eur J Gastroenterol Hepatol*. 2006; 18:233-7.
31. Poortahmasebi V, Alavian SM, Keyvani H, Norouzi M, Mahmoodi M, Jazayeri SM. Hepatic steatosis: prevalence and host/viral risk factors in Iranian patients with chronic hepatitis B infection. *Asian Pac J Cancer Prev*. 2014; 15:3879-84.
32. El Raziky M, Fathalah WF, El-Akel WA, Salama A, Esmat G, Mabrouk M, et al. The Effect of Peginterferon Alpha-2a vs. Peginterferon Alpha-2b in Treatment of Naive Chronic HCV Genotype-4 Patients: A Single Centre Egyptian Study. *Hepat Mon*. 2013; 13:e10069.
33. Awad Mel-D, Shiha GE, Sallam FA, Mohamed A, El Tawab A. Evaluation of liver stiffness measurement by fibroscan as compared to liver biopsy for assessment of hepatic fibrosis in children with chronic hepatitis C. *J Egypt Soc Parasitol*. 2013; 43:805-19.
34. Yin XM, Ding WX. Death receptor activation-induced hepatocyte apoptosis and liver injury. *Curr Mol Med*. 2003; 3:491-508.