The expression of bcl–2 in chronic liver diseases

Ruya Mutluay, *MD,* ***Seren Ozenirler,*** *MD,* ***Aylar Poyraz,*** *MD.*

ABSTRACT

Objective: Bcl-2 is an oncogene that prevents apoptosis (programmed cell death). Expression of bcl-2 protein has been reported in association with a variety of human tumors.

Methods: This study was conducted in the Department of Gastroenterology and Pathology, Faculty of Medicine Gazi University, Ankara, Turkey during the period 1996 to 2000 on formalin-fixed paraffin embedded tissue specimens of 69 liver biopsy with chronic liver disease. To evaluate the clinical importance of bcl-2 expression in chronic liver disease and its correlation with biochemical parameters, underlying liver disease types and histopathological parameters; we studied the bcl-2 expression in 69 biopsy proven patients. These were diagnosed with chronic liver disease, and had no other disease or had not received any treatment. Of these patients, 30 were diagnosed as having hepatitis C, 20 with hepatitis B, 19 with liver cirrhosis.

Results: The bcl-2 expression was significantly higher in the hepatitis C group when compared with the hepatitis B group ($p<0.001$). No significant correlation was found among serum transaminase, bilirubin, albumin, hepatitis C virus - RNA, hepatitis B virus - DNA levels, prothrombin time and bcl-2 expression ($p>0.05$).

Conclusions: The reason for the increased expression of bcl-2 in hepatitis C is unclear and may be related to difference in the injury mechanism of the virus, differences in the infection period, and immunology.

Saudi Med J 2005; Vol. 26 (8): 1245-1249

The oncogene bcl-2 was first reported by Tsujimoto et al in 1985.[1] The human bcl-2 gene is a protein consisting of 239 amino acids with a mass of 26 kDa, localized mainly in the outer mitochondrial membrane, endoplasmic reticulum and nuclear membrane.[2-13] The protein encoded with the bcl-2 protooncogene has been implicated in the prolongation of cell survival by blocking programmed cell death, namely, apoptosis.[2-7,14-16] The bcl-2 is mainly expressed in basal cells in the skin, pharynx and bronchial mucosa and intestinal crypt epithelium. Although the bcl-2 gene was initially described in non-Hodgkin's lymphomas, it also plays a crucial role in the pathogenesis of other malignancies, such as prostate, colorectal, stomach, and lung cancers. In addition, it is found in melanomas, acute and chronic lymphocytic and non-lymphocytic leukemia.[2,10,13,15,17-20]

There are few studies, which investigated bcl-2 expression in different benign or malignant liver diseases and their results were controversial.[2,21-26] Additionally, previous studies did not show any correlation between biochemical parameters and the bcl-2 expression, or the severity of liver disease. In normal liver, bcl-2 is described in the small bile duct epithelium but not in large bile duct epithelium.[21] In cirrhotic liver, ductular proliferation was found to be bcl-2 positive.[21] However, many

From the Department of Internal Medicine (Mutluay), Department of Gastroenterology (Ozenirler) and the Department of Pathology (Poyraz), Faculty of Medicine, Gazi University, Besevler, Ankara, *Turkey.*

Received 22nd December 2004. Accepted for publication in final form 5th June 2005.

Address correspondence and reprint request to: Dr. Ruya Mutluay, Department of Internal Medicine, Gazi University Hospital, Besevler, Ankara 06510, *Turkey*. Tel. +90 (312) 2025229. Fax. +90 (312) 2129006. E-mail: ruya_pretty@hotmail.com

studies of neoplastic liver diseases did not report bcl-2 expression in the dysplastic and neoplastic hepatocytes.[21-23,27] Frommel at al[23] reported bcl-2 protein expression in hepatocytes of patients with cirrhosis due to hepatitis.[22]

Chronic infection with the hepatitis virus is a predisposition for both cirrhosis and hepatocellular carcinoma. The destruction of the liver parenchyma most likely results from the combined effect of direct viral cytopathogenicity and immune related cytopathogenity.[28] To evaluate the effect of chronic inflammation due to hepatitis C and B we studied the bcl-2 expression in patients with hepatitis and cirrhosis, as well as its possible correlation with biochemical findings of these patients with histopathologic parameters.

Methods. This study was conducted in the Department of Gastroenterology and Pathology, Faculty of Medicine Gazi University, Ankara, Turkey during the period 1996 to 2000 on formalin-fixed paraffin embedded tissue specimens of 69 liver biopsy with chronic liver disease. The study was performed on 69 patients diagnosed as having chronic liver disease, liver cirrhosis without any other known disease, and who had not received any treatment. Of these patients, cirrhosis was classified as A and B, according to the Child-Pugh Score. Transaminase, total serum protein, albumin and bilirubin levels, and prothrombin time were determined for each patient. In addition, the hepatitis C virus (HCV) RNA (mEq/ml) levels for the hepatitis C group, and hepatitis B virus (HBV) DNA (hybridization-pg/ml) levels for the hepatitis B group were determined. Sections of 10% formalin fixed paraffin embedded tissues were stained with hematoxylin and eosin (H&E), periodic acid-Schiff and Masson's trichrome and iron stain. All H&E slides were examined and biopsies were graded according to the Knodell Scoring System.[29]

For hepatitis B immunohistochemical hepatitis B surface and core antigen were performed in all cases (HBV surface antigens, monoclonal, Clone T9, NeoMarkers, 08-0022, ZYMED). For hepatitis C biopsies stained with HCV antigen (Clone: TORDJI-22, Signet).

The immunohistochemical streptavidin-biotin immuno-peroxidase (SAB) method was used on 4 µm thick paraffin sections for detection of bcl-2 oncoprotein. The sections were de-waxed in xylene and hydrated with graded concentrations of alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes. Antigen retrieval was achieved with microwave pretreatment (HIAR) for 15 minutes in a 10 mmol/L citrate buffer, pH 6. Protein blocking was performed for 20 minutes. Sections were incubated with anti bcl-2 (Signet 124 monoclonal mouse antibody) for 2 hours. The 3-amino-9 ethylcarbazide (Signet AEC chromogen system code no: 1060-1080) was used as chromogen. Sections were rinsed, counterstained with Mayer's hematoxylin. Human tonsils were used as positive controls. For the negative control, the primary antibody was omitted. Cytoplasmic staining for bcl-2 was considered as positive. The bcl-2 expression was evaluated semiquantitatively. Bcl-2 positivity was graded as negative; no staining, (+) 25% of the cells, stained; (++) 26-60% of the cells, stained; (+++) 61-100% of the cells, stained. Antigen expression was assessed independently for hepatocytes, Kupffer cells, bile duct epithelium, bile ductular epithelium and mononuclear cells.

Statistical methods. Results were reported as mean ± standard deviation. Differences between groups were compared using the Mann-Whitney U test. Ratios were compared by using chi-square and Fisher's exact tests. Correlation analysis, using the Spearman rank test, $p<0.05$ was considered significant.

Results. This study included 69 patients, of whom 26 were women and 43 were men. Mean age was 43 years. Of these patients, 20 had chronic hepatitis B, 30 had chronic hepatitis C and 19 had cirrhosis (10 due to hepatitis C, 9 due to hepatitis B) (**Table 1**). For hepatitis C and hepatitis B, the correlation between transaminase levels, bilirubin and albumin levels, the quantity of HCV RNA, HBV DNA levels and prothrombin time and bcl-2 expression is shown in **Table 2**. The number of portal areas was 5.96 ± 4.21 as average for patients with HBV and 5.82 ± 3.65 for the HCV. There were no significant statistical differences in the average of portal areas for each group ($p>0.05$). **Table 3** shows the total necroinflammatory grade (HAI) score and degree of fibrosis. There were bile duct epithelial changes (epithelial irregularity and vacuoles) in 10 patients with HBV (34%). Also in 27 cases (67%) of HCV showed similar bile duct changes. Although germinal center formation was obvious in patients with HCV, lymphoid aggregates were present in the portal area in 27 biopsies with HBV (93%) and 35 biopsies with HCV (87%). Steatoses was obvious in 26 cases with HBV and 35 patients with HCV. Sinusoidal inflammatory cells were accompanied in 19 cases by HBV (65%) and in 38 cases (95% of patients) with HCV. Focal necrosis was present in 27 patients with HBV (93%) and 38 patients with HCV (95%). Eosinophils were observed in 21 patients in the portal area with HBV (72%) and 24 patients with HCV (60%). Piecemeal necrosis was mild in 11 biopsies (37%), moderate in 4 biopsies (13%) and severe in 5 biopsies (17%) with HBV; however, it was mild in 14 biopsies (35%), moderate in 6 biopsies (15%) and severe in 8 biopsies (20%) with HCV. Portal hemosiderin was

Table 1 - Clinical details of patients.

Patient characteristics	Hepatitis B virus	Hepatitis C virus
Male	22	21
Female	7	19
Age (mean ± SD)	44 ± 13	41 ± 13
Chronic active hepatitis	20	30
Child A	7	6
Child B	2	4
Total patients	**29**	**40**

Table 4 - Bcl-2 expression in the chronic active hepatitis and liver cirrhotic patients.

Group	Bcl-2 expression							
	0		1		2		3	
	n	(%)	n	(%)	n	(%)	n	(%)
HCV								
CAH	4	(13.3)	19	(63.3)	5	(16.7)	2	(6.7)
Child A	0		5	(83.3)	0		1	(16.7)
Child B	0		3	(75)	1	(25)	0	
HBV								
CAH	14	(70)	5	(25)	1	(5)	0	
Child A	1	(14.3)	6	(85.7)	0		0	
Child B	1	(50)	1	(50)	0		0	

HCV - hepatitis C virus, HBV - hepatitis B virus, CAH - chronic active hepatitis, 0 - no staining, 1 - 1-25% of the cells stained, 2 - 26-60% of the cells stained, 3 - 61-100% of the cells stained.

Table 2 - Bcl-2 expression in the hepatitis B and hepatitis C patients.

Immunohisto-chemistry	ALT (IU/ml)	AST (IU/ml)	Bilirubin (mg/dl)	Albumin (g/dl)	Prothrombin time (s)	HBV DNA (pg/ml)	HCV RNA (mEq/ml)
HBV							
bcl 0	136.56 ± 93.1	106.68 ± 73.56	1.25 ± 0.46	3.88 ± 0.59	13.85 ± 0.79	773 ± 695.9	
	(p=0.41)	(p=0.36)	(p=0.12)	(p=0.10)	(p=0.16)	(p=0.30)	
bcl 1	146.23 ± 64.86	111.15 ± 40.15	1.54 ± 0.49	3.6 ± 0.41	14.31 ± 0.83	698 ± 694.2	
HCV							7.35 ± 2.8
bcl 0	53.25 ± 25.48	58 ± 22.73	1.65 ± 0.47	3.72 ± 1.04	13.62 ± 0.45		(p=0.65)
	(p=0.10)	(p=0.53)	(p=0.84)	(p=0.72)	(p=0.67)		7.29 ± 1.98
bcl 1	103.11 ± 87.1	75.61 ± 55.51	1.69 ± 0.43	3.55 ± 0.62	13.75 ± 1.12		

HCV - hepatitis C virus, HBV - hepatitis B virus, AST - aspartate aminotransferases, ALT - alanine aminotransferase, Bcl 0 - bcl-2 expression absent, Bcl 1 - bcl-2 expression exist.

Table 3 - Total necroinflammatory score and degree of fibrosis.

N of cases	Necroinflammatory grade				Stage			
	1-3	4-8	9-12	13-18	1	2	3	4
HCV	6	11	13	10	21	4	5	10
HBV	4	9	7	9	5	11	4	9

HCV - hepatitis C virus, HBV - hepatitis B virus.

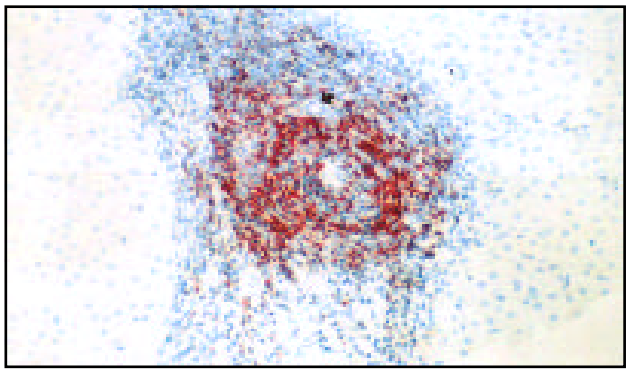

Figure 1 - Bcl-2 expression in hepatitis C with portal lymphoid aggregate. Bile duct did not show any staining (arrow) (streptavidin-biotin-immunoperoxidase-3-amino-9 ethyl carbazide x 100).

present in 6 biopsies with HBV (20%) and 7 biopsies with HCV (17%). All the cases of HBV were positive for hepatitis surface antigenes. Only 15 cases (37%) showed nuclear positivity for core antigenes. The bcl-2 expression was observed in sinusoidal lymphocytes, portal lymphocytes, and areas of ductular proliferation and lobular mononuclear cells. The bcl-2 expression is summarized in **Table 4**.

Bile ductule epithelium staining was observed in 5 biopsies (17%) with hepatitis B; additionally in 4 biopsies (13%) portal lymphocytic infiltration stained with bcl-2. Whereas in HCV, 9 biopsies were positive for bile ductule epithelium (22%) and portal inflammation was positive in 12 biopsies (30%) (**Figure 1**).

Statistical analysis shows that bcl-2 expression is higher in cases of chronic hepatitis C compared to those with chronic hepatitis B ($p<0.001$). There was no statistical significant correlation between bcl-2 expression and age, serum transaminase, total bilirubin and serum albumin levels, prothrombin time and HCV RNA levels in the hepatitis C group (**Table 2**). There was no statistical significant correlation between the bcl-2 expression and age, serum transaminase, total bilirubin and serum albumin levels, prothrombin time and HBV DNA levels in hepatitis B group (**Table 2**).

Discussion. In many studies the bcl-2 expression was evaluated in benign and malignant liver diseases.[4,21-27] In most of them, the bcl-2 expression was reported in bile ductules, small interlobar bile ducts, mononuclear cells but not in hepatocytes or dysplastic hepatocytes. However, Frommel et al[23] reported the bcl-2 expression in hepatocytes in cirrhosis due to hepatitis C, we did not find any bcl-2 expression in hepatocytes in cirrhosis either due to hepatitis C or due to hepatitis B. Zhao et al[24] evaluated the bcl-2 expression in cirrhosis and hepatocellular carcinomas. They described the bcl-2 expression in 5 hepatocellular carcinomas. Similarly Charrlotte et al[21] studied the bcl-2 expression in cholangiocarcinomas, hepatocellular carcinomas and metastatic adenocarcinomas but they did not describe any expression in hepatocellular carcinomas. Despite suggestions that bcl-2 may not involved in hepatocarcinogenesis, limited studies have been carried out to research the role of bcl-2 in hepatocellular carcinomas, and some of them reported that bcl-2 may play a role.[4,21,23,24]

In our study we found bcl-2 positivity in 26 of 30 biopsies (86.7%) with chronic hepatitis C and 6 of 20 biopsies (30%) with hepatitis B. The bcl-2 expression was significantly higher in hepatitis C than in hepatitis B patients. A possible explanation for this could result from different immunologic stimulation induced by HCV and HBV. However, in situ hybridization studies (ISH) for bcl-2 did not describe any different expression between hepatitis C and hepatitis B; ISH is an alternative, more sensitive method for assessing the expression of bcl-2 mRNA.[21] Studies with ISH also have shown some different results of expression in hepatocytes and hepatocellular carcinomas.[13,22,26] Controversial reports on bcl-2 protein expression and its role in hepatocarcinogenesis may be related to the sensitivity of the immunohistochemical technique, fixation or the limited numbers of cases examined and the primary antibody that is used for staining.

In our study, we observed a higher bcl-2 expression in hepatitis C than hepatitis B cases. This may be related to different viral mechanisms and bcl-2 may play a role in the development of cirrhosis and hepatocellular carcinomas in hepatitis C patients. In conclusion, further studies, more sensitive methods and a larger number of cases are indicated to confirm the role of the bcl-2 expression in the progression to cirrhosis and it is role in hepatitis C related hepatocarcinogenesis.

References

1. Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. ***Science*** 1985; 228: 1440-1443.
2. Desoize B. Anticancer drug resistance and inhibition of apoptosis. ***Anticancer Res*** 1994; 14: 2291-2294.
3. Abe-Dohmae S, Harada N, Yamada K, Tanaka R. Bcl-2 gene is highly expressed during neurogenesis in the central nervous system. ***Biochem Biophys Res Comm*** 1993; 191: 915-921.
4. Skopelitou A, Hadjiyannakis M, Alexopoulou V, Krıkoni O, Kamına S, Agnantıs N. Topographical Immunohistochemical Expression of bcl-2 protein in Human Liver Lesions. ***Anticancer Res*** 1996; 16: 975-978.
5. Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. ***Blood*** 1992; 80: 879-886.
6. Boıse LH, Gottschalk AR, Quintans J. Bcl-2 related proteins in apoptosis regulation. ***Curr Top Microbiol Immunol*** 1995; 200: 107-121.
7. Monaghan P, Robertson D, Amos TA, Dyer MJ, Mason DY, Greaves MF. Ultrastructural localization of bcl-2 protein. ***J Histochem Cytochem*** 1992; 40: 1819-1825.
8. Jacobson MD, Burne JF, King MP, Miyashita T, Reed JC, Raff MC. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. ***Nature*** 1993; 361: 365-369.
9. Hockenbery D, Nunez G, Milliman C, Schreıber RD, Korsmeyer ST. Bcl-2 as an inner mitochondrial membrane protein that blocks programmed cell death. ***Nature*** 1990; 348: 334-336.
10. Blandino G, Strano S. Bcl-2: the Pendulum of the Cell Fate. J. Exp. Clin. ***Cancer Res*** 1997; 16: 3-10.
11. Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer S. J.Bcl-2 Functions in an Antioxidant Pathway to prevent apoptosis. ***Cell*** 1993; 75: 241-251.
12. Itoh N, Tsujimoto Y, Nagata S. Effect of bcl-2 on fas antigen-mediated cell death. ***J Immunol*** 1993; 151: 621-627.

13. Fiorentino M, D'Errico A, Altimari A, Barozzi C, Grigioni WF. High levels of bcl-2 messenger RNA detected by in situ hybridization in human hepatocellular and cholangiocellular carcinomas. ***Diagn Mol Pathol*** 1999; 8: 189-194.
14. Emily HY, Beth L, Lawrence HB. Bax-independent inhibition of apoptosis by Bcl-XL. ***Nature February*** 1996; 379: 554-556.
15. Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. ***Blood*** 1993; 81: 151-157.
16. Nakamoto Y, Kaneko S, Kabayashi K. Increased susceptibility to apoptosis and attenuated bcl-2 expression in T lymphocytes and monocytes from patients with advanced chronic hepatitis C. ***J Leukoc Biol*** 2002; 72: 49-55.
17. Dolcetti R, Boıocchı M. Cellular and molecular bases of B-cell clonal expansions. ***Clin Exp Rheumatol*** 1996; 14 (Suppl 14): 3-13.
18. Panayıotıdıs P, Ganeshaguru K, Jabbar SA, Hoffbrand AV. Interleukin-4 inhibits apoptotic cell death and loss of the bcl-2 protein in B-chronic lymphocytic leukaemia cells in vitro. ***Br J Haematol*** 1993; 85: 439-445.
19. Thomas A, El Rouby S, Reed JC, Krajewski S, Silber R, Potmesil M, et al. Drug-induced apoptosis in B-cell chronic lymphocytic leukemia: relationship between p53 gene mutation and bcl-2/bax proteins in drug resistance. ***Oncogene*** 1996; 12: 1055-1062.
20. Bhalla K, Ibrado AM, Tourkina E, Tang C, Mahoney ME, Huang Y. Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. ***Leukemia*** 1993; 7: 563-568.
21. Charlotte F, L' Hermine A, Martin N, Geleyn Y, Nollet M, Gaulard P, et al. Immunohistochemical detection of bcl-2 protein in normal and pathological human liver. ***Am J Pathol*** 1994;144: 460-465.
22. Tsamandas A, 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. ***Dig Dis Sci*** 2002; 47: 1678-1624.
23. Frommel TO, Yong S, Zarling EJ. Immunohistochemical evaluation of bcl-2 gene family expression in liver of hepatitis C and cirrhotic patients: A Novel Mechanism to explain the high incidence of hepatocarcinoma in cirrhotics. ***Am J Gastroenterol*** 1999; 94: 178-182.
24. Zhao M, Zhang N-X, Economou M, Blaha I, Laıssue JA, Zımmermann A. Immunohistochemical detection of bcl-2 protein in liver lesions: bcl-2 protein is expressed in hepatocellular carcinomas but not in liver cell dysplasia. ***Histopathology*** 1994; 25: 237-245.
25. Koga H, Sakısara S, Ohıshı M, Sata M, Tanıkawa K. Nuclear DNA fragmentation and expression of bcl-2 in primary biliary cirrhosis. ***Hepatology*** 1997; 25: 1077-1084.
26. Ravazoula P, Tsamandas A, Kardamakıs D, Gogos C, Karatza C, Thomopoulos K, et al. The potential role of bcl-2 mRNA and protein expression in hepatocellular carcinomas. ***Anticancer Res*** 2002; 22: 1799-1806.
27. Iwata M, Harada K, Kono N, Kaneko S, Kobayashı K, Nakanuma Y. Expression of bcl-2 familial proteins ıs reduced in small bile duct lesions of primary biliary cirrhosis. ***Hum Pathol*** 2000; 31: 179-184.
28. Gonzalez-Peralta RP, Davis GL, Lau JYN. Pathogenetic mechanisms of hepatocellular damage in chronic hepatitis C virus infection. ***J Hepatol*** 1994; 21:255-259.
29. Knodell RG, Ishak KG, Black WC, Chen TS. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. ***Hepatology*** 1981; 1: 431-435.