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• LIVER CANCER •

Effect of bax, bcl-2 and bcl-xL on regulating apoptosis in tissues of normal liver and hepatocellular carcinoma

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Abstract

AIM: To investigate the expression of bax, bcl-2 and bcl-xL mRNA in the tissues of normal liver and hepatocellular carcinoma (HCC), and analyze the relationship between the expression of bax, bcl-2 and bcl-xL mRNA and clinical parameters of HCC patients.

METHODS: The expression of bax, bcl-2 and bcl-xL mRNA of normal liver and HCC was measured by Northern blot. Statistical analyses were made by *t* test and correlation analysis.

RESULTS: A very low mRNA level was indicated at bax, bcl-2 and bcl-xL in the HCC tissues in contrast to the tissues of normal liver by Northern blot analysis. The analyses of mRNA level revealed that HCC tissues exhibited a mean 7.6-fold decrease in bax, 4.2-fold in bcl-2 and 3.5-fold in bcl-xL in comparison with normal control tissues, respectively. Positive correlation was found between bax and bcl-xL (r=0.7061,P<0.01). There was no significance between the mRNA expression of these three genes and age, gender, tumor differentiation and tumor stage of HCC patients .

CONCLUSION: The results are consistent with the fact that apoptosis rarely occurs in normal livers but increases in HCC, indicating that bcl-2 and bcl-xL may play a very important role in regulating the apoptosis of normal liver and HCC.

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INTRODUCTION

Apoptosis is a highly regulated form of programmed cell death defined by distinct morphological and biochemical features. Programmed cell death is involved in a type of cell death, in which the cell actively uses a genetically controlled program to cause its own demise during the tissue remodeling of embryogenesis[1]. Apoptosis is a key mechanism causing cell death and organ diseases, failure of apoptosis is now understood to contribute to the development of human malignancies[2-5].

The bcl-2 family is the best characterized group of apoptosis-mediating factors, which include bcl-2, mcl-1, bcl-x, bax, bak, and several others. Although its members share close structural homologies, their biologic functions differentiate into apoptosis-promoting (bax, bak, bcl-xS) or apoptosis-inhibiting (bcl-2, mcl-1, bcl-xL) properties[1, 6]. The bcl-2 related genes regulate cell death and are considered to correlate with the pathogenesis and progression of cancers[7-14].Since the relationship between bcl-2 family and HCC is still unclear, we investigated the expression of the three genes in HCC and normal controls and evaluated the mediating action in HCC and the relationship between the genes and clinical parameters as well.

MATERIALS AND METHODS

Patients

Ten cases of (4 women, 6 men) normal liver tissues were obtained. The median age in the control group was 57 years, with a range of 39-75 years. HCC tissues were obtained from 21 patients (9 women, 12 men) undergoing surgery for HCC. The median age of the HCC patients was 64 years (a range of 33-76 years). According to the TNM classification of the International Union against Cancer, there were 6 patients with stage Ⅱ,13 with stage Ⅲ,and 2 with stage Ⅳ disease. Tissues destined for RNA extraction were frozen in the operating room in liquid nitrogen immediately on surgical removal and maintained at -80 ℃ until use.

Northern blot analysis

Total RNA was extracted by the guanidinium isothiocyanate method, fractionated on 1.2 % agarose. 1.8 mol· L formaldehyde gels, and stained with ethidium bromide for verification of RNA integrity and loading equivalence[15]. The RNA was electrotransferred to Nylon membranes (GeneScreen; DuPont, Boston, Massachusetts, USA) and cross linked by UV irradiation. The filters were then prehybridized, hybridized, and washed under conditions appropriate for digoxigenin labelled antisense riboprobes (bcl-2 and bax) or the ^{32}P labelled antisense riboprobe (bcl-xL) and cDNA probe (7S) as previously described[15,16].

In the case of the digoxigenin-labelled bcl-2 and bax cRNA probes, the filters were prehybridized and hybridized overnight at 68 ℃ (bcl-2) or 65 ℃(bax) in a buffer containing 50 % formamide, 2×SSC (1×SSC is 0.15M NaCl/0.015 M sodium citrate buffer) (bcl-2) or 5×SSC (bax), 2 % blocking reagent (Boehringer Mannheim, Mannheim, Germany), 0.1 % N-lauroylsarcosine, and 0.02 % sodium dodecyl sulphate (SDS). The filters were then washed in 2×SSC/0.1 % SDS at room temperature, followed by three 15 minute washing at 68 ℃ (bcl-2) or 65 ℃ (bax) in 0.065× SSC/0.065% SDS/35% formamide (bcl-2) or 0.1×SSC /0.1 % SDS (bax). The filters were then incubated in a blocking buffer (1 % blocking reagent in 100 mmol· L maleic acid/150 mmol· L NaCl, pH 7.5) for 30 minutes, and in blocking buffer containing anti-digoxigenin alkaline phosphatase antibody (1:20 000; Boehringer

Mannheim) for 30 minutes, washed three times with maleic acid buffer for 15 minutes, and incubated with 25 mmol/L CDP-Star (Boehringer Mannheim). The membranes were then exposed to X ray films.

For the ^{32}P labelled antisense riboprobe (bcl-xL), the blots were prehybridized for 6 hours in 50 % formamide,0.5 % sdiumm dodecyl sulfate (SDS), 5×SSC (sodium chloride/ sodium citrate buffer), 5×Denhardt' s solution (1×Dennhard' s solution=0.02 % ficoll, 0.02 % polyvinylpyrrolidone, and 0.02 % bovine serum albumin), 250 mg/L salmon sperm DNA, and 50 mmol/L sodium phosphate buffer (pH 6.5). The blots were then hybridized for 18 hours at 65 ℃ in the presence of 1×10^6 cpm/ml labeled antisense riboprobe, washed twice at 65 ℃ in a solution containing 1×SSPE (150 mmol/1 NaCl, 10 mmol/1 NaH_2PO_4, and 1 mmol/1 EDTA) and 0.5 % SDS, and twice at 65 ℃ in a solution containing 0.1×SSPE and 0.5% SDS.

In the case of the 7S cDNA probe, blots were prehybridized for eight hours at 42 ℃ in a buffer which contained 50 % formamide, 1 % SDS, 0.75 mol/L NaCl, 5 mmol/L EDTA, 5 ×Denhardt' s solution, 100 mg/L salmon sperm DNA, 10 % dextran sulfate, and 50 mmol/1 phosphate buffer (pH 7.4). The hybridization was carried out at 42 ℃ for 18 hours by adding the ^{32}P labelled cDNA probe (1×10^5 cmp/mL). The blots were rinsed twice in 2×SSC at room temperature and washed three times at 55 ℃ in 0.2× SSC/2 % SDS under conditions appropriate for cDNA probes. Blots were then exposed at -80 ℃ to Fuji X ray films with intensifying screens (DuPont).

For statistical analysis of the Northern blot results, the intensity of the radiographic bands was quantified by laser densitometry (Bio-Rad 620; Richmond, Califirnia, USA). The ratio between the bax, bcl-2 or bcl-xL and the corresponding 7s signal was calculated in each sample.

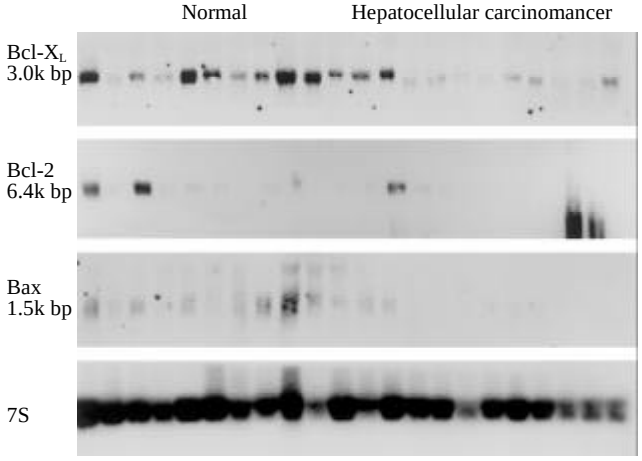
Statistical analysis

The data were expressed as median and range. Statistical analyses were carried out using the SPLM software (Statistical Department of Fourth Military Medical University). For statistical analysis, the *t* test and correlation analysis were used. Significance was defined as $P<0.05$.

RESULTS

Northern blot analysis

Northern blot analysis was carried out to determine bax, bcl-2 and bcl-xL mRNA expression in the normal and the cancerous liver. In contrast, hybridization signals of mRNA of three genes were higher in normal liver tissues as compared with the signals in cancerous samples (Figure 1). A low mRNA level of bax, bcl-2 and bcl-xL mRNA was almost present in all HCC tissue samples. In some HCC tissues the expression levels for all three genes were very faint and were only visible on the original autoradiographs. None of the normal or cancer samples showed any aberrant bax, bcl-2 or bcl-xL mRNA transcripts, and bax and bcl-2 mRNA were reduced in 100 % of cancer samples, and bcl-xL mRNA was reduced in 95 % of cancer samples in contrast with the normal controls. Densitometric analysis of the Northern blots indicated that the bax and bcl-2 mRNA levels in all cancer samples were 7.6- and 5.4-fold (P=0.0002; P=0.00887) higher than those in the matched control samples; bcl-xL mRNA levels in all cancer samples were 3.5-fold lower (P=0.0002) than those in the normal samples (Table 1). The expression of bax and bcl-xL mRNA was positively correlated in HCC (r=0.7061, $P<0.01$), but there was no correlation between bax and bcl-2 or bcl-2 and bcl-xL (r=0.1637, r=0.4830).



Normal Hepatocellular carcinoma
Figure 1 result of Northern blot analysis

Table 1 The expression of bax, bcl-2 and bcl-xL mRNA in hepatocellular carcinoma and normal liver

	Hepatocellular carcinoma (21 cases)	normal liver (10 cases)	*t* value	*P* value
Bax	0.929±1.233	7.060±6.574	4.197	0.0002
Bcl-2	1.414±1.331	5.930±7.227	2.815	0.0087
Bcl-xL	2.433±2.218	8.500±5.743	4.277	0.0002

Correlation of Northern blot analysis with clinicalpathological parameters

To determine whether the presence of bax, bcl-2 or bcl-xL mRNA in the HCC tissues is of clinical significance, the Northern blot data were statistically analysed in patient data (sex, age) and clinical data (tumor stage, tumor differentiation). No significance was found between the expression of bax,bcl-2 or bcl-xL and these parameters (Table 2).

Table 2 The relationship between the expression of bax,bcl-2 and bcl-xL and clinical parameters of patients with hepatocellular carcinoma

		n	bax	bcl-2	bcl-xL
Age	≤65	10	0.980±1.530	1.960±1.678	3.020±2.549
	>65	11	0.882±0.962	0.918±0.663	1.900±1.825
gender	male	13	1.100±1.471	1.654±1.532	2.600±2.645
	female	8	0.650±0.699	1.025±0.871	2.163±1.386
grading	well and moderate	12	0.975±1.384	1.592±1.540	2.775±2.453
	poor and undifferentiated	9	0.867±1.076	1.178±1.028	1.978±1.901
tumor stage	Ⅰ Ⅱ	6	0.583±0.454	0.700±0.616	2.000±1.616
	Ⅲ Ⅳ	15	1.067±1.423	1.700±1.445	2.607±2.445

Statistical analysis revealed no relationship between bax,bcl-2 or bcl-xL expression and these parameters.

DISCUSSION

Apoptosis is a central regulator of tissue homeostasis. It contributes to the elimination of damaged cells in normal tissues and balances the appropriate cell number under the circumstances of physiologic cell proliferation and tissue repair.

In the past few years, scientific interest has focused on the process of apoptosis. Like cell replication, apoptosis is controlled by the network of positive and negative growth signals. Based on the currently prevailing views, it is assumed that malignant cells should be incapable of apoptosis and /or not responsive to death signals, thereby allowing unrestrained growth of cancer. Recently, more and more studies indicated that apoptosis is of importance in the growth and development of many tumors, but the pattern of apoptosis varies in different tumors[11-19]. In contrast with normal tissues, there is often reduction of apoptosis in most cancerous tissues[20-23], but hepatic cancer is different. HCC is one of the most common and aggressive tumors in the world today, and little is known about the cellular pathogenesis[24-34]. The apoptosis is rare in normal liver tissues (there is only 2-4 apoptotic cells per 10 000 hepatic or biliary cells[35]), while the HCC tissues have higher rates of apoptosis[37].These findings indicate that the apoptosis-related genes are expressed in various frequencies in different cancers, and a general pattern of activation or inactivation of these genes in malignant tumors cannot be defined. Therefore, the function of apoptotic genes in different human cancers must be evaluated individually.

In this study, we analysed the concomitant expression of bax, bcl-2 and bcl-xL in the HCC tissues and normal liver. These genes belong to the same family of apoptotic genes. Although the structures are similar, they exert opposite effects on apoptosis. bcl-2 and bcl-xL inhibit apoptosis and contribute to cell survival and the resistance of cells against damaging influences. In contrast, bax, which is considered to be a central regulator of apoptosis, is a promoter of programmed cell death[37]. The relationship between bcl-2 related genes and HCC is still unclear.

By the analysis of 21 HCC patients , reduced bax and bcl-2 signals were present in all samples and reduced bcl-xL signals were present in 95 % of the cancer samples. The low expression of bcl-2 and bcl-xL is consistent with enhanced apoptosis in the HCC, but the low expression of bax does not account for this phenomenon. The findings revealed that anti-apoptotic genes, but not apoptosis-promoting genes, might play a more important role in regulating the apoptosis of normal liver and HCC. There was no correlation between bax, bcl-2 and bcl-xL and age, gender, differentiation or stage of tumor in HCC patients. We can not give a reasonable explanation for the finding that the expression of bax and bcl-xL was positively correlated in HCC. We concluded that anti-apoptotic genes do not influence the differentiation and development of HCC, but these genes can increase the rate of apoptosis in HCC by reducing their expression or changing the ratio with other genes. The influencing factors of apoptosis increased in HCC are not known, which may include 1) inherent metabolic process of tumor tissues;2) hypoxia of tumor tissues;3) some cytokines such as tumor necrosis factor;4) attack of CTL, etc.

The low expression of anti-apoptotic genes, which increase apoptosis in HCC, may have negative impact on growth of tumors as a homeostasis mechanism that inhibits cell group with extensive growth, and this effect can delay the development of tumors. But it may also be a selective pressure, which removes the aging tumor cells or some tumor cells with phenotype similar to normal cells and selects more aggressive and prosperous clone of tumor cells, accelerating the development of tumors. Further studies on the regulating action of apoptotic genes will deepen the understanding about the growth and development of HCC and offer valuable information to genetic therapy of HCC, thus enhancing the sensibility to radiotherapy and chemotherapy.

REFERENCES

1 **Que FG**, Gores GJ. Cell death by apoptosis: basic concepts and disease relevance for the gastroenterologist. *Gastroenterology* 1996; **110**: 1238-1243

2 **Friess H**, Lu Z, Graber HU, Zimmermann A, Adler G, Kore M, Schmid RM, Buchler MW. Bax, but not bcl-2, influences the prognosis of human pancreatic cancer. *Gut* 1998; **43**: 414-421

3 **Deng LY**, Zhang YH, Xu P, Yang SM, Yuan XB. Expression of interleukin 1β converting enzyme in 5-FU induced apoptosis in esophageal carcinoma cells. *World J Gastrornterol* 1999; **5**: 50-52

4 **Zhao AG**, Yang JK, Zhao HL. Chinese Jianpi herbs induce apoptosis of human gastric cancer grafted onto nude mice. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 737-740

5 **Xin ZP**, Xu CF, Huang YX, Wen QS, Xu CZ, Zhao YF. Experimental study on induction of apoptosis by cisplatin in human gastric carcinoma cell line SGC-7901. *Huaren Xiaohua Zazhi* 1998; **6**: 844-846

6 **Friess H**, Lu Z, Andren-Sandberg A, Berberat P, Zimmermann A, Adler G, Schmid R, Buchler MW. Moderate activation of the apoptosis inhibitor bcl-xL worsens the prognosis in pancreatic cancer. *Ann Surg* 1998; **228**: 780-787

7 **Xiao B**, ShiYQ, Zhao YQ, You H, Wang ZY,Liu XL, Yin F, Qiao TD, Fan DM. Transduction of Fas gene or bcl-2 antisense RNA sensitizes cultured drug resis tant gastric cancer cells to chemotherapeutic drugs. *World J Gastroenterol* 1998; **4**: 421-425

8 **Wu JY**, Zhou XF, Jiang WX, Wang JL, Yang F, Cai XS, Zhang ZG. The impact of chemotherapeutic drugs on the expression of bcl-2,p53 and Ki-67 in gastric cancer cells. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 589

9 **Yuan RW**, Ding Q, Jiang HY, Tan XF, Zou SQ, Xia HS. Apoptosis and the expression of bcl-2 and p53 in pancreatic cancer. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 851-854

10 **Qiao Q**, Wu JS, Zhang J,Ma QJ, Lai DN. The expression of bcl-2 and bax in human colon cancer. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 936-938

11 **Chen XJ**, Ai ZL, Liu ZS. Apoptosis-study on the mechanism of hepatic cell injury by mitomycin. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 746-750

12 **Yang JQ**, Yang LE, Zhu HC. Mitomycin induces apoptosis of human hepatic cancer cell. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 268-272

13 **Guo WJ**, Yu EX, Zheng SG, Shen ZZ, Luo JM, Wu GH, Xia SA. Study on Jianpi Liqi medicine inducing apoptosis in human hepatic cancer cell SMMC7721. *Shijie Huaren Xiaohua Zazhi* 2000; **8**:52-55

14 **Chen HY**, Liu WH, Qin SD. Induction of arsenic trioxide on apoptosis of hepatic cancer cell strain. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 532-535

15 **Liang WJ**, Huang ZY, Ding YQ, Zhang WD. Lovo cell line apoptosis induced by cycloheximide combined with TNFα . *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 326-328

16 **Lin JK**, Chou CK. *In vitro* apoptosis in the human hepatoma cell line induced by transforming growth factor β_1. *Cancer Res* 1992; **52**: 385-388

17 **Tu SP**, Jiang SH, Tan JH, Jiang XH, Qiao MM, Zhang YP, Wu YL, Wu YX. Proliferation inhibition and apoptosis induction by arsenic trioxide on gastric cancer cell SGC-7901. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 18-21

18 **Thompson CB**. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995; **267**: 1456-1462

19 **Fukuda K**, Kojiro M, Chiu JF. Demonstration of extensive chromatin cleavage in transplanted morris hepatoma 7777 tissue: apoptosis or necrosis? *Am J Pathol* 1993; **142**: 935-946

20 **Xiao B**, Xiao LC, Lai ZS, Zhang YL, Zhang ZS, Zhang WD. In vitro experimental study on Zhen'ailing inhibiting growth of gastric cancer cells. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 951-954

21 **Li LP**, Zhang Z, Han SX. Retinoic acid inhibits proliferation of tumor cells and induces apoptosis. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 437-440

22 **Liu HF**, Liu WW, Fang DC. Anti-Fas monoclonal antibody induces apoptosis of human gastric cancer cell line SGC-7901. *Shijie*

Huaren Xiaohua Zazhi 1999; **7**: 476-478
23 **Zhan J**, Xie DR, Yao HR, Lin XG, Liang XW, Xiang YQ. Arsenic trioxide induces apoptosis of colon cancer line SW620. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 228-229
24 **Liu HF**, Liu WW, Fang DC, Yang SM, Wang RQ. The expression of bax in gastric precancerous and cancerous tissues and its relation with apoptosis. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 665-668
25 **Xu AG**, Li SG, Liu JH, Gan AH. The function of apoptosis and protein expression of bcl-2, p53 and C-myc in the development of gastric cancer. *World J Gastroenterol* 2000; **6**(Suppl 3):27
26 **Lai DN**, Wu JS, Wu YZ. The relationship between the expression of bcl-2 and differentiation of colon and rectum cancer. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 91-92
27 **Soini Y**, Virkajarvi N, Lehto VP, Paakko P. Hepatocellular carcinomas with a high proliferation index and a low degree apoptosis and necrosis are associated with a shortened survival. *Br J Cancer* 1996; **73**: 1025-1030
28 **Friess H**, Yamanaka Y, Buchler M. Enhanced expression of the type II transforming growth factor beta receptor in human pancreatic cancer cells without alteration of type III receptor expression. *Cancer Res* 1993; **53**: 2704-2707
29 **Guo XZ**, Friess H, Graber HU. Kai1 expression is up-regulated in early pancreatic cancer and decreased in the presence of metastases. *Cancer Res* 1996; **56**: 4876-4880
30 **Zang GQ**, Yu H, Zhou XQ, Liao D, Xie Q, Wang B. TNF-α in vitro induces apoptosis and necrosis of rat hepatic cell. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 303-306
31 **James SJ**, Muskhelishvili L. Rates of apoptosis and prliferation vary with caloric intake and may influence incidence of spontaneous hepatoma in C57BL/6×C3H F_1 mice. *Cancer Res* 1994; **54**:5508-5510
32 **Mills JJ**, Chari RS, Boyer IJ, Gould MN, Jirtle RL. Induction of apoptosis in liver tumors by the monoterpene perillyl alcohol. *Cancer Res* 1995; **55**: 979-983
33 **Tanaka S**, Wands JR. Insulin receptor substrate 1 overexpression in human hepatocellular carcinoma cells prevents transforming growth factor β1. *Cancer Res* 1996; **56**: 3391-3394
34 **Patel T**, Gores GJ. Apoptosis and hepatobiliary disease. *Hepatology* 1995; **21**: 1725-1741
35 **Peng LM**, Wang ZL. Foundation and clinic of apoptosis. 1st eds. *Beijing:Renmin Weisheng Chubanshe*, 2000: 394
36 **Grasl-Kraupp B**, Ruttkay-Nedecky B, Mullauer L, Taper H, Huber W, Bursch W, Schulte-hermann R. Inherent increase of apoptosis in liver tumors: implication for carcinogenesis and tumor regression. *Hepatology* 1997; **25**: 906-912
37 **Walton MI**, Whysong D, O'Connor PM, Hockenbery D, Korsmeyer SJ, Kohn KW. Constitutive expression of human bcl-2 modulates nitrogen mustard and camptothecin induced apoptosis. *Cancer Res* 1993; **53**: 1853-1861

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