PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2004;10(22):3251-3254
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

Detection of bcl-2 and bax expression and bcl-2/JH fusion gene in intrahepatic cholangiocarcinoma

Lin-Lang Guo, Sha Xiao, Ying Guo

Lin-Lang Guo, Sha Xiao, Ying Guo, Department of Pathology, Zhujiang Hospital, Guangzhou 510282, Guangdong Province, China
Correspondence to: Dr. Lin-Lang Guo, Department of Pathology, Zhujiang Hospital, Guangzhou 510282, Guangdong Province, China. linlangg@yahoo.com
Telephone: +86-20-61643495 **Fax:** +86-20-84311872
Received: 2003-05-10 **Accepted:** 2003-06-04

Abstract

AIM: To investigate the relationship between bcl-2 gene and its related protein bax and intrahepatic cholangiocellular carcinoma (CCC).

METHODS: Semi-nested *in situ* PCR (SNISPCR) and immunohistochemistry were performed to detect bcl-2/JH fusion gene and bcl-2, bax protein expression in 29 cases of CCC.

RESULTS: No bcl-2/JH fusion gene was found in all cases of CCC, 72.4% of 29 cases expressed bcl-2 protein. Bcl-2 protein expression was related to histopathological grades ($P<0.05$). There was no corresponding relationship between bcl-2/JH fusion gene formation and bcl-2 protein expression in CCC ($P<0.05$). Bax was expressed in 10.3% of 29 cases. The ratio of bcl-2 to bax in normal liver tissues (3.5 to 1) was different from that in tumor tissues (7.0 to 1).

CONCLUSION: It is suggested that bcl-2/JH fusion gene formation is not a frequent event and may not play an important role in the pathogenesis of CCC. However, aberrant ratio of bcl-2 to bax protein expression may be involved in the course of tumorigenesis of CCC. Abnormal bcl-2 protein expression may not be solely resulted from bcl-2/JH fusion gene.

Guo LL, Xiao S, Guo Y. Detection of bcl-2 and bax expression and bcl-2/JH fusion gene in intrahepatic cholangiocarcinoma. *World J Gastroenterol* 2004; 10(22): 3251-3254
http://www.wjgnet.com/1007-9327/10/3251.asp

INTRODUCTION

Bcl-2 gene was first identified in B-cell leukemia and follicular lymphoma which confered a survival advantage on B-cells by inhibiting apoptosis. Bcl-2 gene at chromosome 18 is juxtaposed with the immunoglobulin heavy chain gene (JH) on chromosome 14[1]. The t (14; 18) chromosome translocation was present in 70% to 85% of follicular lymphoma and in 20% to 30% of diffuse large-cell lymphomas[1-4]. While aberrant expression of bcl-2 protein was also found in follicular lymphoma. In the early study, bcl-2 gene was mainly investigated in lymphoid tissues. Recently, bcl-2 gene aberrant expression has been observed in a variety of tumors, such as adenocarcinoma of the prostate, bladder carcinoma, squamous cell carcinoma of the lung, nasopharyngeal carcinoma, breast carcinoma and cholangiocarcinoma[5-17]. However, bcl-2/JH fusion gene and its relationship with bcl-2 protein expression have not been detected in cholangiocellular carcinoma (CCC).

Bax, an important homologue of bcl-2, is a promoter of apoptosis. It has been proposed that the sensitivity of cells to apoptosis stimuli be closely related to the ratio of bcl-2/bax and other bcl-2 homology. When bcl-2 is in excess, cells are protected. However, when bax is in excess and bax homodimers dominate, cells are susceptible to apoptosis[18]. Whether bcl-2/bax expression ratio is involved in the course of tumorigenesis of CCC remains unknown.

In this study, we demonstrated that bcl-2/JH fusion gene, bcl-2 and its related protein bax expression with semi-nested *in situ* PCR (SNISPCR) and immunohistochemistry in 29 cases of CCC to understand the relationship between bcl-2 gene and its related protein bax in CCC.

MATERIALS AND METHODS

Tissue preparation

A total of 29 samples were obtained by surgical resection in our department from 1995-06-01 to 1998-02-28. All samples were independently reviewed by two pathologists and graded as recommended by WHO. The cases of CCC were classified as follows: 6 cases of well differentiated, 14 cases of moderately differentiated and 9 cases of poorly differentiated carcinomas. Undamaged liver tissues from surgical resection specimens of young adults with minor liver injury who underwent partial hepatectomy were used as normal controls. All tissues were fixed in 40 g/L formaldehyde (pH 7.0) for 12-24 h and embedded in paraffin and then 4 μm thick serial sections were cut and mounted on poly-l-lysine coated slides.

Immunohistochemical staining

The sections were deparaffinized and rehydrated routinely. Antigens were retrieved by heating the sections in a microwave oven at 700 W in 10 mmol/L citrate buffer (pH 6.0) for 10 min. After blocked with 3 mL/L H_2O_2 and swine serum, specimens were then incubated with the primary antibodies, directed against bcl-2 (Maxim Biotech Co., China) and bax (Senta Cruz). The staining was performed by streptavidin peroxidase enzyme conjugate method using a S-P kit (Zymed). Reaction products were visualized by DAB. Brown-yellow granules in cytoplasm were recognized as positive staining.

Semi-nested in situ PCR

Pretreatment: Sections were deparaffinized and dehydrated routinely, then washed in 0.1 mol/L HCI and PBS, digested with proteinase K (10 mg/L), and placed on the platform in a DNA thermal cycler at 98 ℃ for 2 min.

Amplification *in situ*: The final volume of 20 μL (200 μmol/L each of dATP, dCTP, dGTP and dTTP, 1.5 U Taq polymerase,10× buffer). Two and half sets of primers specific for the two hot breakpoint regions on bcl-2 gene were synthesized according to the DNA sequences published by Gribben[19]. The following oligonucleotides were used as primer pairs: A, 5'-CAGCCTTGA AACATTGATGG-3' for the mbr (in major breakpoint region); B, 5'-CGTGCTGGTACCACTCCTG-3' for the mcr (in minor breakpoint region); C, 5'-TATGGTGGTTTGACCTTTAG-3' for the mbr (in major breakpoint region); D, 5'-GGACCTTCCTTGG

TGTGTTG-3' for mcr (in minor breakpoint region); Immunoglobulin heavy chain (JH), 5'-ACCTGAGGAGACGGTGACC-3'. At first, mbr-JH fusion gene was amplified by primer A and JH, and mcr-JH fusion gene was amplified by primer B and JH. The slides were covered after adding reaction mixture. The samples were subjected to 25 amplification cycles, each cycle consisiting of denaturation at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min and extension at 72 ℃ for 1 min. The final extension period was extended to 10 min. Reamplification was performed for 30 cycles using primer C, JH for mbr-JH fusion gene and D, JH for mcr-JH fusion gene. The conditions were the same as stated above.

In situ hybridization

The covers were removed and dehydrated routinely. The slides were incubated in 40 g/L poly formeldehyde for 10 min. Forty μL hybridization solution (probe concentration 50 pmol/L) was added on slides overnight at 37 ℃ in a wet box. The oligonucleotide probe was labeled with biotin. Sequences of the probes were 5'-CCCTCCTGCCCTCCTTCCG-3' for mbr and 5'-GGACCTTC CTTGGTGTGTTG-3' for mcr.

Non-specific antigen was blocked with 20 mL/L bovine serum and 3 g/L Triton X-100, followed by incubation with anti-biotin antibody alkaline phosphates mixture for 1 h. Slides were then visualized with BCIP/NBT. Purple-blue granules in nuclei were regarded as positive.

Controls

Follicular lymphoma was used as positive control. Negative controls included blank control with no primers, dNTP or Taq polymerase omission in PCR reaction solution, and probe omission in hybridization solution. To exclude false positive or negative results, each sample was analyzed at least twice. Non-corresponding tissues such as normal skin tissue were also treated to exclude the false positive possibility.

Statistical analysis

Statistical significance was calculated by χ^2 test, which was used to analyze the relation between bcl-2 and bax protein staining and positive bcl-2/JH fusion gene and histopathological grades. $P<0.05$ was considered as statistically significant.

RESULTS

Expression of bcl-2 protein in CCC

Twenty-one of 29 cases expressed bcl-2 protein, induding 5 well differentiated, 13 moderately differentiated and 3 poorly differentiated carcinomas. There was a statistically significant difference between moderately differentiated and poorly differentiated carcinomas in Bcl-2 expression ($P<0.05$, $\chi^2 = 5.58$). Bcl-2 was also expressed in 87.5% (14/16) in normal liver tissues of small bile ducts (Figures 1, 3).

Detection of bcl-2/JH fusion gene in CCC

Bcl-2/JH fusion gene was not detected in any case of CCC and normal liver tissues.

Relationship between bcl-2 protein expression and bcl-2/JH fusion gene

No bcl-2/JH fusion gene was detected in bcl-2 protein positive and negative groups. There was no correlation between bcl-2 protein expression and bcl-2/JH fusion gene.

Expression of bax protein in CCC

Bax was expressed in 3 (10.3%) of 29 cases , in which 1/6 was well differentiated carcinoma, 2/14 were moderately differentiated carcinomas and none was poorly differentiated carcinoma. There was no statistically significant difference between bax expression and histological grades. Four (25%) of 16 normal liver tissue samples expressed bax protein (Figures 2, 4).

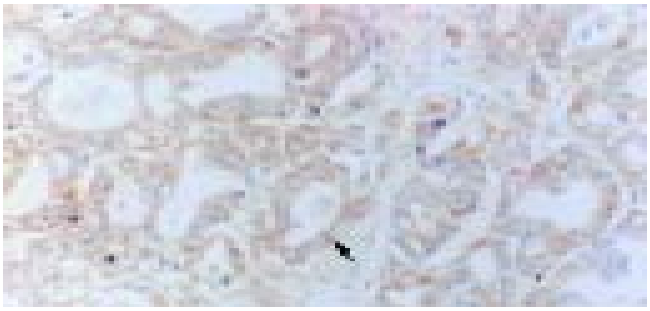

Figure 1 Primary hepatic cholangiocarcinoma. Brown-yellow staining in cytoplasm of carcinoma cells for bcl-2 protein (arrowhead) Immunohistochemistry staining ×200.

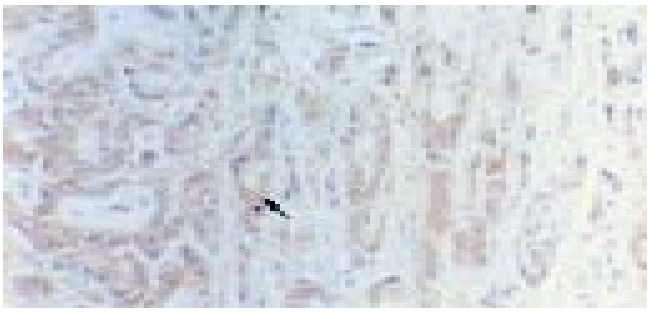

Figure 2 Primary hepatic cholangiocarcinoma. Brown-yellow staining in cytoplasm of carcinoma cells for bax protein (arrowhead) Immunohistochemistry staining ×200.

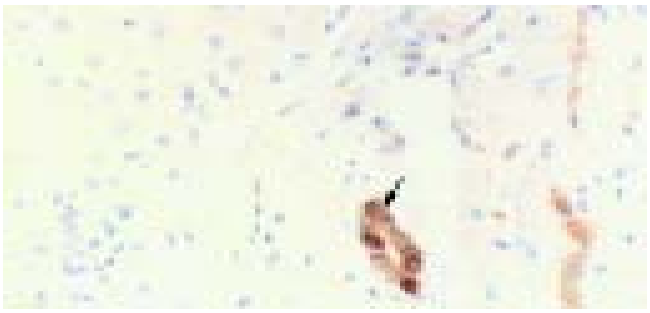

Figure 3 Normal liver. Brown-yellow staining in cytoplasm of small bile duct epithelial cells for bcl-2 protein (arrowhead) Immunohistochemistry staining ×200.

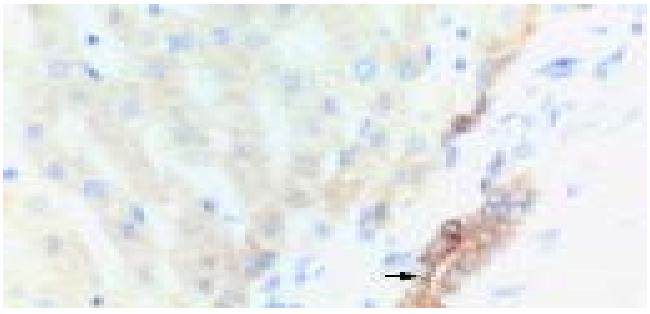

Figure 4 Normal liver. Brown-yellow staining in cytoplasm of small bile duct epithelial cells for bax protein (arrowhead) Immunohistochemistry staining ×200.

Ratio of bcl-2 to bax in normal liver and CCC

In normal liver tissues, the expression rates of bcl-2 and bax

were 87.5% and 25%, while positive rates of bcl-2 and bax were 72.4% and 10.3% in CCC respectively. The ratio of bcl-2 to bax was 3.5 to 1 in normal liver tissues and 7.0 to 1 in CCC (Figure 5).

Figure 5 Non-Hodgkin's lymphoma. Purple-blue deposition in nuclear of lympoma cells (positive for mbr, arrowhead). In situ PCR ×400.

DISCUSSION

Varying results of bcl-2 protein expression in CCC were reported. Bcl-2 protein expression in normal and pathological human liver has been reported by Charlotte *et al*.[11]. In the report, Charlotte *et al.* described that bcl-2 protein was expressed by small bile duct epithelia in normal liver, but not by hepatocytes or large bile duct epithelia. Eight of 11 CCCs were stained positively for bcl-2, whereas all 15 hepatocellular carcinomas were bcl-2 negative. So the authors suggested that bcl-2 protein appeared to be a diagnostic marker in distinguishing CCC from hepatocellular carcinoma[11]. Skopelitou *et al.* found all ten samples of CCC were positive for bcl-2 protein staining[12]. Ito *et al.* reported 31.7% (13/41) of CCCs were positive for bcl-2 protein[13]. However, Terada *et al.* showed low or negative expression of bcl-2 protein in bile duct epithelia and CCC[14-17]. In our report, 21 of 29 CCCs expressed bcl-2 protein (72.4%), which was similar to that reported by Charlotte *et al*. Significant differences of bcl-2 expression in CCC have not been known yet, probably because of the different methods used, and the difference of intrahepatic cholangiocarcinoma specimens from the liver[13,16].

To clarify the role of bcl-2/JH in the pathogenesis of CCC, mbr-JH and mcr-JH which have been shown to be the important molecular event in the genesis of follicular lymphoma, were also studied in 29 cases of CCC. This is the first study to examine bcl-2/JH fusion gene in CCC by *in situ* PCR. Mbr-JH or mcr-JH was negative in all cases of CCC. It is suggested that bcl-2/JH fusion gene formation was not a common event and might not play an important role in the pathogenesis of CCC.

In the present study, bcl-2 protein expression did not correlate with bcl-2/JH in CCC. Similar results were also found in mucosa-associated lymphoma, large cell lymphoma, Hodgkin's disease and nasopharyngeal carcinoma[20-25]. These findings suggested that abnormal bcl-2 protein expression might not be solely resulted from bcl-2/JH fusion sequences. Whether other types of bcl-2 gene aberrance such as mutation or methylation corresponding to bcl-2 protein expression are existed in CCC requires further investigation.

Compared to a high expression rate of bcl-2 in CCC (72.4%), only 10.3% (3/29) samples expressed bax. To understand the significance of the ratio of bcl-2 to bax in the regulation of apoptosis, Oltvai *et al*.[18] found that over-expressed bcl-2 in two cell lines with high levels of bax and in cells with the lowest bcl-2 to bax ratio (0.55) was 73% viable at 24 h, but lost viability by d 7. When cells with high bcl-2 to bax ratios (2.04 and 1.65), they possessed viable cells over 2 wk following IL-3 deprivation. These data suggested a model in which the ratio of bcl-2 to bax could determine survival or death following an apoptotic stimulus. Similar results were also found by Lilling *et al.* and others[26-29]. The alteration of Bcl-2 and Bax might be an important mechanism in the pathogenesis and progression of some tumors[30-32]. Yue *et al*.[30] showed that Bcl-2/Bax in acute myelogenous leukemia was significantly higher than that in normal control ($P<0.001$). Madewell *et al*.[31] found that the bax:bcl-2 ratio was lower in skin tumors than that in normal skin. But a lowered bcl-2/bax ratio and increased apoptosis were observed in oral squamous cell carcinomas, compared with normal oral epithelium[32]. In this study, the ratio of bcl-2 to bax (7.0 to 1) in CCC was different from that in normal small bile ducts (3.5 to 1). We suppose that the changes of the ratio between bcl-2 and bax protein may influence the susceptibility of bile duct cells to apoptosis and would be involved in the tumorigenesis of CCC. Further evidence would be obtained from further investigations.

REFERENCES

1 **Tsujimoto Y,** Finger LR, Yunis J, Nowell PC, Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with the t (14; 18) chromosome translocation. *Science* 1984; **226**: 1097-1099

2 **Weiss LM,** Warnke RA, Sklar J, Cleary ML. Molecular analysis of the t (14; 18) chromosomal translocation in malignant lymphomas. *N Engl J Med* 1987; **317**: 1185-1189

3 **Bskhshi A,** Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ. Cloning the chromosomal breakpoint of t (14; 18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* 1985; **41**: 899-906

4 **Aisenberg AC,** Wilkes BM, Jacobson JO. The bcl-2 gene is rearranged in many diffuse B-cell lymphomas. *Blood* 1988; **71**: 969-972

5 **Mcdonnell TJ,** Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, Tu SM, Campbell ML. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res* 1992; **52**: 6940-6944

6 **Nakopoulou L,** Vourlakou C, Zervas A, Tzonou A, Gakiopoulou H, Dimopoulos MA. The prevalence of bcl-2, p53, and Ki-67 immunoreactivity in transitional cell bladder carcinomas and their clinicopathologic correlates. *Hum Pathol* 1998; **29**: 146-154

7 **Lu QL,** Elia G, Lucas S, Thomas JA. Bcl-2 proto-oncogene expression in Epstein-Barr-virus-associated nasopharyngeal carcinoma. *Int J Cancer* 1993; **53**: 29-35

8 **Pezzella F,** Turley H, Kuzu I, Tungekar MF, Dunnill MS, Pierce CB, Harris A, Gatter KC, Mason DY. Bcl-2 protein in non-small-cell lung carcinoma. *N Engl J Med* 1993; **329**: 690–694

9 **Papadimitriou CS,** Costopoulos JS, Christoforidou BP, Kotsianti AJ, Karkavelas GS, Hytiroglou PM, Koufogiannis DJ, Nenopoulou HE. Expression of Bcl-2 protein in human primary breast carcinomas and its correlation with multifocality, histopathological types and prognosis. *Eur J Cancer* 1997; **33**: 1275-1280

10 **Tjalma W,** De Cuyper E, Weyler J, Van Marck E, De Pooter C, Albertyn G, van Dam P. Expression of bcl-2 in invasive and in situ carcinoma of the uterine cervix. *Am J Obstet Gynecol* 1998; **178**(1 Pt 1): 113-117

11 **Charlotte F,** L'Hermine A, Martin N, Geleyn Y, Nollet M, Gaulard P, Zafrani ES. Immunohistochemical detection of bcl-2 and protein in normal and pathological human liver. *Am J Pathol* 1994; **144**: 460-465

12 **Skopelitou A,** Hadjiyannakis M, Alexopoulou V, Krikoni O, Kamina S, Agnantis N. Topographical immunohistochemical expression of bcl-2 protein in human liver lesions. *Anticancer Res* 1996; **16**: 975-978

13 **Ito Y,** Takeda T, Sasaki Y, Sakon M, Monden M, Yamada T, Ishiguro S, Imaoka S, Tsujimoto M, Matsuura N. Bcl-2 expression in cholangiocellular carcinoma is inversely correlated with biologically aggressive phenotypes. *Oncology* 2000; **59**: 63-67

14 **Terada T,** Nakanuma Y. Utility of pancreatic digestive enzyme immunohistochemistry in the differential diagnosis of hepatocellular carcinoma, cholangiocarcinoma and metastatic adenocarcinoma of the liver. *Pathol Int* 1996; **46**: 183-188

15 **Okaro AC,** Deery AR, Hutchins RR, Davidson BR. The expres-

sion of antiapoptotic proteins Bcl-2, Bcl-X(L), and Mcl-1 in benign, dysplastic, and malignant biliary epithelium. *J Clin Pathol* 2001;**54**: 927-932

16 **Arora DS,** Ramsdale J, Lodge JP, Wyatt JI. p53 but not bcl-2 is expressed by most cholangiocarcinomas: a study of 28 cases. *Histopathology* 1999; **34**: 497-501

17 **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

18 **Oltvai ZN,** Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993; **74**: 609-619

19 **Gribben JG,** Freedman A, Woo SD, Blake K, Shu RS, Freeman G, Longtine JA, Pinkus GS, Nadler LM. All advanced stage non-Hodgkin's lymphomas with a polymerase chain reaction amplifiable breakpoint of bcl-2 have residual cells containing the bcl-2 rearrangement at evaluation and after treatment. *Blood* 1991; **78**: 3275-3280

20 **Tang SC,** Visser L, Hepperle B, Hanson J, Poppema S. Clinical significance of bcl-2-MBR gene rearrangement and protein expression in diffuse large-cell non-Hodgkin's lymphoma: an analysis of 83 cases. *J Clin Oncol* 1994; **12**: 149-154

21 **Isaacson PG,** Wotherspoon AC, Diss TC, Pan LX. Bcl-2 expression in lymphomas. *Lancet* 1991; **337**: 175-176

22 **Huebner-Chan D,** Fernandes B, Yang G, Lim MS. An immunophenotypic and molecular study of primary large B-cell lymphoma of bone. *Mod Pathol* 2001; **14**: 1000-1007

23 **Kneba M,** Eick S, Herbst H, Pott C, Bolz I, Dallenbach F, Hiddemann W, Stein H. Low incidence of mbr bcl-2/JH fusion genes in Hodgkin's disease. *J Pathol* 1995; **175**: 381-389

24 **Nolte M,** Werner M, Spann W, Schnabel B, von Wasielewski R, Wilkens L, Hubner K, Fischer R, Georgii A. The bcl-2/JH gene rearrangement is undetectable in Hodgkin's lymphomas: results from the German Hodgkin trial. *Virchows Arch* 1995; **426**: 37-41

25 **Harn HJ,** HO LI, Liu CA, Liu GC, Lin FG, Lin JJ, Chang JY, Lee WH. Down regulation of bcl-2 by p53 in nasopharyngeal carcinoma and lack of detection of its specific t(14; 18) chromosomal translocation in fixed tissues. *Histopathology* 1996; **28**: 317-323

26 **Lilling G,** Hacohen H, Nordenberg J, Livnat T, Rotter V, Sidi Y. Differential sensitivity of MCF-7 and LCC2 cells, to multiple growth inhibitory agents: possible relation to high bcl-2/bax ratio? *Cancer Lett* 2000; **161**: 27-34

27 **Chresta CM,** Masters JR, Hickman JA. Hypersensitivity of human testicular tumors to etoposide-induced apoptosis is associated with functional p53 and a high Bax: Bcl-2 ratio. *Cancer Res* 1996; **56**: 1834-1841

28 **Perlman H,** Zhang X, Chen MW, Walsh K, Buttyan R. An elevated bax/bcl-2 ratio corresponds with the onset of prostate epithelial cell apoptosis. *Cell Death Differ* 1999; **6**: 48-54

29 **Raisova M,** Hossini AM, Eberle J, Riebeling C, Wieder T, Sturm I, Daniel PT, Orfanos CE, Geilen CC. The Bax/Bcl-2 ratio determines the susceptibility of human melanoma cells to CD95/fas-mediated apoptosis. *J Invest Dermatol* 2001; **117**: 333-340

30 **Yue B,** Chen Y, Yu D, Xiang Z. Study on the relationship between the Bcl-2/Bax ratio and the growth types of leukemic cells and drug resistance in acute myelogenous leukemia. *J Tongji Med Univ* 1998; **18**: 101-104

31 **Madewell BR,** Gandour-Edwards R, Edwards BF, Matthews KR, Griffey SM. Bax/bcl-2: cellular modulator of apoptosis in feline skin and basal cell tumours. *J Comp Pathol* 2001; **124**: 115-121

32 **Loro LL,** Vintermyr OK, Liavaag PG, Jonsson R, Johannessen AC. Oral squamous cell carcinoma is associated with decreased bcl-2/bax expression ratio and increased apoptosis. *Hum Pathol* 1999; **30**: 1097-1105

Edited by Zhang JZ and Wang XL **Proofread by** Xu FM