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

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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 expression of bcl-2 protein may be involved in the course of tumorigenesis of CCC. Abnormal bcl-2 protein expression cannot be solely resulted from bcl-2/JH fusion gene.

Introduction

Bcl-2 gene was first identified in B-cell leukemia and follicular lymphoma which conferred 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[15]. 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[14-16]. 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[17-19]. 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 H2O2 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 HCl and PBS, digested with proteinase K (10 mg/L), and placed on the platform in a DNA thermal cycler at 98°C 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, 10X 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’-CACGCCTTGG AACATTGATGG-3’ for the mbr (in major breakpoint region); B, 5’-CGTGGTGTCACACCTCTG-3’ for the mcr (in minor breakpoint region); C, 5’-TATTTGTGGTTACCTTTAG-3’ for the mbr (in major breakpoint region); D, 5’-GGACCTTCCTTGG
TGTGTG-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 consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C 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 formaldehyde for 10 min. Forty µL hybridization solution (probe concentration 50 pmol/L) was added on slides overnight at 37 °C in a wet box. The oligonucleotide probe was labeled with biotin. Sequences of the probes were 5’-CCCTCCTGGCCTCCTTCCG-3’ for mbr and 5’-GGACCTTCCTTGGTGTGTTG-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 χ2 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, including 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, χ2 = 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).

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 lymphoma 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, 15]. 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 [16, 17].

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 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 [26-31]. These findings suggest 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, 31]. Yue et al. [32] showed that Bcl-2/Bax in acute myelogenous leukemia was significantly higher than that in normal control (P < 0.001). Madewell et al. [33] 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 [34]. 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.
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Edited by Zhang JZ and Wang XL. Proofread by Xu FM