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Expression of regulating apoptosis gene and apoptosis index in primary liver cancer

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INTRODUCTION

Programed cell death plays an important role in the genesis of cancer[1-5]. Certain cancer genes can regulate apoptosis. Recently, several proteins that are structurally related to Bcl-2, an inhibitor of apoptosis, have been identified[6-11]. Therefore, novel strategies and agents that target specific molecular pathways, as well as triggering a process of cell death, are being evaluated in the treatment of several neoplasms. The homologous Bcl- 2 and Bcl-x1 proteins can extend cell survival by suppressing apoptosis[12,13], whereas the proapoptotic proteins (e.g. Bax, Bak, Bik) act as dominant cell death inducers when overexpressed[14,15]. C-myc not only can promote cell growth, but also induce apoptosis. Many studies have shown that oncogene plays an important role in the growth, progression and metastasis of solid tumors. Recently, several oncogene factors have been identified. Primary liver cancer remains one of the most common malignancies worldwide, with an annual incidence of approximately 1 million cases[16,17]. It bears the character of portal venous invasion, so metastases are often presented when the cancer is discovered. We use immunohisto - chemical method to detect the protein expression o f Bcl-2, Bax, c-myc and use TdT-mediated dUTP nick end labeling (TUNEL) method to detect the apoptosis index. The purpose of our study was to evaluate the function of these proteins and their relationship with the clinical characteristics of hepatoma.

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MATERIALS AND METHODS

Clinical meterial

Twenty-two resected hepatoma specimens in our hospital from 1994 to 1998 were studied. All the specimens were confirmed by pathology. Twenty-seven were men, and 5 women, average age 50.26 years. No patient had received chemotherapy or radiation therapy before surgery. All the specimens together with some paracance rous tissues were fixed in 100mL/L formaldehyde solution and embedded in paraffin. Five μm thick sections were cut and mounted on glass slides.

Immunohistochemical determination and in situ hybridization detection

Immunohistochemical determination of Bcl-2, Bax and c-myc Immunohistochemical studies were performed using ABC method. The antibodies of Bcl-2, Bax and c-myc were bought from Boshide Biotechnology, Inc (Wuhan). Immunohist-ochemical assay was performed as described by the manufacturer. Immunohistochemical studies were performed using the streptoavidinbiotin method. Sections were dewaxed in xylene, taken through ethanol, and then incubated with 3mL/L hydrogen peroxide in methanol for 30min to block endogenous peroxidase activity. Sections were then washed in phosphate-buffered saline and incubated in 100mL/L normal goat serum for 20min to reduce nonspecific antibody binding. Specimens were then incubated with a 1:50 dilution of a primary antibody overnight at 4℃, followed by three washes with PBS. Sections were then incubated with biotinylat ed goat antirabbit immunoglobulin G at a dilution of 1:100 for 30min followed by 3 washes slides were then treated with streptoavidin-peroxidase reagent for 30min at a dilution of 1:100 and were washed with PBS 3 times. Finally, slides were incu bated in PBS containing diaminobenzidine and 10mL/L hydrogen peroxide for 10min, counterstained with methyl green, and mounted. Normal rabbit immunoglob ulin-G was substituted for primary antibody as the negative control. Slides were interpreted for antigen expression by two investigators without knowledge of the corresponding clinicopathologic data. Ten optical fields, about 500-1000 cells were counted in each slide under the high power (×400) microscopy. Results presented as -, indicated 25% slightly positively stained cells; +, 25%-50% positive cells; ++, 50%-75% positive cells; +++, >75% positive cells.

In site hybridization dectetion

Apoptosis was further confirmed by the TdT-mediated DUTP nick end labeling (TUN EL) method using a detection kit from Boshide Biotechnology, INC (Wuhan).

Sections were dewaxed in Xylene, taken through ethanol, and then incubated with 30mL/L hydrogen peroxide in methanol for 30min, washed with PBS, incubated in dialysate solution for 2min on ice, and incubated with TUNEL reaction mixture at 37℃ for 30min. Samples were washed with PBS, incubated with converter PO D at 37℃ for 1h and stained with 3, 3 '-diaminobenzidine tetrahydroc hloride. A negative control using all reagents except terminal transferase was performed in parallel. The nucleus of positive cells was stained brown as detected under light microscopy. Ten optical fields, about 500-1000 cells were counted in each slide under the high power (×400) microscopy. The apoptosis index is the percentage of positive cells in 1000 cells.

Statistical analysis

Statistical significence was determined by using χ^2 test or Student's *t* test.

RESULTS

The protein expressions of Bcl-2, Bax, c-myc and apoptosis

The rate of Bcl-2 protein positive expression in human hepatocellular carcinoma was 22.73% and it was 18.18% in paracancerous tissues. No difference was seen as compared with each other ($P>0.05$). The rate of Bax positive expression was 45.45% in carcinoma tissue and 77.27% in paracancerous tissues ($P<0.01$). The rate of c-myc protein expression was 81.82% in carcinoma tissues and 72.73% in paracancerous tissues ($P>0.05$). The apoptosis index in hepatocellular carcinoma was 9.55% and was lower than that in paracancerous tissues ($P<0.01$).

The relationship between the protein expressions of Bcl-2, Bax and apoptosis index in cancerous tissues and paracancerous tissues

The apoptosis index of Bcl-2 positive group was higher than that of Bcl-2 negative group, but no difference was found between them ($P>0.05$); The apoptosis index of Bax positive group was significent higher than that of Bax negative group ($P<0.01$) (Table 1).

Relationship between protein expressions of Bcl-2, Bax and protein expression of c-myc in cancerous tissues

In the carcinoma tissues, the expression of c-myc was not related to the expression of Bcl-2 and Bax (Table 2).

Table 1 The relationship between the protein expressions of Bcl-2, Bax and apoptosis index in cancerous tissues and paracancerous tissues

	Case	Apoptosis index %
Cancerous tissues		
Bcl-2 negative	17	9.12±5.37
Bcl-2 positive	5	11.00±5.48
Bax negative	12	5.83±1.95
Bax positive	10	14.00±4.59[b]
Paracancerous tissues		
Bcl-2 negative	18	16.39±5.89
Bcl-2 positive	4	12.50±5.00
Bax negative	5	10.00±3.54
Bax positive	17	17.35±5.62[b]

[b]$P<0.01$ *vs* compared with Bax negative group.

Table 2 The relationship between the protein expressions of Bcl-2, Bax and the protein expression of c-myc in cancerous tissues

	c-myc positive	c-myc negative
Bcl-2 positive	3	1
Bcl-2 negative	15	3
Bax positive	14	3
Bax negative	4	1

DISCUSSION

Apoptosis, a normal cellular process that provides the orderly existence and death of cells in a programmed fashion, may be positively or negatively modulated by several external or internal factors[18-22]. Modern molecular biology investigations have indicated that proliferative inhibition of some neoplasm cells is related to apoptosis induction regulated by the oncogene expression of these cells[23-26]. Recently, regulating apoptosis gene is divided into two groups, existence gene and death gene. Living gene includes improving cell proliferation gene, c-myc and improving cell existence gene, Bcl-2[27-29]. Death gene includes supressing cell proliferation gene i.e. p53 and promoting cell death gene, i.e. Bax[30,31]. It is generally considered that Bcl-2 is an important gene for cell survival, the Bcl-2 gene can inhibit the apoptosis by blocking the last tunnel of apoptotic signal transmitted system[32-37], C-myc is a kind of effective protein of the karyomitosis signal, which can trigger and regulate the transcription of the genes related with proliferation[38]. Some recent study showed that Bcl-2 and Bax play important roles in regulating apoptosis in prostate cancer, mammary cancer and gastric carcinoma[39-41]. But there has been no report on the relationship between regulating apoptosis gene and apoptosis index in human hepatocellular carcinoma.

We detected the protein expression of Bcl-2, Bax and c-myc in hepatocellular carcinoma and paracancerous tissues using immunohistochemical stain. The rate of Bcl-2 protein positive expression in human hepatocellular carcinoma was 22.73 %,

and 18.18% in paracancerous tissues. No statistical difference was seen between them (P>0.05). The result was similar to that of Guo *et al*[42]. The rate of Bax protein positive expression in hepatocellular carcinoma was 45. 45%, and 77.27% in paracancerous tissues. We have found that Bcl-2 and Bax play a crucial role in the genesis of hepatoma. The higher expression of Bcl-2 and the lower expression of Bax supressed apoptosis. These broke the balance between cell proliferation and apoptosis, and resulted in carcinogenesis. Bcl-2 protein blocks apoptosis by binding to and suppressing the action of Bax, a positiver egulator of cell death. The expression of c-myc was higher in the carcinoma tissue than that in paracancerous, but no difference was seen as compared with each other. The result was similar to the report of Yang[43]. The c-myc proto-oncogene, usually implicated in cell transformation, differentiation and cell cycle progression, also has a central role in some forms of apoptos is[44,45]. These opposite roles of c-myc in cell growth and death requi re other gene products to dictate the outcome of c-myc expression on a cell. We thought that the higher expression of Bcl-2 could not effect cell proliferation, the higher expression of c-myc could improve cell proliferation. The paracancerous tissues may be in the precancer state. But the mechanism of regulating cell apoptosis is intact. The balance of cell proliferation and death was not broken, cell was still in normal state. The paracancerous tissues could become cancer following the enhancing of cell proliferation and the imbalance in regulating apoptosis. Bissonnette RP found that Chinese hamster ovary cell apoptosed due to the proliferation of c-myc gene, but it would be suppressed by Bcl-2 gene[35], and cell would be in a balanced state of proliferation and apoptosis resulting from the appropriate expression of c-myc gene. The balance would be broken by some factors. A 'two signal' model emerges, in which c - myc can provide the first signal, leading either to apoptosis or proliferation, and certain growth factors may provide a second signal, to inhibit apoptosis and allow c-myc to drive cells into the cell cycle. Bcl-2 may substitute for the putative signal, as suggested by the abilities of Bcl-2 to delay apoptosis after growth-factor withdrawal and to cooperate with c-myc in transforming cells[46]. In our study, no relation ship was seen between the expressions of c-myc and Bcl-2 or Bax. We propose that c-myc activation affords the advantage of proliferation is essential for carcinogenesis, because the proliferative and apoptosis functions of c-myc are tightly coupled, it is impossible to select only for one without the other. In our study, the number of cases was insufficient to reflect the relationship.

Now, there are a lot of methods to detect apoptosis. But TdT-mediated dUTP nick end labeling method is the most accurate one[47]. It could find the DNA broken in the early stage and accurate localization, so it is most accurate to reflect the status of apoptosis. Using this method, the apoptosis index of paracancerous tissue was 15.68%, which was higher than that of cancer tissues, 9.55%. There was no difference of apoptosis index between the positive and negative expression of Bcl-2. There was significant difference between the apo ptosis index in Bax positive and negative group. We thought the balance of the expression of Bcl-2 and Bax may decide whether the cell is alive or dead. The isodipolymer with Bax and Bcl-2 could decide the protein molecules of active Bcl -2 or not. Bcl-2 could have different effects on inducing or inhibiting apoptosis. So isodipolymer Bcl-2/Bax is more accurate in reflecting the status of a poptosis. There were significant differences between the apoptosis index in cancer tissues and paracancerous tissues. It is valuable to study whether apoptosis index could become an index in judging canceration or not.

In conclusion, our study demonstrated that apoptosis plays an important role in the genesis of hepatoma, Bcl-2/Bax could reflect accurately the status of apoptosis. Apoptosis index was the most accurate index to reflect apoptosis, and may become an index for judging carcinogenesis. It is also valuable to study whether c-myc is affected by Bcl-2 and Bax expression.

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