Dynamic expression of apoptosis-related genes during development of laboratory hepatocellular carcinoma and its relation to apoptosis

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INTRODUCTION
Occurrence and biological characteristics of tumors are related not only to over-proliferation of carcinoma cells but also to decrease of apoptosis. Investigation of apoptosis helps to disclose the biological characteristics of tumors, and seeks new methods of diagnosis and treatment for tumors. Product of gene bax (bcl-2 associated X gene) expression is a 21-ku protein, which is able to combine bcl-2 protein to form a heterogeneous dimer, thus inhibiting the function of bcl-2 and promoting the occurrence of apoptosis. p53 is universally accepted as a tumor suppressor, whose mutation and deficiency accelerate cancer growth. Survivin is a new member of the inhibitor of apoptosis inhibiting protein family. It is believed that cell apoptosis induced by most of apoptosis signals is realized through the effect of a cascade protease, namely caspase. Survivin can act directly on caspase, thus inhibiting the activity of caspase-3 and caspase-7[1] and eventually blocking the terminal pathway of apoptosis. In this research, expression of p53, bcl-2, bax, and survivin in different stage liver tissues during the development of laboratory tree shrew HCC were explored by the immunohistochemistry method and the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) technique. The relationship between expression of these genes, its impact on HCC formation, and its relation to cell apoptosis were discussed.

MATERIALS AND METHODS

Animal experiment
Adult laboratory tree shrews, weighing 100-160 g, were raised in separate stainless cages under a laboratory temperature of 25±2 °C. After 2-4 wk of feeding, all animals were examined for serum ALT and HBsAg. Thus, 61 healthy animals were
selected and divided into experimental group and control group.

Forty-eight tree shrews in the experimental group received aflatoxin B1 (AFB1) at the beginning of the experiment, 150 µg/(kg·d), five times/wk. AFB1 was solved into dimethyl sulfoxide, then added into milk for lapping by the animals. The animals received AFB1 for 105 wk then common animal food for 45 wk when the experiment was completed.

Thirteen tree shrews in the control group did not receive AFB1 but common animal food, milk and fruits.

Liver tissue biopsy

During the whole experiment, liver tissue biopsy was carried out in both groups of tree shrews once in every 15 wk. Biopsy tissues were fixed in 10% formaldehyde solution.

Tissue specimens

Specimens of liver biopsy tissues taken at wk 30 and 60 and HCC tissues from the experimental animals and those of the liver biopsy tissues taken at wk 30, 60, and 90 were embedded with paraffin wax and cut into 5-µm consecutive sections, then subjected to the routine HE staining. Expression of p53, bcl-2, bax, and survivin was detected by immunohistochemistry method. Apoptotic cells were subjected to in situ detection by the TUNEL technique.

Reagents

Mouse anti-human p53, bcl-2, bax, and survivin mAbs were from Beijing Zhongshan Company. Streptavidin-peroxidase (S-P) kits and DAB developers were from Fuzhou Maixin Company; TUNEL staining kits (Cat. No. 1684809) were from Beohringer Mannheim GmbH, Deutschland. DNAase I, proteinase K, and 3% bovine serum albumin were from Dalian Baosheng Company.

Immunohistochemistry staining and in situ detection of apoptosis

S-P method was applied. Sections before addition of mAb were subjected to microwave antigen repairing, all other procedures were according to the kit manual. PBS was substituted for the first antibody to act as the negative contrast, and the positive contrast sections from Fuzhou Maixin Company were used as the positive contrast. In situ detection of apoptosis was carried out as previously described\(^2\). Positive contrast sections were subjected to 30-min digestion with DNAase I before TUNEL reacting solution was added, whereas negative contrast sections were subjected to TdT-free TUNEL reacting solution.

Result assessment and statistical analysis

p53 and survivin protein positive reactions were located in nuclei, presenting light or dark brown. Bcl-2 and bax protein positive reactions were located in cytoplasm, appearing as dark brown.

Immunohistochemical staining was divided into five grades: grade 0: <5%; grade I: 5-25%; grade II: 25-50%; grade III: 50-75%; and grade IV: >75%. Five percent was taken as the criterion for the judgment of positive cells.

Staining intensity of positive cells was divided into three grades: grade I: low; grade II: medium; grade III: strong. The staining intensity of all the positive contrast sections was determined as grade III for reference. Immunohistochemical staining score = grading for percentage of positive cells \(\times\) staining intensity\(^2\).

Apoptotic cells were calculated under five high power fields randomly selected, at least than 1 000 cells per field. Percentage of positive cells to total cells was taken as apoptosis index.

Statistical analysis was carried out by variance analysis, \(\chi^2\) test, etc., with the statistical software package PEMS.

RESULTS

Occurrence of HCC

At the 150\(^{th}\) wk of the experiment, all dead animals were subjected to etiological diagnosis of HCC. The result showed that occurrence of HCC was seen in 35 of 48 experimental tree shrews, with an incidence rate of 72.9%, and in none of 13 control tree shrews. The earliest time of carcinoma occurrence was 88 wk, and the latest time was 150 wk. The average time was 117 wk.

Pathological tissues’ change at each stage

In the liver biopsy tissues from the control animals at each stage, liver cells were arrayed in good order, lobular structure was clear and complete, and no inflammation and hepatocyte hyperplasia appeared. In the experimental animals, the liver biopsy tissues taken at wk 30 and 60 appeared with hepatocellular degeneration and hyperplasia change. The former was primarily vacuole degenerative, the latter presented hepatocyte array in two lines, multi-lines, even pieces, with clear lobular structure. In HCC animals, liver cells were poorly and moderately differentiated presenting diffusive, nodular, or girder-like types.

Expression of apoptosis-related genes and cell apoptosis

The expression of p53, bcl-2, bax, and survivin in HCC and other liver tissues is listed in Table 1; the apoptosis indices in HCC and other liver tissues are shown in Table 2; the relationship between expression of p53, bcl-2, bax, survivin, and cell apoptosis in HCC tissues is summarized in Table 3.

DISCUSSION

Studies\(^3,15,18,20\) have shown that genes p53, bcl-2, bax, and survivin play an important role in HCC formation, but these studies are confined to the resected HCC, employing carcinoma-adjacent tissues as control liver tissues. This study was carried out in the tree shrew HCC model established at the Department of Pathology of the Affiliated Tumor Hospital of Guangxi Medical University. The HCC model allows repeated biopsy with no animals being killed during the induction of HCC, so that liver tissues at different stages of carcinogenesis are available. These tissues were utilized for the investigation on the dynamic expression of p53, bcl-2, bax, and survivin during the development of HCC, and for the exploration of its impact on HCC development.

Relationship between cell apoptosis and HCC

Apoptosis is a process of programmed cell death under certain physiological or pathological conditions. This process is active, highly ordered, multi-gene controlled, and serial-
enzyme catalyzed. As an important modulating mechanism maintaining the stability of organ itself, apoptosis not only plays a role in physiological courses such as embryo developing, tissue shaping, hematopoiesis modulating, growing and ageing, but also has a close relation to cancer occurrence and development. The occurrence of cancer is related to the over-proliferation of carcinoma cells and the relative destitution of apoptosis.

It was reported that the apoptosis index in HCC is apparently lower than that in tissues of normal liver, chronic hepatitis, hepato-cirrhosis, and adjacent cancer tissues. The occurrence of cancer is related to the over-proliferation of carcinoma cells and the relative destitution of apoptosis.

### Table 1: Expression of p53, bcl-2, bax, and survivin in HCC and other liver tissues

| Groups | Biopsy at | Cases | p53 | Bcl-2 | Bax | Survivin |
|--------|-----------|-------|-----|------|-----|---------|
|        |           |       | Positive (%) | Positive (%) | Positive (%) | Positive (%) |
| Control | 30 wk     | 13    | 0 (0)         | 0 (0)          | 0 (0)         | 4 (30.8)     |
|         | 60 wk     | 13    | 0 (0)         | 0 (0)          | 0 (0)         | 5 (38.4)     |
|         | 90 wk     | 13    | 0 (0)         | 0 (0)          | 0 (0)         | 5 (38.4)     |
| Trial   | 30 wk     | 35    | 0 (0)         | 3 (8.6)        | 2 (5.7)       | 14 (40)      |
|         | 60 wk     | 35    | 21 (60)       | 7 (20)         | 10 (28.6)     | 13 (37.1)    |
|         | HCC       | 35    | 25 (71.4)     | 14 (40)        | 25 (71.4)     | 19 (57.1)    |

### Table 2: Apoptosis indices in HCC and other liver tissues

| Groups | Biopsy at | Cases | Apoptosis indices |
|--------|-----------|-------|-------------------|
|        |           |       | Maximum | Minimum | Average |
| Control | 30 wk     | 13    | 0.25     | 0        | 0.01±0.02 |
|         | 60 wk     | 13    | 0.25     | 0        | 0.01±0.02 |
|         | 90 wk     | 13    | 0.3      | 0        | 0.02±0.04 |
| Trial   | 30 wk     | 35    | 1.5      | 0        | 0.57±0.47 |
|         | 60 wk     | 35    | 2.1      | 0        | 0.70±0.60 |
|         | HCC       | 35    | 2.5      | 0.1      | 0.71±0.51 |

### Table 3: Relationship between expression of p53, bcl-2, bax, survivin, and cell apoptosis in HCC tissues

| Observation indications | Cases | Apoptosis indices (%) | P |
|-------------------------|-------|-----------------------|---|
|                         |       | Maximum | Minimum | Average |     |
| p53                     | +     | 25      | 2.5     | 0.1     | 0.70±0.57 | >0.05 |
|                         | -     | 10      | 1.0     | 0.2     | 0.72±0.31 | <0.05 |
| Bcl-2                   | +     | 14      | 2.5     | 0.4     | 1.02±0.45 | <0.01 |
|                         | -     | 21      | 1.5     | 0.1     | 0.50±0.40 | <0.05 |
| Bax                     | +     | 25      | 2.5     | 0.2     | 0.74±0.25 | <0.01 |
|                         | -     | 10      | 0.5     | 0.1     | 0.24±0.16 | <0.05 |
| Survivin                | +     | 19      | 1.5     | 0.1     | 0.51±0.40 | <0.05 |
|                         | -     | 16      | 2.5     | 0.2     | 0.86±0.54 | <0.01 |
| Bcl-2 Bax               | +     | 14      | 2.5     | 0.5     | 1.02±0.45 | <0.01 |
|                         | -     | 10      | 0.5     | 0.1     | 0.25±0.15 | <0.05 |

### Relationship between p53, bcl-2, bax, and survivin expression, HCC, and apoptosis

Fifty percent of human tumors are related to p53 mutation.
The wild-type p53 has a very short half life, and cannot be detected by immunohistochemistry method. The half life of mutant p53 is long enough to allow detection by the same method[11]. The possible reason is that the control animals are free from cancer-inducible factors so that the liver tissues do not present any substantial change. At the late stage of our experiment, p53 was significantly expressed (0.60% and 71.4%), showing that the mutant p53 participate in occurrence of liver cancer. It is also found that p53 protein expression does not relate to apoptosis index level. The reason may be that during the development of idiopathic HCC, liver cell apoptosis increases with the augment of liver cell proliferation. In addition, in this experiment p53-positive tissues accompanied the expression of the anti-apoptosis-promoting gene bax, which may counteract the influence of p53 on apoptosis. Therefore, in AFB1-induced HCC p53 expression does not inhibit cell apoptosis but stimulates cell proliferation, thus promoting the malignant conversion of cells.

It was reported that bax can significantly promote cell apoptosis[15], yet bcl-2 has anti-apoptosis effects. Bax distributes in tissues and cells in human body, and its expression is high in liver and kidney tissues, but low in most carcinoma tissues. This study found that bax did not express in tissues of the control animals, but expressed apparently with an increasing tendency in tissues of the HCC-infected animals. Presently, there are still some controversies over the relationship between bcl-2 and HCC. Results from this study indicate that bcl-2 is significantly expressed in HCC tissues but not in tissues of control animals. The scores of bcl-2 expression present an increasing trend, suggesting that both bax and bcl-2 proteins take part in the formation of HCC.

The anti-apoptosis effect of bcl-2 is affected by bax. The bcl-2/bax ratio is a key factor for determining apoptosis. When bcl-2 expresses excessively, bcl-2-bax heterogeneous dimer predominates, thus inhibiting apoptosis[18]. When bax expresses excessively, bax-bax homogeneous dimer or monomer predominates, thus promoting apoptosis. This study found that apoptosis index for bcl-2-positive HCC tissues was higher than that for bcl-2-negative tissues. In bcl-2-positive tissues, bax appeared positive. Because of the overexpression of bax, bax-bax homogeneous dimer predominates, thus speeding up apoptosis.

Survivin, a recently discovered anti-apoptosis gene, bears the following anti-apoptosis mechanisms: (1) directly inhibiting the activity of the terminal-responsive enzymes caspase-3 and caspase-7, which act during the apoptosis process, thus blocking up apoptosis[16]; (2) combining the cell cycle modulator CDK4 to form survivin–CDK4 complex, thus causing CDK4 complex releasing p21 that further combines with caspase-3 within mitochondrion, so as to inhibit the activity of caspase-3, eventually blocking apoptosis[19].

Although survivin inhibits various kinds of stimulant-induced apoptosis through a direct action on caspase, survivin does not significantly present any anti-apoptosis effect in HCC. This study found that survivin rendered high expression in normal liver tissues, experimental animal liver tissues, and HCC tissues, showing no significant difference between these three tissues. This is in accordance with the previous report[16]. In HCC, apoptosis index for survivin-positive tissues is higher than that for survivin-negative tissues, suggesting that during the formation of HCC, enhancement of survivin expression is a late incident. The possible reason is that tumor formation activates through a certain mechanism, then holds down apoptosis of carcinoma cells, and at last results in consecutive proliferation and differentiation of carcinoma cells. Therefore, the primary effect of survivin in HCC is to inhibit cell apoptosis and promote cell proliferation and malignant conversion.

**Interrelationship between p53, bcl-2, bax, and survivin in HCC**

Carcinoma occurrence is an extremely complicated course. Though it is known that genes have a close relation to carcinoma occurrence, but whether they relate to each other is still unknown. p53 is an important apoptosis-regulating gene and modulates apoptosis through many pathways. It was reported that p53 serves as a very important regulator, that promotes apoptosis through the bax pathway[17,18]. p53 also causes downregulation of bcl-2[19,20] and survivin[21] at protein and mRNA level. This study showed that there was no significant interrelation between p53, bcl-2, bax, and survivin in HCC. This may result from the fact that the mutation of p53 has lost effect on expression of bax, bcl-2, and survivin. In HCC tissues of 14 bcl-2-positive cases, bax was positive for all cases; in HCC tissues of 21 bcl-2-negative cases, bax was positive for 10 cases; in HCC tissues of 25 bax-positive cases, bcl-2 was positive for 14 cases; in HCC tissues of 11 bax-negative cases, bcl-2 negative for all cases. Inter-group comparison showed that the difference was rather significant, suggesting that there is a mechanism of co-activation or mutual activation between bcl-2 and bax.

In conclusion, apoptosis rate in normal liver cells is extremely low and increases during the development of HCC. Apoptosis-related genes p53, bcl-2, bax, and survivin are all related to HCC occurrence, and their expression renders an increasing tendency during the formation of HCC. There is no distinct relationship between bcl-2, bax, and survivin, but there is a close relationship between bcl-2 and bax. Bax can facilitate apoptosis, and the anti-apoptosis effect of bcl-2 is affected by bax. Therefore, the bax/bcl-2 ratio reflects more accurately the extent of cell apoptosis. Survivin relates negatively to the apoptosis index, and its expression inhibits apoptosis. In HCC, p53 is not noticeably related to cell apoptosis, and its high expression may relate to the proliferation of liver cells.

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