Alteration of The Survivin Gene During The Hepatocarcinogenesis in Rats

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Abstract **Objective** At present,studies on hepatocellular carcinoma (HCC) are mostly confined to those on human HCC and the surrounding liver tissues.The most deficiency of these kinds of studies is incapable of observing dynamically various changes of different periods during the hepatocarcinogenesis. Our aim was to clarify the expression and variation of the survivin gene in tumorigenesis of rats induced by aflatoxin B1 (AFB1).**Methods** Seventy-eight rats were divided into two groups. The experimental group were treated with AFB1 and the control were not.Liver and HCC tissues were detected by immunohistochemistry assay and reverse transcriptase-polymerase chain reaction (RT-PCR).**Results** The earliest hepatocarcinogenic incidence occurred in the 46 th week after the rats treated with AFB1. HCC incidence was 54.90%(28/51)in 46 th week and 64.86%(24/37)in 58 th week.The positive rates of survivin protein expression in 24 HCC, para-carcinomous liver tissues of experimental group were 41.67% and 54.17%, respectively, with no significantly difference between them (P>0.05). But no survivin expression was detected in the experimental group before 46th week or the group without HCC occurrence or the normal controls. The level of survivin mRNA expression in HCC was significantly higher than that in pre-HCC, no-HCC and normal liver tissues in control group (P <0.01). The level of survivin mRNA expression in para-carcinoma tissues was significantly higher than that in no-HCC and liver tissues of the control (P <0.01). The level of survivin mRNA in pre-HCC 12^{th},20^{th},36^{th},46^{th} week were significantly higher than those in the liver tissues from control group of the same stages (P <0.01). **Conclusion** The survivin gene is related to the occurrence of HCC and might play an important role in the carcinogenesis of HCC.

Key words Survivin; Hepatocarcinogenesis; Alterarion

Hepatocellular carcinoma (HCC) as one of the worlds most common cancers is predominant in Africa and Southeast Asia. It has a multistage and multifunctional process. Epidemiological studies indicate that contamination of food with aflatoxin B1 (AFB1) and chronic infection with hepatitis B virus (HBV) are the major risk factors for human liver cancer. Several investigations in different species of experimental animals have demonstrated synergistic effects of AFB1 in hepatocarcinogenesis[1].

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Recently,novel proteins which suppress apoptosis through caspase-dependent and caspase-independent mechanisms have been characterized, and named inhibitors of apoptosis (IAPs). In humans, six members of the IAP family have been described: HIAP1, HIAP2, XIAP, NIAP, survivin and livin. Within the IAP family survivin has also been recently described. Despite its limited expression in normal tissues, survivin has been found over-expressed in a variety of human tumors, including breast,colon, pancreas and prostate carcinoma, neuroblastoma,melanoma and non-Hodgkins lymphom [2]. Studies performed by immunohistochemistry described presence of survivin in a variable percentage of tumors, ranging from 30% of gastric cancers to 90% of melanomas[3]. Most of these studies found a positive correlation between survivin expression and prognosis

of disease, which is more evident in neuroblastoma and in colorectal cancer,where a multivariate statistical analysis revealed that survivin expression is an independent prognostic factor for disease progression[4].

Expression of survivin has been evaluated in HCC by immunohistochemistry and survivin protein was found to express not only in most HCC tissues but also in some cirrhotic nodules. In non-cancerous regions, the levels of survivin mRNA increased in proportion to their stage of progression.Survivin protein was expressed mainly in periportal areas, where proliferating cells were localized[5].Furthermore, an important role was suggested for survivin in progression, recurrence, and treatment of hepatocellular carcinoma[6].

Using rats as an animal model for the development of HCC after AFB1 treatment, we investigated the expression and alteration of the survivin gene in rat hepatocarcinogenesis by immunohistochemistry assay and real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

METHODS AND MATERIALS

Animal experiment

Seventy-eight adult laboratory purebred male sprague-dawley (SD) rats weighing 70±4.8g were obtained from the Animal Centre, Guangxi Medical University, China. They were held according to the rules of the local ethical guidelines and all animals received humane care as approved by the Animal Care Committee at Guangxi Medical University according to the Guidelines of the National Academy of Science. They were housed in an individual stainless steel cage at room temperature of 25±1 ℃ and fed with fruit, milk, and egg. Drinking water was distilled and given ad libitum. The 78 rats were randomly divided into groups A(66) and B(12). The animals of group A were given with AFB_1 50-100μg /kg.bw/d, while group B was used as control.

AFB1

AFB_1 was purchased from Sigma Chemical Co. ,USA.It was dissolved into dimethylsuphoxide (DMSO) and mixed with milk to be sipped by the rats.

Reagents

Rabbit polyclonal antibody against human survivin protein was purchased from Santa Cruz Biotechnology Inc., California. and EnVision kit were purchased from DAKO. RT-PCR kit was bought from Invitrogen Inc.,USA.

Liver tissue biopsy

During the whole experiment, the liver tissue biopsies were performed in each group at 12^{th}, 20^{th}, 36^{th} and 46^{th} week under anaesthesia .The liver biopsy tissue (0.8cm×0.4cm×0.4cm) was taken every time. The survival animals were sacrified at 58^{th} week and the autopsy specimens were cut (experimental group 30 rats, control 11 rats).All liver biopsy tissues were cut into two pieces. One, for histological and immunohistochemistry examinations, was fixed in 10% buffered formalin and embedded with paraffin within 3 days; and the other was kept at -80℃ refrigerator after immersing in liquid nitrogen for RNA extraction.

Immunohistochemistry

Briefly, the paraffin-embedded tissues (4μm thick) were dewaxed using xylene and transferred to graded alcohol, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min. After washing with PBS, slides were incubated with the primary antibodies overnight at 4℃. After washing the slides with PBS, EnVision was applied as the secondary antibody for 30 min. The reaction products were visualized with diaminobenzidine (DAB) as the chromogen, and the slides were counterstained with hematoxylin. PBS was substituted for the first antibody to act as the negative control and the positive contrast sections from Maxim company was used as the positive contrast.

Immunohistochemistry result assessment

Each positive control staining sections should be positive and the negative ones negative. The survivin proteins revealed in immunohistochemical staining light brown or dark brown and located in the cytoplasm. The survivin protein staining, as per the percentage of the positive cells to the total ones, were divided into

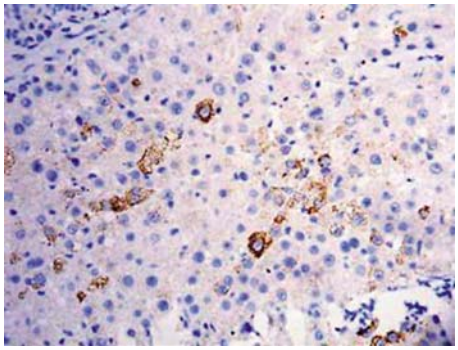

Fig.1 Expression of survivin was detected in HCC tissues by the immunohistochemistry method in HCC in 58th week (200×).

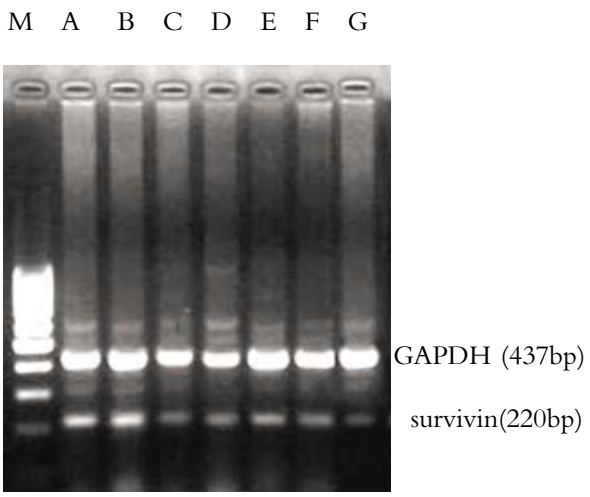

Fig.2 mRNA levels of survivin was examined by RT-PCR in different liver and HCC tissues. (M) Marker. (A)para-HCC tissue. (B)HCC tissue. (C) Liver tissue in 46w. (D) Liver tissue in 36w. (E) Liver tissue in 20w. (F)Liver tissue in 12w. (G)Normal control.

five grades: grade 0: <5%; grade Ⅰ: 5%-25%; grade Ⅱ: 25%-50%; grade Ⅲ: 50%-75%, grade Ⅳ :>75%. ≥5% served as the standard for positive. The staining intension of the positive cells was divided into 3 grades: grade 1, low; grade 2, moderate; grade 3, high. The staining intension of each positive control section was regarded as grade 3 for reference. The composite score for immunohistochemical staining=the percentage grade of positive cells from every tissue×staining intension.

RT-PCR

Total RNAs were extracted from 10mg of frozen tumors and normal liver tissues. Total RNA from tissues same cDNA were amplified with GAPDH and survivin primers. Each amplification was performed for 30 cycles; a cycle profile consisted of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. Sequences of the primers used are as follows: GAPDH upstream,5'-TCATTGACCTCAACTACATG-3'; GAPDH downstream, 5'-GCAGTGATGGCATGGACTGT -3';survivin upstream, 5'-GCTGGCTTCATCCACTGC-3';survivin downstream, 5'-CAATTTTGTTCTTGGCTCT-3'; The size of the amplified products were 437 and 220 bp for GAPDH, survivin, respectively. A sample without RNA was included in each RTPCR as a negative control; for positive controls, RNA extracted from M14 cell line for survivin. PCR products were analyzed on 1.5% (w/v) agarose gel containing ethidium bromide. GAPDH mRNA was quantified to adjust the amount of mRNA in each sample.

Data analysis

Chi-square and Students t-test were used by SPSS version 13.0 for Windows. Significance was defined as $P< 0.05$.

RESULTS

Occurrence of HCC

The first case of HCC occurred in the 46th week of experiment in experimental group. HCC incidence was 54.90%(28/51)in 46th week and 64.86%(24/37)in 58 th week.

Immunohistochemistry

In 46th week, 7(7/24, 29.17%)rats had positive expression of survivin. In 58th week, the positive rates of survivin protein expression in 24 HCC, para-carcinoma liver tissues of experimental group were 41.67%(10/24) and 54.17%(13/24), respectively, with no difference between them ($P >0.05$). No expression of survivin protein in pre-HCC and no-HCC of experimental and control tissues. There were statistical significance between HCC and no-HCC, para-carcinoma iver tissues and no-HCC respectively(Fig. 1).

RT-PCR

Tab 1 The expression of survivin mRNA in the liver and HCC tissues in rats(MEAN±SD)

group	tissues	cases	12th	20th	36th	46th	58th
Experimental group	HCC	24	0.382±0.161*★	0.397±0.137*★	0.398±0.197*★	0.379±0.175*★	0.539±0.176*▲★
	Para-HCC	24	-	-	-	-	0.533±0.160*▲
	Non-HCC	6	0.304±0.137	0.294±0.072	0.312±0.152	0.294±0.072	0.303±0.052
Control group	Normal liver	11	0.234±0.088	0.241±0.102	0.224±0.081	0.209±0.094	0.202±0.088

*vs the expression of survivin mRNA in control group in the same time: $P<0.01$;

▲vs the expression of survivin mRNA in non-HCC group in the same time : $P<0.01$;

★vs the expression of survivin mRNA in the same group but different time : $P<0.01$.

Since in 46thweek, there was no biopsy of para-carcinoma tissues, so the expre`ssion of mRNA was not detected.

The level of survivin mRNA expression in HCC was significantly higher than those in pre-HCC, no-HCC and normal liver tissues from control group ($P<0.01$). The survivin mRNA expression in para-carcinoma was significantly higher than that in no-HCC and control ($P<0.01$). The level of survivin mRNA in pre-HCC 12th, 20th, 36th, 46th week were significantly higher than those in control group of the same stages ($P<0.01$)(Fig. 2, Table 1).

DISCUSSION

IAPs might have a major role in the apoptotic resistance that marks many cancers. The studies on IAPs in human HCC have focused on survivin or XIAP, indicating that their new or increased expression in this tumor is associated with a more unfavorable prognosis [7]. Survivin is a novel inhibitor of apoptosis. It is detected in fetal and neoplastic adult tissue, but not in normal tissue. Several recent studies have shown that survivin not only inhibits apoptosis, but also accelerates cancer cell proliferative activity. Expression of the protein may be of prognostic significance and therapeutic relevance in many cancers [6,8-9]. In our study, the dynamical changes of survivin expression was observed and the level of survivin mRNA expression in HCC was significantly higher than those in pre-HCC, no-HCC and normal liver tissues from control group, which suggests that the survivin expression happen increases in the late period of the development of HCC in rats. The survivin gene was expressed in a most of HCC tissues, which indicates survivin gene might play an important role in the carcinogenesis and the diagnosis of HCC. Fields *et al.*[6]has also reported that survivin mRNA expression could be used as an independent prognostic factor for patients with HCC after hepatectomy. The survivin gene in our study was also expressed in the para-carcinoma tissues ,which indicates that there might be precancerous cells there, which might later develop into HCC. This result is in accordance with that of the research by Morinaga *et al.*[10] Further more, survivin is associated with reduced tumor cell apoptosis, increased tumor cell proliferation, and histologically aggressive tumor features, and may play an important role in tumor progression of HCC. However, further examination is needed to clarify its predictive significance for HCC patients.

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Meeting Information

Dear editorial,colleagues and friends:

The 21th International Congress of Lymphology will be hold from September 26th to 29th, 2007, in shanghai of China. Many our friends and colleagues will attend the meeting. By this opportunity, as the chief−editor of US−Chinese Journal of Lymphology and Oncology. Firstly, thank all of our editors, colleagues and friends who give us many support and help for efforts of our journal. I am greatly honored to extend my warm welcome and gracious greeting to all of our editors and all friends who works on lymphology and oncology. We will meet each other and talk about the advantages and developments of our journal in the future.

Zhiyu Liu MD.

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