

# The Expression of Insulin-like Growth Factor II, Hepatitis B Virus X Antigen and p21 in Experimental Hepatocarcinogenesis in Tree Shrews

J J Su,\*<sup>MD</sup>, G Z Qin,\*\*<sup>BSc</sup>, R Q Yan,\*\*\*<sup>MD, MS</sup>, D R Huang,\*\*\*\*<sup>BSc</sup>, C Yang,<sup>†</sup><sup>BSc</sup>,  
P D Lotlikar,<sup>‡</sup><sup>PhD</sup>

## Abstract

The purpose of this paper was to study the mechanism of synergistic effect in hepatocarcinogenesis induced by hepatitis B virus (HBV) infection and aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) intake. Immunohistochemical staining was used in formalin-fixed, paraffin-embedded sections of cancer and liver tissues. The incidence of hepatocellular carcinomas (HCCs) was 52.9% in experimental tree shrews that received both HBV and AFB<sub>1</sub>. It was significantly higher than that of animals exposed to HBV (11.1%, Group B), or (AFB<sub>1</sub>) (15.8%, group C) alone. HCC was not found in the control animals (group D). The expressions of insulin-like growth factor II (IGF-II) were 82.4%, 22.2%, 24.3% and 0 in groups A, B, C and D, respectively. The significant differences of IGF-II were observed between groups A and B, C and D ( $P < 0.05$ ). The expressions of p21 were 29.4%, 11.1%, 15.8% and 0 in group A, B, C and D, respectively. The positive rate of hepatitis B x antigen (HbxAg) was significantly higher in the group A than that in the group B (52.9% vs. 11.1%,  $P < 0.05$ ). The parallel relations between the incidence of HCC and the overexpressions of these genes/protein have been found in each group. On the other hand, the expressions of these genes in tumour-bearing tree shrews were significantly higher than that in nontumour-bearing animals. These findings suggest a synergistic effects of HBV and AFB<sub>1</sub> in activation of these genes in tree shrews. Overexpressions of these genes may take an important role in the course of hepatocarcinogenesis in tree shrews.

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**Key words:** Hepatitis B x antigen, Hepatocellular carcinoma, Insulin-like growth factor II, p21, Tree shrew

## Introduction

Hepatocarcinogenesis is a multistage and multi-functional process. Epidemiological studies indicate that contamination of food with aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and chronic infection with hepatitis B virus (HBV) are the major risk factors for human primary liver cancer.<sup>1,2</sup> Several investigations in different species of experimental animals have demonstrated synergistic effects of AFB<sub>1</sub> and HBV in hepatocarcinogenesis.<sup>3-5</sup>

IGF-II plays an important role in fetal growth and development.<sup>6,7</sup> Its synthesis rapidly decreases after birth although it remains detectable at low levels throughout life, and it is regulated in a tissue-specific and development-dependent way. The carcinogenic effect of IGF-II has been observed in the transgenic mice in which the circulating levels of IGF-II were increased 20- to 30-fold

and in which diverse types of tumours developed after 18 months.\* High levels of fetal IGF-II transcripts had also been observed in some human hepatocellular carcinomas (HCCs) and in the adjacent cirrhotic tissue.<sup>9</sup>

The ras gene codes for 21 KD protein (p21), which binds guanine nucleotides and possesses GTPase activity. Through this mechanism, the ras p21 participates in the control of cell proliferation, possibly as a signal transducer from cell surface receptors to the nucleus.<sup>10</sup> The mutations of ras genes in codons 12, 13, and 61 leading to the increased expression of normal or mutant form of the p21 protein, have been observed in human hepatocellular carcinoma and several other tumours.<sup>11,12</sup>

HBx gene encodes a protein of 154 amino acids, its sequences are well conserved in other hepadna virus such as woodchuck hepatitis virus (WHV) and ground

\* Professor, Department of Experimental Pathology and Director, Department of Pathology

\*\*\*\* Deputy Chief Technician and Director, Department of Clinical Laboratories

† Deputy Chief Technician, Department of Pathology

Guangxi Cancer Institute, Nanning, Guangxi, P R China

\*\* Deputy Chief Technician, Department of Experimental Pathology

‡ Professor of Molecular Biology

Fels Institute for Cancer Research and Molecular Biology and Department of Biochemistry

Temple University School of Medicine, Philadelphia, USA

\*\*\* Professor, Department of Pathology

Cancer Institute, Sun Yet-sen University of Medical Sciences, Guangzhou, P R China

Address for Reprints: Dr Jianjia Su, Department of Pathology, Guangxi Cancer Institute, Nanning 530021, Guangxi, P R China.

squirrel hepatitis virus (GSHV). A variety of genes are known to be activated by HBx protein. The recent finding that HBx protein complexes with the wild-type p53 protein and inhibits its sequence-specific DNA binding *in vitro*, also inhibits p53-mediated transcription activation *in vivo* and the *in vitro* association of p53 and ERCC3, a general transcription factor involved in nucleotide.<sup>13,14</sup> HBx protein may be as a multifunction regulatory protein that affects transcription, cell growth and cell viability.<sup>15</sup> However, the role of HBx gene in HBV-associated hepatocarcinogenesis is still debated. In the present article, we examined the expressions of IGF-II, p21 and HBx protein in tree shrews subjected to experimental carcinogenesis.

## Materials and Methods

### Animals and Experimental Design

Fifty-three liver samples were taken from a previous study in tree shrews.<sup>16</sup> Seventeen animals were inoculated with human HBV and intake AFB<sub>1</sub> (group A); 9 were infected with HBV only (group B); 19 were administered AFB<sub>1</sub> alone (group C) and 9 without treatment were used as controls (group D). AFB<sub>1</sub> dissolved in dimethyl sulfoxide (DMSO) was mixed into the milk to be sipped by the animals. The experiment was terminated at 158th week. During this period, the liver tissues and tumours of experimental animals that died or sacrificed were fixed in 10% buffered formalin, and paraffin-embedded. Serial 5 µm sections were cut, and were examined pathohistologically by haematoxylin and eosin staining. Nine, 1 and 3 cases of HCCs were observed in groups A, B and C, respectively. The liver tissues and tumours were examined for IGF-II, p21 and HBxAg expressions using immunohistochemical technique.

### Reagents

- (1) Primary antibody, monoclonal anti-human IGF-II, was purchased from Bio Source International (AHG0024). The working dilution was 1:100.
- (2) Primary antibody, monoclonal antibody to Pan-ras, was purchased from Oncogene Science, Inc (p21). The working dilution was 1:50.
- (3) Avidin-biotin-peroxidase complex kit (ABC kit) was purchased from Vector Laboratories, Burlingame, CA, Inc.
- (4) Primary antibody, rabbit polyclonal anti-HBxAg was kindly provided by Dr Feitelson M A (Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania, USA). The Working dilution was 1:800.

### Immunohistochemistry

IGF-II, p21 and HBxAg were detected using immunohistochemical staining by the avidin-biotin complex (ABC) method. In brief, formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and were

passed through ethanol series. After quenching the endogenous peroxidase activity, the sections were rinsed in 0.01 M PBS. Non-specific binding was blocked by treatment with 5% normal horse or goat serum for 20 min. Primary antibody was applied to the sections and incubated in a moist chamber overnight. After washing in 0.01 M PBS, biotinylated horse or goat anti-mouse or rabbit immunoglobulin G was applied and sections were incubated for 50 min at room temperature. After washing, the sections were incubated with avidin-biotin-peroxidase complex for 50 min. After washing, the chromogen, 3-3'-diaminobenzidine (DAB) was added for 5 to 10 min. Finally, the sections were washed and mounted. A negative control section, to which normal horse or goat serum was applied, was included in each staining.

The data were evaluated for statistical significance using X<sup>2</sup> test.

## Results

### Expressions of IGF-II, p21 and HBxAg

Twenty-one positive cases of IGF-II were detected in 53 experimental animals. Fourteen of them were in group A, 2 in group B and 5 in group C. IGF-II expression was not found in the control group (Table I). Thirteen cases developed HCCs during the experimental period from 83rd to 158th weeks. All of them were positive for the expressions of IGF-II, except 1 from group B which was not examined. In groups A and C, the positive rates for IGF-II were shown to be much higher in tumour-bearing livers than that in nontumour-bearing ones. The first case of IGF-II appeared earlier in group A than in the other groups. Only 9 cases of positive p21 were expressed in all the experimental animals. Five, 1 and 3 cases were detected in groups A, B and C, respectively. The positive rate for p21 was higher in the tumour-bearing livers than those in nontumour-bearing livers (58.3% vs 6.1%,  $P < 0.05$ ), but did not differ significantly in tumour-bearing animals between groups A and C (55.5% vs 66.6%) (Table II). Among the 12 tumour-bearing samples, 7 (58.3%) were positive for IGF-II and p21 (5 in group A and 2 in group C). Ten cases (38.5%) were positive for HBxAg among the 26 HBV infected animals [9 (52.9%) in group A and only 1 (11.1%) in group B ( $P < 0.05$ )]. In group A, HBxAg were expressed in 7 of 9 HCCs (77.8%), and only in 2 of 8 cases (25%) of nontumour animals. One case which did not express HBxAg developed HCC in group B. The expression of HBV markers in tumour-bearing animals was more often than that in nontumour-bearing animals (Table III).

### Cellular and Tissue Distribution

IGF-II positive cells were found to be exclusively localized in the nucleus of 8 samples. Eleven were detected both in the nucleus and cytoplasm. Among 12

TABLE 1: THE STATE OF IGF-II EXPRESSION IN EACH GROUP

| Group | No. | HCC<br>n (%)          | IGF-II<br>n (%)        | The time for<br>IGF-II to appear (weeks) | IGF-II in nonHCC-<br>bearing animals (%) | IGF-II in HCC-<br>bearing animals (%) |
|-------|-----|-----------------------|------------------------|------------------------------------------|------------------------------------------|---------------------------------------|
| A     | 17  | 9 (52.9) <sup>*</sup> | 14 (82.4) <sup>†</sup> | 85                                       | 5/8 (63.5) <sup>‡</sup>                  | 9/9 (100) <sup>‡</sup>                |
| B     | 9   | 1 (11.1) <sup>*</sup> | 2 (22.2) <sup>†</sup>  | 137                                      | 2/9 (22.2)                               |                                       |
| C     | 19  | 3 (15.8) <sup>*</sup> | 5 (26.3) <sup>†</sup>  | 88                                       | 2/16 (12.5) <sup>§</sup>                 | 3/3 (100) <sup>§</sup>                |
| D     | 8   | 0                     | 0                      | 0                                        |                                          |                                       |
| Total | 53  | 13                    | 21 (39.6)              |                                          | 9/33 (27.3) <sup>*</sup>                 | 12/12 (100) <sup>  </sup>             |

\*  $\chi^2 = 4.026$ ,  $P < 0.05$ ;  $\dagger \chi^2 = 6.6285$ ,  $P < 0.05$ ;  $\ddagger \chi^2 = 1.924$ ,  $P > 0.05$ ;  $\S \chi^2 = 4.276$ ,  $P < 0.05$ ;  $\parallel \chi^2 = 15.89$ ,  $P < 0.01$

TABLE II: THE STATE OF P21 EXPRESSION IN EACH GROUP

| Group | No. | HCC<br>n (%)          | p21<br>n (%) | Time for p21<br>to appear (weeks) | p21 in nonHCC-<br>bearing animals (%) | p21 in HCC-<br>bearing animals (%) |
|-------|-----|-----------------------|--------------|-----------------------------------|---------------------------------------|------------------------------------|
| A     | 17  | 9 (52.9) <sup>*</sup> | 5 (29.4)     | 85                                | 0/81-                                 | 5/9 (55.5) <sup>†</sup>            |
| B     | 9   | 1 (11.1)              | 1 (11.1)     | 137                               | 1/8 (12.5)                            | 0/1                                |
| C     | 19  | 3 (15.8) <sup>*</sup> | 3 (15.8)     | 88                                | 1/16 (6.25) <sup>‡</sup>              | 2/3 (66.6) <sup>‡</sup>            |
| D     | 8   | 0                     | 0            |                                   | 0                                     | 0                                  |
| Total | 53  | 13 (24.5)             | 9 (16.9)     |                                   | 2/132 (6.3) <sup>§</sup>              | 7/13 (53.8) <sup>§</sup>           |

\*  $\chi^2 = 4.026$ ,  $P < 0.05$ ;  $\dagger \chi^2 = 3.905$ ,  $P < 0.05$ ;  $\ddagger \chi^2 = 3.135$ ,  $P > 0.05$ ;  $\S \chi^2 = 11.93$ ,  $P < 0.01$

TABLE III: HBxAg EXPRESSION IN DIFFERENT GROUPS OF TREE SHREWS

| Group | No. | HCC<br>n (%)          | HBxAg<br>n (%)        | With tumours<br>HBxAg (%) | Nontumours<br>HBxAg (%) |
|-------|-----|-----------------------|-----------------------|---------------------------|-------------------------|
| A     | 17  | 9 (52.9) <sup>*</sup> | 9 (52.9) <sup>*</sup> | 7/9 (77.8)                | 2/8 (25.0)              |
| B     | 9   | 1 (11.1) <sup>*</sup> | 1 (11.1) <sup>*</sup> | 0/7                       | 1/8 (12.5)              |

\*  $P$  value  $< 0.05$

HCC: human hepatocellular carcinoma

cases of HCC, IGF-II was distributed in the tumour nodules of 6 cases and the remainder (6) were expressed in the tumour nodules and adjacent hyperplastic nodules. In 9 nontumour-bearing samples, the positive cells for IGF-II only appeared in hyperplastic nodules. IGF-II positive cells were distributed mostly in clumps pattern, some in diffuse pattern.

All 9 cases of positive p21 were detected in tumour nodules or proliferative foci. p21 expression was not found in normal liver cells. It was localized in the nucleus in 7 cases, and 2 localized both in the nucleus and cytoplasm. They were distributed in clumps or diffuse pattern.

HBxAg was detected in the cellular plasma in 4 cases, 3 in the nucleus and another 3 cases were distributed both in the cellular plasma and nucleus. Among 7 animals of positive HBxAg which had HCCs, 3 (42.84%) appeared in tumour cells, and 4 (57.16%) in the surrounding nontumour liver cells. The positive cells for HBxAg were distributed mostly in clumps pattern.

## Discussion

The positive staining of IGF-II, p21, or HBxAg was not found in the negative control section.

In this report, out of the 12 HCCs examined, 4 cases were well-differentiated while 8 were poorly-differentiated grade II to III. IGF-II was expressed in all HCCs. In addition, the IGF-II expression was also found in the proliferated nodules surrounding liver tumours and in non cancerous liver tissues. It indicates that IGF-II expressed earlier during HCC development, and suggested that IGF-II may act as an autocrine or paracrine growth factor and play an important role in the development of HCC.<sup>17,18</sup> The expression of IGF-II and HBxAg were significantly higher in animals exposed to both HBV and AFB<sub>1</sub> than those that received either HBV infection or AFB<sub>1</sub> intake only. HCCs also developed more in group A than that in other groups.<sup>19</sup> This indicated that the HCC development was parallel to the expression of both IGF-II and HBxAg. Results from humans are similar to that of the tree shrew model. There, the tree shrew is an ideal model for the study of

the mechanism of HCC development. Some authors<sup>19</sup> postulated a non-stochastic mechanism for the role of HBV in the regulation of c-myc, ets 2 and IGF-II in hepatic proliferation by suggesting that these factors were triggered by HBV or chemical carcinogens, such as AFB<sub>1</sub>. In addition, IGF-II was expressed in both HCV positive and HBV positive HCCs,<sup>18</sup> suggesting that these viruses may share common mechanisms by which they activate the IGF-II transcription factor. Besides, IGF-II expression was localized in the cells of neoplastic and proliferated nodules, but not revealed in normal liver cells; it is possible that IGF-II overexpression accompanies this process in the tree shrew's hepatocarcinogenesis. We support the hypothesis that IGF-II in HCC may constitute an early phase marker of cell alteration to cancer. Others reported that IGF-II acts as a liver mitogen to promote cell growth and as a factor that inhibits apoptosis.<sup>20</sup> Apoptosis is a generally encoded cell death programme with defined morphologic and biochemical character.<sup>21</sup> The apoptotic process is a mechanism of self-annihilation that can prevent tumour progression following an oncogenic event. Growth factors may be involved in this process.<sup>22</sup> The IGF-II expression was mainly localized in the nucleus in the present study in contrast to results from human studies. The difference may be species-related.

Although the incidence of p21 expression was not shown to be significantly different in the various groups, all the positive p21 expression occurred only in the tumour-bearing liver tissues (5/9, 55.5%). No p21 was detected in 8 nontumour-bearing animals in group A. It suggested that the expression of ras gene is important in carcinogenesis.

In the present study, 4 cases of well-differentiated HCCs showed no positive p53 expression (the data is shown in another paper<sup>23</sup>), whereas 2 of them showed HBx protein expression in tumour nodules and surrounding liver cells. It suggests that the persistence of HBxAg is important to the pathogenesis of early HCC and that HBxAg expression in the liver during chronic HBV infection may be an important prognostic marker for the development of HCC.<sup>24</sup> The incidence of HCC and HBxAg expression in group A was significantly higher than that in group B. Moreover, the expression of HBxAg in tumour-bearing animals was also higher than in nontumour-bearing animals. It provided evidence of HBxAg in hepatocarcinogenesis.

The HBx gene was considered to be a viral transactivator. A variety of genes had been shown to be activated by HBx protein.<sup>24</sup> Activating a broad spectrum of gene, the HBx gene is implicated in carcinogenesis as one of the causative factors.<sup>25</sup> This is supported by the present study in which, the rate of HBxAg expression was higher in the tree shrews that have developed HCC than those without HCC. Koike et al<sup>26</sup> have observed the

natural course of HCC development in HBx transgenic mice in their life span. The results showed that the liver of the transgenic mice appeared normal at one month of age. At two months of age, the mice began to show foci of altered hepatocytes, concentrated mainly in the centrilobular areas. At the age of 7 to 9 months, small clusters of dysplastic hepatocytes with large nuclei and increased chromatin appeared with the foci. Around 12 months of age large areas of dysplastic hepatocytes, accompanied by small nodules that were presumably precursors of adenoma. In these cells, the levels of HBx gene expression were higher than in the surrounding areas. Adenoma and HCC began appearing at 13 months of age. These results demonstrated a close aetiologic relationship between HBx gene existence and HCC development.

Others have pointed out that transactivation protein HBx is considered essential for establishing a chronic infection<sup>27</sup> and as a putative oncoprotein mediating hepatocarcinogenesis,<sup>28,29</sup> but the mechanism of carcinogenesis was not clear. The occurrence of HCC in animals infected with corresponding hepadna virus (WHV or GSHV) is very frequent. In contrast, duck hepatitis B virus (DHBV) lacks the HBx gene and no HCC induced by DHBV in its host.<sup>30</sup> Although HBV as well as WHV, GSHV and DHBV belong to the hepadna virus family, there are some differences in their molecular structures. The animal hepadana virus cannot replace the role of human HBx gene in the research of the mechanism of carcinogenesis. This study was performed in the tree shrew's model that had been infected with human HBV, and evidence of synergistic hepatocarcinogenic effect of HBV and AFB<sub>1</sub> was demonstrated. The pattern of HBxAg expression was shown to be similar in tree shrews and humans,<sup>31</sup> and it is possible therefore research on the oncogenic mechanism using this species.

These findings suggest a synergistic effect of HBV and AFB<sub>1</sub> in activation of these genes in tree shrews. Overexpression of these genes may have an important role in the course of hepatocarcinogenesis in tree shrews.

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