EXPRESSION OF *ras* GENE IN EXPERIMENTAL HEPATOCARCINOGENESIS IN TREE SHREWS

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Abstract

Objective: In order to investigate the relationship between the expression of *ras* gene and the development of hepatocellular carcinoma (HCC). Materials and Methods: The experimental tree shrews were divided into four groups: group A, infected human hepatitis B virus (HBV) and exposed to aflatoxin B1 (AFB1); group B, infected human HBV alone; group C, only exposed to AFB1; group D, use as controls. The serial bioptic liver tissues were detected for *ras* p^{21} protein using immunohistochemical method. Results: The total p^{21} protein positive rates in group A, B, C and D were 35.3%, 5.3%, 13.3%, 0, respectively, thus the significant difference were showed between group A and group B ($P<0.05$); The HCC incidences in group A, B, C and D were 47.1%, 0, 13.3%, 0, respectively, and there was a significant difference between group A and C ($P<0.05$). The incidences of HCC in the animals with and without p^{21} protein positive in group A were 100% and 18.2%, respectively, and there was a significant difference among them ($P<0.01$). Conclusion: HBV and AFB1 play a remarkable synergistic role in the development of HCC; they can enhance the expression of *ras* gene. The over-expression of *ras* gene is closely related to pathogenesis of HCC in tree shrews.

Key words: Hepatocellular carcinoma, P^{21} Aflatoxin B1, Hepatitis B virus.

Hepatocellular carcinoma (HCC) occurs at high frequency in Southeast Asia and Sub-Saharan Africa. The major risk factors involved in the development of liver cancer in these areas are hepatitis B virus (HBV) and the food contaminated with aflatoxin B1 (AFB1).[1,2] Although epidemiological evidence as well as animal HCC models suggest that there is a synergistic relationship between HBV and AFB1 in hepatocarcinogenesis,[3,4] the specific synergistic oncogenic mechanisms mediated by HBV and AFB1 are unknown. Many investigations have confirmed that *ras* gene is related to the development of HCC.[5,6] This stimulated us to study the relationship between expression of *ras* gene and HCC in tree shrews exposed to human HBV and AFB1.

MATERIALS AND METHODS

Animals

Adult tree shrews (*Tupaia belongri Chinensis*), were obtained from the Kunming Institute for Zoology Research, Chinese Science Academy. The animals were housed with one metal cage for each animal under conventional conditions (room temperature: 23±2°C; relative humidity, 60%-70%). The animals were fed with basal diet supplemented with milk, eggs, silkworm chrysalis and fruits such as apple and pear.

HBV Sera for Infection

Human sera with positive for hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) determined by ELISA and with Dane particles observed under the electric microscopy were obtained from blood donors.

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Inoculation Methods

0.5 ml of human HBV serum was injected through the femoral vein or artery for each tree shrew. After 3 days, another 0.5 ml of the same serum was injected peritoneally.

HBV Markers and Assay

Before inoculation, blood sample was drawn control examination of every animal. After inoculation, blood was drawn repeatedly for detecting HBV markers including HBsAg, HBeAg and anti-HBc by ELISA. HBsAg was detected by immunohistochemical method and HBV DNA was examined by *in situ* hybridization in liver tissues.

Reagents

Reagents for detecting serum markers by ELISA were purchased from Shanghai SIIC Kehua Biotech Co., LTD, Shanghai. Reagents for the immunohistochemical detection of HBsAg and p^{21} were obtained from Zhongshan Biological Technic Co., Ltd, Beijing. P^{21} (pan-*ras*) was from Oncogene Research Products, USA. The HBV DNA probe reagents were purchased from ENZO Diagnostics Inc., USA. AFB1 was a product of Sigma Chemical Co., USA.

Experimental Design and AFB1 Treatment

120 of tree shrews were screened with HBV marker. 35 animal with HBV marker negative were randomly divided into two groups: group C, 20 animals; group D, 15 animals. The other animals with HBV marker negative were inoculated with human HBV. After the HBV infection had been confirmed, animals with serological HBV marker positive were randomly divided into another two groups: group A and B, both of groups were 20 animals, respectively. Group A and group C were fed with $150\mu l/kg^{-1}/bw/d^{-1}$ of AFB1 mixed in the milk for 105 weeks. Liver biopsy was taken every 14 or 15 weeks from every animal. The liver tissues were fixed in 10% buffered formalin, then paraffin-embedded. In this study, we detected the expression of p^{21}, HBsAg and HBV DNA at 44th, 105th and 119th weeks.

RESULTS

The Total AFB1 Uptake

The total AFB1 uptake for each animal in group A and C were 11.5926±0.8567mg and 11.3773±0.7701 mg respectively, and there was not significant difference in the total amount of AFB1 ingested among them ($P>0.05$).

HBV Marker in Liver Tissue

The results on markers of HBV infection in the two HBV infection groups with and without AFB1-treatment are given in Table 1.

Expression of *ras* Gene

Liver tissues were stained for the expression of *ras* gene by immunohistochemical method. A total of 9 animal were positive expression of *ras* gene (Table 1 and Table 2).

HCC Incidences

A total of 10 animals developed HCCs throughout the experimental period of 119 weeks. Each case of HCC was confirmed by histopathology. The distribution of HCCs among various groups are showed in Table 1. The incidence of HCC in group A was statistically significant higher than that in groups C. None HCC was found in either group B or group D.

Table 1. HBsAg, HBV DNA and p^{21} in liver tissues and HCC incidence of tree shrews in experimental hepatocarcinogenesis

Groups		Effective animal numbers*	No. of cases positive for HBV marker in liver tissues (%)		No. of animals positive for p^{21} protein in liver tissues (%)	No. of HCC cases (%)
HBV	AFB1		HBsAg	HBV DNA		
(A) +	+	17	13 (76.5)[a**]	9 (52.9)[c]	6 (35.3)[e]	8 (47.1)[h]
(B) +	-	19	18 (94.7)[b]	11 (57.9)[d]	1 (5.3)[f]	0 (0)
(C) -	+	15	0 (0)	0 (0)	2 (13.3)[g]	2 (13.3)[I]
(D) -	-	11	0 (0)	0 (0)	0 (0)	0 (0)

*The number of animals surviving when the first case of HCC was discovered (at the 99th weeks)

** a versus b, $P>0.05$; c versus d, $P>0.05$; e versus f, $P<0.05$; e versus g, $P>0.05$; h versus I, $P<0.05$.

Table 2. The expression of ras gene and the development of HCC in group A

P^{21}	No. of animals	No. of HCC case (%)
+	6	6 (100%)[a*]
-	11	2 (18.2%)[b]

*a versus b, $P<0.01$

DISCUSSION

In 1987 we have successfully established the animal model in tree shrews infected with human HBV.[7] In the present study, HBsAg and/or HBV DNA were detected in the sera and/or liver tissues, this demonstrated that tree shrews had been successfully infected with human HBV.

The incidence of HCC was significantly higher in the animals both infected with HBV and exposed to AFB1 (group A, 47.1%) than that in those only exposed to AFB1 (group C, 13.3%), and no HCC appeared in those infected HBV alone (group B) and in those as controls (group D) until the end of the experiment. This suggested that HBV and AFB1 play a remarkable synergistic role in the development of HCC; Under our experimental conditions, the contribution of AFB1 to the carcinogenic process was much stronger than that of HBV infection, which did not produce any HCC when infected with HBV alone. The results are in agreement with our previous studies.[4]

Accumulating evidence suggested that in HCC, ras gene may play a role in the early stages of carcinogenesis.[6,8] To develop a more understanding of the role of molecular oncogenic mechanism in the process of HCC development, we detected the liver bioptic tissues taken at 44th, 104th and 119th weeks for p^{21} protein. The total positive rates in group A (35.3%) was higher than that in group B (5.3%, $P<0.05$), and group C (13.3%). Our finding first indicated that HBV and AFB1 may play a synergistic role in the expression of *ras* gene.

We examined the expression of *ras* gene in bioptic liver tissues of group A (HBV+AFB1). The results showed that the incidences of HCC in the animals those with and without p^{21}expression in liver tissues were 100% and 18.2%, respectively, and there was a significant difference between those animals ($P<0.001$). The results demonstrated that there is a closely relationship between p^{21} expression in liver tissues and HCC under the HBV infection and AFB1 uptake.

These results provided conclusive evidence that HBV and AFB1 can enhance expression of *ras* gene and over-expression of ras gene is closed related to pathogenesis of HCC in tree shrews.

In the present article, we first addressed that the study on the relationship between the status of genes in liver tissues and the outcome using the serial biopsies technique in experimental hepatocarcinogenesis.

REFERENCES

[1] Buendia MA. Hepatitis B virus and hepatocellular carcinoma. Adv Cancer Res 1992; 59:167.

[2] Ross PK, Yuan JM, Yu MC, et al. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. Lancet 1992; 339:943.

[3] Yeh FS, Yu MC, Mo CC, et al. Hepatitis B virus, aflatoxin, and hepatocellular carcinoma in Southern Guangxi, China. Cancer Res 1989; 49:2506.

[4] Yan RQ, Su JJ, Huang DR, et al. Human hepatitis B virus and hepatocellular carcinoma II. Experimental induction of hepatocellular carcinoma in tree shrews exposed to hepatitis B virus and aflatoxin B1. J Cancer Res Clin Oncol 1996; 122:289.

[5] Soman NR, Wogan GN. Activation of the c-ki-ras oncogene in aflatoxin B1-induced hepatocellular carcinoma and adenoma in the rat: Detection by denaturing gradient gel electrophoresis. Proc Natl Acad Sci USA 1993; 2045.

[6] Zheng J, Ruan YB, Bing L, et al. The role of platelet-derived growth factor and ras p^{21} in experimental hepatocarcinogenesis. Chin J Pathol 1996; 25:79.

[7] Su JJ, Yan RQ, Gan YQ, et al. Experimental infection of human hepatitis B virus (HBV) in adult tree shrews. Chin J Pathol 1987; 16:103.

[8] Lin YZ, Ding L, Chen ZY. The expression of c-*myc*, N-ras mRNA and its relations to the differentiation of preneoplastic altered hepatocytes. Chin J Oncol 1993; 15:97.