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Expression of p53 gene in hepatocellular carcinomas induced by aflatoxin B1 with or without human hepatitis B virus in tree shrews

Jianjia Su,[1,2] Guozhong Qin,[1] Ruiqi Yan,[2] Dingrui Huang,[2] Chun Yang,[2] Guohua Huang[2] and Prabhakar D. Lotlikar[1,3]

1 Fels Institute for Cancer Research and Molecular Biology and Department of Biochemistry, Temple University School of Medicine, 3420 North Broad Street, Philadelphia, PA 19140, USA

2 Department of Pathology, Guangxi Cancer Institute, Nanning 530021, Guangxi, China

3 Corresponding author

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Abbreviations: HCC, hepatocellular carcinoma; AFB_1, aflatoxin B_1; HBV, hepatitis B virus; HHBV, human HBV; DHBV, duck hepatitis B virus; WHV, woodchuck hepatitis virus; GSHV, ground squirrel hepatitis virus

Abstract

Using tree shrew as an animal model, our previous studies have demonstrated synergistic effects of aflatoxin B_1 (AFB_1) and human hepatitis B virus (HHBV) in the induction of hepatocellular carcinoma (HCC). In the present study, we have examined expression of p53 gene in HCCs induced by AFB_1 with or without HHBV infection in tree shrews. Avidin-biotin-peroxidase complex immunohistochemical method with human p53-CM_1 polyclonal antibody has been used to detect p53 expression in serial sections of paraffin-embedded liver and HCC tissues. Five out of 9 animals with HCCs (55.6%) induced by AFB_1 with HHBV infection and 2/3 animals with HCCs (66.7%) induced by AFB_1 alone expressed the p53 protein. Out of 18 HCCs examined, expression of p53 protein was observed in 9/10 moderately and poorly differ-entiated HCCs (90.0%). None of the well differentiated HCCs (0/8) expressed p53 (0%). Similarly, no p53 expression was observed in either non-tumorous or hyperplastic liver tissues or nodules. These results suggest that p53 expression associated with p53 mutation is a late event occurring probably during tumor progression in AFB_1 and HHBV induced hepa-tocarcinogenesis in the tree shrew. This report is the first example of an experimental animal model where combination of human HBV and AFB_1-induced HCCs demonstrate p53 expression.

Keywords: hepatocellular carcinoma; hepatitis B; aflatoxin B_1; p53 expression

Introduction

Hepatocellular carcinoma (HCC) is one of the world's most common cancers, occurring especially in Africa and South-East Asia (Beasley, 1982; Parkin *et al*., 1988). Epidemiological studies indicate that contamination of food with aflatoxin B_1 (AFB_1) and chronic infection with hepatitis B virus (HBV) are the major risk factors for human liver cancer (Buendia, 1992; Ross *et al*., 1992; Yeh *et al*., 1989). Several investigations in different species of experimental animals have demonstrated synergistic effects of AFB_1 and HBV in hepatocarcinogenesis (Sell *et al*., 1991; Cova *et al*., 1994; Bannasch *et al*., 1995). Thus, synergy between HBV expression and AFB_1 hepatocarcinogenesis has been established in transgenic mice (Sell *et al*., 1991). In duck and woodchuck studies, in addition to AFB_1, duck hepatitis B virus (DHBV) and woodchuck hepatitis virus (WHB) were employed respectively as infectious agents (Bannasch *et al*., 1995; Cova *et al*., 1994).

p53, the tumor suppressor gene located on the short arm of chromosome 17 (Isobe *et al*., 1986) normally regulates the activity of the cell cycle machinery (Marx, 1993). Its gene product is critical for guarding the genome from incorporation of damaged DNA (Lane, 1992). Mutation of the *p53* gene has been observed with a high prevalence in diverse types of human cancers (Hollstein *et al*., 1991), and frequently occurs with point mutation in the coding sequence of tumor cellular gene. Mutation of G to T transversion at codon 249 position 3 of *p53* gene has been detected in human HCCs associated with high exposure to AFB_1 in Africa and in Qidong, China (Bressac *et al*., 1991; Hsu *et al*., 1991; Scorsone *et al*., 1992). The frequency of *p53* mutations in HCCs in different studies are variable from 18% to 67%. However, these *p53* mutations are more frequent in HBV-infected HCC patients exposed to AFB_1 (Bressac *et al*., 1991; Goldblum *et al*., 1993; Hosono *et al*., 1993; Hsu *et al*., 1991; Livni *et al*., 1995; Nagao *et al*., 1995; Scorsone *et al*., 1992).

Using tree shrew (Tupaia glis) as an animal model, it was shown (Reddy *et al*., 1976) that feeding of AFB_1 to these animals produced HCCs in this species. On the basis of their results, we investigated the hepatocarcinogenesis of AFB_1 and human HBV (HHBV) in this species (Yan *et al*., 1996a, 1996b). The results indicated that the HCC incidence was significantly higher in the tree shrews exposed to both HHBV and AFB_1 than in those which

received either HHBV infection or AFB_1 ingestion only. Thus, our data suggested a synergistic effect of HHBV and AFB_1 in induction of HCCs in tree shrews. We have also examined expression of *p53* gene in various grades of HCCs of tree shrews using immunohistochemical method. These results suggest that p53 expression associated with *p53* mutation is a late event occurring probably during tumor progression in AFB_1 and HHBV induced hepatocarcinogenesis in the tree shrew. This report is the first example of an experimental animal model where combination of human HBV and AFB_1-induced HCCs demonstrate p53 expression.

Materials and Methods

Animals and experimental design

Carcinogenicity studies were carried out in China as described previously (Yan *et al*., 1996b). In brief, out of 80 adult tree shrews, 50% were inoculated with HHBV. All of these animals were then given either dimethyl sulfoxide or AFB_1 dissolved in dimethyl sulfoxide added to the diet until the termination of the experiment. Several animals in both (HHBV + and HHBV -) groups died before 80th week without any signs of HCC. First case of HCC was seen in one animal (HHBV + without AFB_1) which died during 83rd week. The experiment was terminated at 158 weeks. The liver tissues of dead or sacrificed animals were fixed in 10% buffered formalin, then paraffin-embedded and were examined pathologically by hematoxylin and eosin staining. Thirteen animals developed HCCs. The tumor differentiation was classified according to Edmonson and Steiner (1954). The distribution of HCCs among various groups is indicated in Table 1. These paraffin-embedded liver tissues including 18 HCCs induced in 12 animals have been examined for *p53* gene expression using immunohistochemistry.

Chemicals

Rabbit polyclonal antibody (CM_1) against human p53 protein and avidin-biotin-peroxidase complex kit (ABC kit) were purchased from Vector Laboratories Inc., Burlingame, CA. 3,3'-Diaminobenzidine was obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were of reagent grade.

Immunohistochemistry

p53 protein was detected using immunohistochemical staining according to published procedures (Shi *et al*., 1991; Resnick *et al*., 1995). In brief, formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and were passed through graded ethanol series. After quenching the endogenous peroxidase activity, sections were heated in a microwave oven followed with sequential treatment with normal goat serum, primary anti-p53 antibody (CM_1) (1:800), biotinylated goat anti-rabbit immunoglobulin G (1:200) and avidin-biotin-peroxidase complex. The sites of peroxidase binding were detected by the diaminobenzidine method. Sections of human colonic carcinoma with p53 expression were used as positive controls. Negative controls consisted of replacement of primary antibody with normal goat serum. The pattern of p53 nuclear expression was classified by estimating the percentage of stained tumor cells as follows: -, <5% positive tumor cells; +, 6-10% positive tumor cells; ++, 11 to 20% positive tumor cells; +++, 21 to 50% positive tumor cells; and ++++, >50% positive tumor cells. We define a tumor as p53 positive if more than 5% of tumor cells showed nuclear p53 expression.

The significance of the data was statistically evaluated by using X^2 test.

Results

Thirteen animals developed HCCs throughout the experimental period of 158 weeks. The distribution of HCCs among various groups is shown in Table 1. The incidence of HCC in group A was significantly higher and statistically significant with a P value of <0.05 when compared with

Table 1. HCC development and p53 expression in various groups

Group and Treatment	No. of animals surviving after 83rd wk	No. of animals with HCCs (%)	p53 expression among animals with HCCs (%)
A (HHBV + AFB_1)	17	9 (52.9)[c]	5 (55.6)
B (HHBV)	9	1 (11.1)[a]	
C (AFB_1)	24[b]	3 (12.5)	2 (66.7)
D (Control)	8	0 (0)	

[a] Not tested for p53 for lack of availability of tumor tissue.

[b] 19 cases were examined for p53.

[c] Statistically significant with P <0.05 when compared with groups C and D.

groups C and D. Fifty three samples of experimental tree shrew liver tissues were stained for the expression of p53 protein (Table 1). Since tumor tissue in group B was not available, it was not tested for p53 expression. The positive expression of p53 in HCC was 7/12 (58.3%), five animals in group A and two in group C. There was, however, no statistical difference in the expression of p53 protein in groups A and C. No p53 immunostaining was observed in either nontumorous or hyperplastic liver tissues or nodules.

Positive p53 immunostaining was seen in nuclei of tumor cells but the intensity of staining was variable from cell to cell. Weak staining was sometimes seen in some tumorous cytoplasm.

Status of 12 cases of HCCs in regards to their size, grade of tumor and degree of p53 expression is presented in Table 2. Among 12 cases, 50% of animals had two nodules per animal. The expression of p53 protein in HCCs of tree shrews was related to the grades of tumor cells' histological differentiation. No positive expression of p53 protein was found in 8 HCCs which were Edmondson and Steiner's (1954) grade I in tumor cells' differentiation. All six grade II HCCs demonstrated p53 expression ranging between 10-22% of tumor cells positive for p53. Among 4 grade III HCCs, two showed high p53 expression (65-85%), one showed low expression (10%) and one without any p53 expression.

Results of grade and p53 expression of HCCs are summarized in Table 3. Thus, 9 out of 10 cases of grade II-III tumors were positive for p53 staining whereas none were positive for p53 staining among grade I tumors. These results are statistically significant with a P value of <0.005.

Discussion

The synergistic effect of HBV infection and intake of AFB_1 has been suggested to be largely responsible for the high frequency of *p53* mutations observed especially at the third base of codon 249 in human HCCs from Africa and China (Bressac *et al*., 1991; Hsu *et al*., 1991; Scorsone *et al*., 1992). Animal model systems have been employed to explore the relationship between mutations of *p53* in HCCs induced by either AFB_1 or/and hepadna viruses (Fujimoto *et al*., 1992; Hulla *et al*., 1993; Duflot *et al*., 1994; Rivkina *et al*., 1994). In contrast to human HCCs, no mutations at codon 249 were detected in HCCs induced by AFB_1 in non-human primates. However, one mutation at the second base of codon 175 with a G to T transversion was observed in one HCC. On the basis of these

Table 2. Status of 12 cases of HCCs

Animal	Treatment		Survival Time	Tumor	HCC		p53[b]	(%)	In the Liver Cells		
					Size	Grade[a]		Positive			
Number	HBV	AFB_1	wks		cm.			Cells	HbsAg[1]	HBcAg[1]	HBV DNA[2]
2644	-	+	88	T1	3.2	III	+	(10%)	-	-	-
3057	+	+	115	T1	1.2	I	-		-	+	+
3050	+	+	120	T1	0.6	III	++++	(85%)	+	-	+
				T2	2.4	III	++++	(65%)	+	-	+
2956	+	+	123	T1	1.8	II	+++	(22%)	-	-	+
3073	+	+	125	T1	0.8	I	-		+	-	+
				T2	3.2	II	++	(12%)	+	-	+
3019	+	+	126	T1	0.3	I	-		+	+	+
				T2	0.4	I	-	(4%)	+	+	+
3052	-	+	132	T1	0.5	III	-		-	-	-
3072	+	+	137	T1	0.8	I	-		+	-	+
				T2	1.3	I	-	(3%)	+	-	+
3077	+	+	137	T1	2.1	II	++	(20%)	+	-	+
				T2	1.5	II	++	(20%)	+	-	+
3070	+	+	142	T1	0.7	II	++	(15%)	+	-	+
3071	+	+	142	T1	1.4	I	-		-	+	+
				T2	1.7	I	-	(3%)	-	+	+
2847	-	+	158	T1	1.5	II	+	(10%)	-	-	-

[a] Grade of HCC differentiation was classified according to Edmondson and Steiner (1954).

[b] Degree of p53 nuclear expression in HCC was graded as follows: -, <5% positive tumor cells; +, 6 to 10% positive tumor cells; ++, 11 to 20% positive tumor cells; +++, 21 to 50% positive tumor cells; ++++, >50% positive tumor cells. We define a tumor as p53 positive if more than 5% of the tumor cells showed nuclear p53 expression.

[1] Unpublished data (Su, J. J., Huang, D. R., Yang, C., Huang, G. H. and Yan, R. Q.).

[2] Data from Su *et al*. (1991).

Table 3. Comparison of p53 positivity in different grades of HCCs

Grade of HCCs	No. of HCCs	No. of HCCs positive for p53 (%)[a]
I	8	0 (0)
II-III	10	9 (90)[b]
Total	18	9 (50.0)

[a] A tumor is defined as p53 positive if more than 5% of the tumor cells show nuclear p53 expression.

[b] Data statistically significant with $P<0.005$ when compared with those of grade I tumors.

data, it was suggested that HBV may be a prerequisite for AFB_1 induction of codon 249 mutation (Fujimoto *et al*., 1992). In the rat, AFB_1-induced preneoplastic hepatic nodules did not demonstrate any *p53* gene mutation at the site corresponding to codon 249 of the human *p53* gene (Hulla *et al*., 1993). Similarly, there was no presence of *p53* gene mutation at 249 codon in HCCs produced in domestic ducks in Qidong, China, irrespective whether these ducks were DHBV positive or negative and treated or not treated with AFB_1 (Duflot *et al*., 1994). In another study, a mutation of *p53* gene was detected in only one out of five ground squirrel HCCs where these animals were GSHV positive and treated with AFB_1. This mutation was at the second base of codon 176 with a G to T transversion. Hepatocellular carcinomas in two ground squirrels without AFB_1 treatment and 11 HCCs in woodchucks persistently infected with WHV were not mutated in the *p53* gene (Rivkina *et al*., 1994).

In the present study, immunohistochemical detection of p53 protein has been used as a surrogate marker for missense mutation due to following reasons: 1) Mutant p53 proteins are more stable than the wild type and thus their accumulation can be detected immunohistochemically (Oren, 1992). 2) Immunohistochemical detection of p53 expression has been shown to be a very reliable indicator of *p53* mutations in several human cancers including HCCs (Hsu *et al*., 1993; Dowell and Hall, 1994; Ng *et al*., 1994; Ojanguren *et al*., 1995; Vet *et al*., 1995). Even though rabbit polyclonal antibody (CM_1) raised against human wild-type and mutant *p53* has been used in the present study, this antibody reacted only with p53 present in grades II and III HCCs produced in tree shrews fed AFB_1 with or without human HBV infection. There was no expression of p53 protein in either non-tumorous or hyperplastic liver tissues or nodules of these animals. Similarly, there was no detection of p53 expression in low grade I, well-differentiated HCCs. Like the present study, cross reactivity of antibody raised against human p53 and hamster pancreatic adenocarcinoma p53 has also been reported recently (Chang *et al*., 1994).

Data reported in the present study indicate that even though 50% of animals bearing HCCs had two tumors, the grade of a tumor determined the p53 expression of that tumor. Thus, well-differentiated (Grade I) HCCs showed no positive p53 expression whereas moderately differentiated (Grade II) HCCs showed p53 expression with 10-22% positive cells and some poorly differentiated Grade III HCCs with p53 expression as high as 65-85% positive cells. These results indicating that p53 expression associated with *p53* mutation is a late event occurring probably during tumor progression of hepatocarcinogenesis in the tree shrew are compatible with data obtained with human HCCs (Oda *et al*., 1992; Hsu *et al*., 1993; Tanaka *et al*., 1993; Hsu *et al*., 1994; Nagao *et al*., 1995; Ojanuren *et al*., 1995). The overall data and no or low expression of p53 in HCCs induced in animals given only AFB_1 in the present study suggest that HHBV infection has a synergistic effect not only on AFB_1-induced HCCs but also on p53 expression. On the basis of our immunohistochemical data, it appears that 50% HCCs induced in tree shrews with AFB_1 and with or without HHBV infection show *p53* mutation. Rates of *p53* mutations in HCCs produced in other experimental animals treated with AFB_1 alone or with hepadna viruses are much lower (Fujimoto *et al*., 1992; Hulla *et al*., 1993; Duflot *et al*., 1994; Rivkina *et al*., 1994) than those reported in the present study. These differences in *p53* mutations may be species specific. On the other hand, on the basis of our present data and those reported with human HCCs (Oda *et al*., 1992; Hsu *et al*., 1993; Tanaka *et al*., 1993; Hsu *et al*., 1994; Ng *et al*., 1994; Nagao *et al*., 1995; Ojanguren *et al*., 1995), it appears that low *p53* mutation rates observed in HCCs induced in ground squirrels may be due to examination of only low grade (well-differentiated) HCCs (Rivkina *et al*., 1994). In addition, experimental tree shrews in the present study were inoculated with human serum containing human HBV. Although, HHBV as well as WHV, GSHV and DHBV belong to hepadnavirus family, there are some differences in their molecular structures. For example, DHBV differs from HHBV such that is lacks the X gene and it induces only mild liver tissue disease in its host (Mandart *et al*., 1984). However, WHV appears to be more oncogenic than HBV (Popper *et al*., 1987; Buendia, 1992). There may be additional differences in infectivity and oncogenicity among these hepadnaviruses.

Our previous studies (Su *et al*., 1992) and recent studies from another laboratory (Walter *et al*., 1996) have demonstrated that HHBV was not only replicated in liver cells but was also integrated into liver cell DNA of the tree shrew. It has been shown recently that HHBV x protein plays an important role in the pathogenesis of early human HCC by interaction with p53 protein (Zhu *et al*., 1993). Recent studies also suggest that functional inactivation of *p53* gene product but not structural mutation of *p53* is responsible for liver cancer causation (Ueda *et al*., 1995). In view of these recent developments, it is important to explore further our p53 studies in HCCs induced in tree

shrews by HHBV and AFB_1 by examining *p53* gene by its amplification by polymerase chain reaction (PCR) and sequence analysis of purified PCR product.

Tree shrews (*Tupaia glis*), squirrel-like mammals occurring throughout Southeast Asia and once considered to be a non-human primate have been colonized successfully (Morris *et al*., 1967). They have been reclassified as Scandentia (Novak, 1991) and are closer to the primates. Since tree shrew has a shorter life span compared to primates and is small, it is an ideal animal for experimental use. On the basis of our studies, it appears that tree shrew is an ideal animal to study the mechanism of HCC development. Data generated with this species may be extrapolated to humans.

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