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Advances in Brief

Low Frequency of *p53* Gene Mutation in Tumors Induced by Aflatoxin B_1 in Nonhuman Primates

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Abstract

Aflatoxin B_1 has been suggested as a causative agent for a G to T mutation at codon 249 in the *p53* gene in human hepatocellular carcinomas from southern Africa and Qidong in China. To test this hypothesis, nine tumors induced by aflatoxin B_1 in nonhuman primates were analyzed for mutations in the *p53* gene. These included four hepatocellular carcinomas, two cholangiocarcinomas, a spindle cell carcinoma of the bile duct, a hemangioendothelial sarcoma of the liver, and an osteogenic sarcoma of the tibia. None of the tumors showed changes at the third position of codon 249 by cleavage analysis of the *Hae*III enzyme site at codon 249. A point mutation was identified in one hepatocellular carcinoma at the second position of codon 175 (G to T transversion) by sequencing analysis of the four conserved domains (II to V) in the *p53* gene. These data suggest that mutations in the *p53* gene are not necessary in aflatoxin B_1 induced hepatocarcinogenesis in nonhuman primates. The occurrence of mutation in codon 249 of the *p53* gene in selective samples of human hepatocellular cancers may indicate involvement of environmental carcinogens other than aflatoxin B_1 or that hepatitis B virus-related hepatitis is a prerequisite for aflatoxin B_1 induction of G to T transversion in codon 249.

Introduction

Dietary exposure to aflatoxin B_1 is an epidemiologically defined risk factor for HCC[3] in southern Africa and China (1–3). Aflatoxin B_1 is also a potent hepatocarcinogen in different species including nonhuman primates (4–6). Recently, a significant number of HCCs in patients from Qidong province in China and from southern Africa, where hepatitis B virus is endemic and dietary exposure to aflatoxin B_1 is high, were found to have a mutation at the third position of codon 249 of the *p53* gene (3, 7, 8). It was suggested that this mutation of the *p53* gene was caused by aflatoxin B_1 and might contribute to the high incidence of HCC in these areas. The absence of mutation at this hot spot in HCCs in patients from Japan, Taiwan, or Australia support this hypothesis since exposure to aflatoxin B_1 is very low in these areas (3, 9–11). The specificity of this mutational event represents the first clue as to how an environmental toxin may contribute to tumor development (12). It is therefore important to characterize the frequency and patterns of *p53* gene mutations in aflatoxin B_1 induced carcinogenesis. We have examined nine tumors previously induced by aflatoxin B_1 in nonhuman primates (4, 5) for mutations in the *p53* gene by restriction enzyme analysis using *Hae*III enzyme site at codon 249 and by sequence analysis for mutations within the highly conserved domains II to V which contain four hot spots for mutations in various tumors (13–15). A predicted amino acid sequence of the *p53* gene of nonhuman primates is 95% identical to the human protein and that of conserved domain IV including codon 249 is completely identical (16).

Materials and Methods

Tumors in Nonhuman Primates. Nine tumors including four HCCs, two cholangiocarcinomas, a spindle cell carcinoma of a bile duct, a hemangioendothelial sarcoma of a liver, and an osteogenic sarcoma of the tibia were derived from eight nonhuman primates composed of four rhesus (*Macaca mulatta*) and four cynomolgus (*Macaca fascicularis*) monkeys (Table 1). Aflatoxin B_1 (Calbiochem, Los Angeles, CA; Makor Chemicals, Ltd., Jerusalem, Israel) was administered according to a variety of schedules from 1964 to 1978 (5). Nonhuman primates that died or were sacrificed were carefully necropsied and the tissues were fixed in buffered formalin and embedded in paraffin (5).

DNA Preparation. Extraction of DNA from paraffin embedded tumor tissue was carried out as described previously (17). Briefly, tumor cell areas from two 10-μ sections of each paraffin block were selectively removed based on corresponding hematoxylin and eosin stained sections. The tissues were deparaffinized by xylene, washed with 95% ethanol, and incubated in lysis buffer (10 mM Tris-HCl, pH 7.5, -5 mM $MgCl_2$, -0.32 M sucrose, -1% Triton X), 0.5% sodium dodecyl sulfate, and 0.1 mg/ml proteinase K at 70°C for 1 h and 42°C overnight. DNA was extracted from lysis solution with phenol/chloroform, precipitated with ethanol, and dissolved in 100 μl of 10 mM Tris-HCl, pH 8.0.

PCR Amplification. Oligonucleotide primers for PCR amplification were synthesized with an Applied Biosystems DNA synthesizer (model 380B). Identification of sequences are:P1, ACGTGAATTCAACTCTGTGTCCTTCCT; P2, TGGATCCAGTCCCAGCTGCTCACC; P3, GGAATTCTGACTGTACCACCATCCA; P4, ACGTGGAATTCAGAGGCAAGCAGAGGCTG; P5, ACGTGAATTCCTTACTGCCTCCTGCTT; P6, ACGTGGAATTCGTGGCAAGGCTCCCCTTT. P1, P2, P3, P4, P5, and P6 were derived from intron 4, intron 5, exon 7, intron 7, intron 7, and exon 8, respectively, of *p53*gene sequence in nonhuman primates (16). Three sets of primers, P1 and P2, P3 and P4, and P5 and P6, were used for PCR amplification for exons 5, 7, and 8, respectively. PCR amplifications were performed in 100-μl volumes with 20 μl of the DNA solutions consisting of 40 cycles of 95°C (1 min), 55°C (1 min), and 70°C (2 min). PCR reactions contained $MgCl_2$ at a final concentration of 2 mM (18). Ten μl of PCR products for exon 7 were digested with restriction enzyme *Hae*III (New England Biolabs, Inc.) at 37°C for 3 h and fractionated by 3% agarose gel electrophoresis to examine for mutation at codon 249 (8).

Sequencing. The six primers were designed to incorporate extra nucleotides comprising *Eco*RI, or *Eco*RI and *Bam*HI sites at their 5′ ends to facilitate cloning. The PCR products were digested with *Eco*RI or *Eco*RI and *Bam*HI, fractionated by 3% low melting agarose gel electrophoresis, and ligated to *Eco*RI or *Eco*RI and *Bam*HI digested pGEM vectors (Promega). At least six individual clones were sequenced for each sample by the dideoxy chain termination method (19) and a total of 180 individual clones were examined for mutations of the *p53* gene. To confirm a mutation, subclones obtained from two individual amplifications were sequenced.

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; HBV, hepatitis B virus.

Table 1 *Mutation of the p53 gene in tumors induced by aflatoxin B_1 in nonhuman primates*

Tumor	Histology	Metastasis[a]	Monkey[b]	Species[c]	Mutation 249[e]	(Codon) others
1	Hepatocellular carcinoma[d]	−	692I	Rh	—	—
2	Hepatocellular carcinoma[d]	+	692I	Rh	—	175 (G to T)
3	Hepatocellular carcinoma	−	497F	Cyno	—	—
4	Hepatocellular carcinoma	−	454F	Rh	—	—
5	Cholangiocarcinoma	+	500F	Rh	—	—
6	Cholangiocarcinoma	+	473F	Rh	—	—
7	Spindle cell carcinoma	−	518G	Cyno	—	—
8	Hemangioendothelial sarcoma	+	498F	Cyno	—	—
9	Osteogenic sarcoma	+	508F	Cyno	—	—

[a] Indicates metastases to distant organs at time of autopsy.
[b] Identification of monkeys corresponding to a previous paper of Sieber *et al.* (5).
[c] Rh, rhesus; Cyno, cynomolgus.
[d] Tumors 1 and 2 are from the same animal. Tumor 1 was from a liver biopsy taken 5 months prior to sacrifice of monkey, whereas tumor 2 was from liver tumor taken at autopsy.
[e] No mutations found at codon 249.

Results and Discussion

Nine tumors derived from eight nonhuman primates treated with aflatoxin B_1 were analyzed for mutations in the *p53* gene, particularly at the third position of codon 249 (7, 8). PCR products for exon 7 were amplified from DNAs extracted from paraffin sections using primers P3 and P4 and were digested with the restriction enzyme *Hae*III. Wild-type *p53* alleles are cleaved by *Hae*III at codon 249, giving three products of 74, 66, and 41 base pairs. A mutation at the second or third position of codon 249 would result in loss of the *Hae*III cleavage site and yield two products of 140 and 41 base pairs. In contrast to human HCCs from Qidong and southern Africa (3, 7, 8), all nine nonhuman primate tumor samples were cleaved by *Hae*III at codon 249 of the *p53* gene (Fig. 1). To test for mutations at different positions within the *p53* gene, exons 5, 7, and 8, which include the highly conserved domains II to V that contain four mutation hot spots (13–15), were amplified from the nine tumors. At least six individual clones for each PCR product were sequenced. A sequence polymorphism was observed at the third position of codon 186, a substitution of T to C, resulting in no amino acid change (Fig. 2). Analysis of the polymorphism revealed no allele loss in eight informative tumors. Only one HCC showed a point mutation at the second position of codon 175, a substitution of G to T, resulting in an amino acid change from arginine to leucine (Fig. 2). The mutation was confirmed by sequence analysis of subclones obtained from two individual amplifications. Although the substitution of G to T at codon 175 might be caused by the preferential binding of aflatoxin B_1 to G residues (20), the frequency of mutations in the *p53* gene in the tumors was very low (Table 1). These data suggest that, unlike the proposed involvement in human HCCs (3, 7, 8), aflatoxin B_1 infrequently induces mutations in the *p53* gene in nonhuman primates. Consequently mutations in the *p53* gene are not necessary in aflatoxin B_1 induced carcinogenesis in nonhuman primates. As to the suggestion that aflatoxin B_1 is responsible for the G to T mutation at codon 249 of the *p53* gene in human HCCs from Qidong and southern Africa (7, 8), we would like to suggest two possible explanations. One is that aflatoxin B_1 may indeed cause the mutation at codon 249 in the *p53* gene in human HCCs. This does, however, not occur in nonhuman primates because of species differences possibly in gene structure (21) metabolism (22, 23), and in the absence of HBV-induced chronic active hepatitis. Since G to T mutation in codon 249 has only been identified in HBV-related human

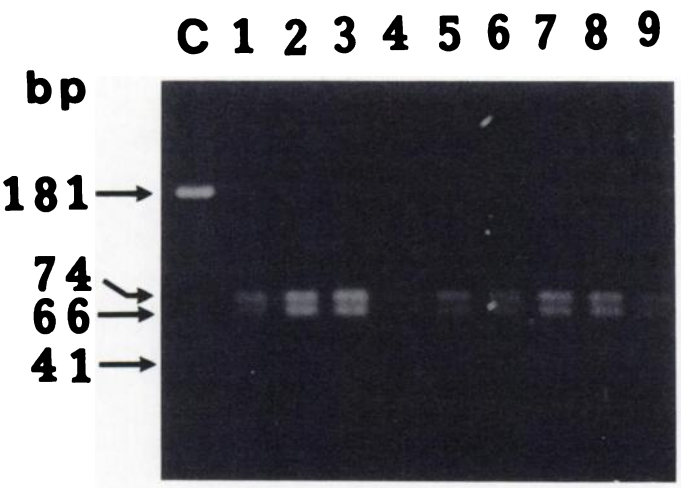

Fig. 1. The digestion profile of amplified fragments for a mutation at codon 249 in the *p53* gene. DNA fragments of 181 base pairs (*bp*) amplified by PCR using P3 and P4 primers were digested with *Hae*III restriction enzyme. Wild-type *p53* alleles resulted in cleavage by *Hae*III at codon 249, giving three products of 74, 66, and 41 base pairs. A mutation at codon 249, which would result in loss of the *Hae*III site giving two products of 140 and 41 base pairs, was not observed. *Lane C*, a control monkey fragment before the *Hae*III digestion; *Lanes 1* to *9*, *Hae*III digested fragments from Tumors 1 to 9, respectively. Electrophoresis was performed using 3% agarose gels and DNA was visualized using ethidium bromide.

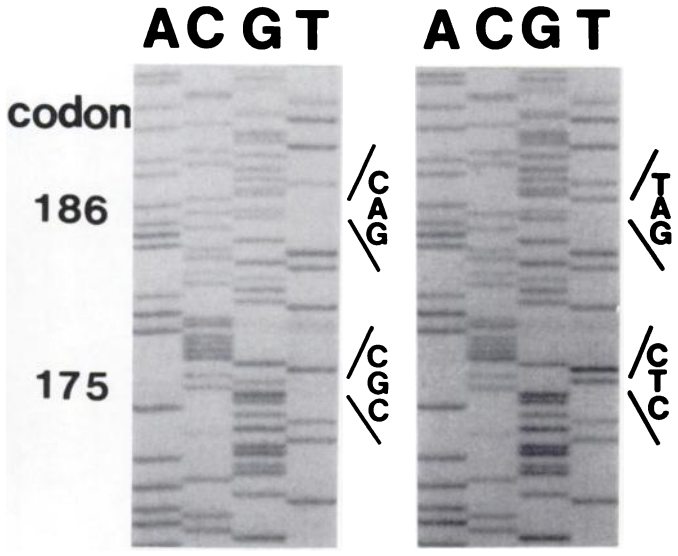

Fig. 2. Identification of a polymorphism and a point mutation of exon 5 in the *p53* gene in nonhuman primates. Shown is representative sequence data obtained from a HCC (Tumor 2) as described in the text. The sequence data illustrate the area surrounding and including codon 186 and 175. *Panel 1*, C-type polymorphism at codon 186 (GAC) and a wild-type sequence at codon 175 (CGC); *Panel 2*, T-type polymorphism at codon 186 (GAT) and a point mutation at codon 175 (CTC).

HCC, it is possible that both aflatoxin B_1 exposure and HBV-induced chronic active hepatitis are necessary for induction of the mutation (3, 7). The other explanation is that environmental carcinogens other than and coincident with aflatoxin B_1 are responsible for the mutation at codon 249 in the *p53* gene in human HCCs.

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References

1. Van Rensburg, S. J., Cook-Mozaffari, P., Van Schalkwyk, D. J., Van Der Watt, J. J., Vincent, T. J., and Purchase, I. F. Hepatocellular carcinoma and dietary aflatoxin in Mozambique and Transkei. Br. J. Cancer, *51:* 713–726, 1985.
2. Yeh, F. S., Yu, M. C., Mo, C. C., Luo, S., Tong, M. J., and Henderson, B. E. Hepatitis B virus, aflatoxins, and hepatocellular carcinoma in southern Guangxi, China. Cancer Res., *49:* 2506–2509, 1989.
3. Ozturk, M. *et al. p53* hotspot mutation in hepatocellular carcinoma is geographically linked to aflatoxin exposure. Lancet, in press, 1992.
4. Adamson, R. H., Correa, P., Sieber, S. M., McIntire, K. R., and Dalgard, D. W. Carcinogenicity of aflatoxin B_1 in rhesus monkeys: two additional cases of primary liver cancer. J. Natl. Cancer Inst., *57:* 67–78, 1976.
5. Sieber, S. M., Correa, P., Dalgard, D. W., and Adamson, R. H. Induction of osteogenic sarcoma and tumors of the hepatobiliary system in nonhuman primates with aflatoxin B_1. Cancer Res., *39:* 4545–4554, 1979.
6. Newberne, P. M., and Wogan, G. N. Sequential morphologic changes in aflatoxin B carcinogenesis in the rat. Cancer Res., *28:* 770–781, 1968.
7. Hsu, I. C., Metcalf, R. A., Sun, T., Welch, J. A., Wang, N. J., and Harris, C. C. Mutational hotspot in the *p53* gene in human hepatocellular carcinomas. Nature (Lond.), *350:* 427–428, 1991.
8. Bressac, B., Kew, M., Wands, J., and Ozturk, M. Selective G to T mutations of *p53* gene in hepatocellular carcinoma from southern Africa. Nature (Lond.), *350:* 429–431, 1991.
9. Murakami, Y., Hayashi, K., Hirohashi, S., and Sekiya, T. Aberrations of the tumor suppressor *p53* and retinoblastoma genes in human hepatocellular carcinomas. Cancer Res., *51:* 5520–5525, 1991.
10. Hosono, S., Lee, C. S., Chou, M. J., Yang, C. S., and Shih, C. Molecular analysis of the *p53* alleles in primary hepatocellular carcinomas and cell lines. Oncogene, *6:* 237–243, 1991.
11. Hayward, N. K., Walker, G. J., Graham, W., and Cooksley, E. Hepatocellular carcinoma mutation. Nature (Lond.), *352:* 764, 1991.
12. Wands, J. R., and Blum, H. E. Primary hepatocellular carcinoma. N. Engl. J. Med., *325:* 729–731, 1991.
13. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, S. H., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C., and Vogelstein, B. Mutations in the *p53* gene occur in diverse human tumor types. Nature (Lond.), *342:* 705–708, 1989.
14. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. *p53* mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.
15. Levine, A. J., Momand, J., and Finlay, C. A. The *p53* tumour suppressor gene. Nature (Lond.), *351:* 453–456, 1991.
16. Rigaudy, P., and Eckhart, W. Nucleotide sequence of a cDNA encoding the monkey cellular phosphoprotein p53. Nucleic Acids Res., *17:* 8375, 1989.
17. Shindo, M., Okuno, T., Arai, K., Matsumoto, M., Takeda, M., Kashima, K., Shimada, M., Fujiwara, Y., and Sokawa, Y. Detection of hepatitis B virus DNA in paraffin-embedded liver tissues in chronic hepatitis B or Non-A, Non-B hepatitis using the polymerase chain reaction. Hepatology, *13:* 167–171, 1991.
18. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science (Washington DC), *239:* 487–491, 1988.
19. Sanger, F., Nicklen, S., and Coulson, A. R. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA, *74:* 5453–5467, 1977.
20. Foster, P. L., Eisenstadt, E., and Miller, J. H. Base substitution mutations induced by metabolically activated aflatoxin B_1. Proc. Natl. Acad. Sci. USA, *80:* 2695–2698, 1983.
21. Soussi, T., Fromentel, C. C., and May, P. Structural aspects of the p53 protein in relation to gene evolution. Oncogene, *5:* 945–952, 1990.
22. Ramsdell, H. S., and Eaton, D. L. Species susceptibility to aflatoxin B_1 carcinogenesis: comparative kinetics of microsomal biotransformation. Cancer Res., *50:* 615–620, 1990.
23. De Flora, S., Bennicelli, C., Camoirano, A., Izzotti, A., Hietanen, E., Bartsch, H., Picciotto, A., and Millman, I. Metabolic activation of food hepatocarcinogens in hepatitis B virus-infected humans and animals. *In:* M. W. Pariz and J. S. Felton (eds.), Mutagens and Carcinogens in the Diet, pp. 167–182. New York: Wiley-Liss, Inc., 1990.