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Low frequency of p53 mutations observed in a diverse collection of primary hepatocellular carcinomas

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ABSTRACT **Recent studies of the p53 tumor suppressor locus (designated *TP53*) in primary hepatocellular carcinoma (PHC) have identified a high frequency of codon 249 mutations. Due to the geographic location from which the samples were obtained and the substitution observed, the mutation was suggested to be attributable to aflatoxin B_1 (AFB_1) exposure. To determine the generality of this phenomenon, we have examined PHC tissues from 107 geographically and ethnically diverse sources. The frequency of p53 gene mutations was evaluated by using PCR/restriction-digest methods, GC-clamp (G+C-rich sequence) denaturing gradient gel electrophoresis, and DNA sequencing. The mutation rate observed in tumors from high-AFB_1-exposure regions (25%) was more than double the rate observed in low-exposure regions (12%) but lower than the 50% frequency previously reported. Codon 249 mutations occurred at a much lower frequency than previously reported (2 of 107 samples examined). These results suggest that changes in DNA encoding p53 may not represent primary oncogenic effects but instead represent genetic changes related to tumor progression. High AFB_1 levels may facilitate the generation of these progressional changes, but not by inducing a specific p53 gene mutation at codon 249 as previously reported.**

Recent molecular genetic studies have implicated the p53 tumor suppressor gene (designated *TP53*) as playing a key role in the genetic etiology of primary hepatocellular carcinoma (PHC) and have elicited speculation of a link with aflatoxin B_1 (AFB_1) exposure in tumors of these types (1, 2). These studies have found that 50% of tumors show a mutation in the p53 gene ("p53 mutation"). In all cases examined, a G-to-T mutation, which resulted in an amino acid substitution, was observed. This type of mutation is consistent with nucleotide substitutions associated with AFB_1 exposure (3). Most provocative among these findings was the observation that 42% (11 of 26) of the tumors examined contained a codon 249 mutation.

In an attempt to determine the generality of this phenomena, we have examined a large collection of tumors from geographically and ethnically diverse sources. The frequency of codon 249 p53 mutations was evaluated by using the PCR/restriction digest method of Bressac *et al.* (1). Mutations at other locations in the p53 gene were assessed by using a G+C-rich sequence (GC-clamp) in conjunction with denaturing gradient gel electrophoresis (DGGE). Direct PCR sequencing was used to identify specific nucleotide substitutions detected by DGGE.

METHODS

Sample Population. For the current study, biosamples have been obtained from PHC patients from five geographically distinct locations: the mainland United States, Alaska, South Africa, Japan, and the People's Republic of China. A total of 22 samples was obtained from cases occurring within the mainland United States. These samples were obtained from a large number of sources and represent an ethnically heterogeneous population. More specifically, samples were obtained from the Fox Chase Cancer Center (Philadelphia), Hospital of the University of Pennsylvania (Philadelphia), Rancho Los Amigos Hospital (Downey, CA), the National Disease Research Interchange (sponsored by the National Cancer Institute's Cooperative Human Tissue Network), and the Liver Transplant Procurement and Distribution System (sponsored by the National Institute of Diabetes, Digestive and Kidney Disease) and represent individuals of European Caucasian, American Black, Japanese, Tonganese, and Vietnamese ethnicity. The seven Alaskan samples were obtained from the Alaska Native Medical Center of the U.S. Indian Health Service from Alaskan natives. Two of the Alaskan samples were obtained from members of a multicase PHC family (4). The samples from China were obtained from two different locations: 45 samples were obtained from the Fourth Military Hospital in Xian, and 12 were from Shanghai Medical University. South African samples were from previous studies of PHC genetics conducted at the University of California, Irvine. The Japanese samples were obtained from the Cancer Institute of Tokyo. In three instances separate tumor samples were available from individuals with recurrences of PHC. These samples could represent new primary tumors or metastatic lesions. In addition to tumor tissue, a sample of uninvolved tissue was also available from each patient. This tissue was either nontumor, surgical margin liver tissue, and/or peripheral lymphocytes. For the majority of cases, information on hepatitis B virus (HBV) infection status, α-fetoprotein level, and other demographic variables (sex, age, etc.) was available. The sample population used is summarized in Table 1. Sample acquisition procedures were approved by the Fox Chase Cancer Center Interval Review Board. Pathology on specimens was performed by the local pathologist from the source of the material.

p53 Mutation Analysis. *DNA preparation and amplification.* DNA was extracted from whole blood and liver tissue by using a modification of the procedure of Poncz *et al.* (5) as described by Murray *et al.* (6). Primers for DNA amplification were selected from the human p53 gene sequence (7) and were prepared by using an Applied Biosystems 391 oligonucleotide synthesizer. Four regions of the p53 gene were selected for analysis based on their high rate of evolutionary conservation: exon 5 (fragment 1), exon 6 (fragment 2), exon

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Abbreviations: PHC, primary hepatocarcinoma; AFB_1, aflatoxin B_1; GC clamp, G+C-rich sequence; HBV, hepatitis B virus; DGGE, denaturing gradient gel electrophoresis.

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Table 1. Results of p53 gene characterization for an ethnically and geographically diverse collection of PHC samples

Sample	Ethnicity (geographic source)*	Sex	Age, yr	Putatuve AFB_1 exposure	HBV status†	p53 gene mutation: Codon 249	p53 gene mutation: Other
922	Ch (1)	M	39	High	+	–	–
923	Ch (1)	M	?	High	+	–	–
924	Ch (1)	M	64	High	–	–	–
925	Ch (1)	F	68	High	+	–	Exon 7
926	Ch (1)	M	65	High	+	–	–
927	Ch (1)	M	37	High	–	–	–
928	Ch (1)	F	37	High	+	–	–
929	Ch (1)	M	26	High	+	–	Exon 7‡
935	Ch (1)	F	49	High	–	+	Exon 7§
936	Ch (1)	M	58	High	–	–	–
941	Ch (1)	M	65	High	–	–	–
942	Ch (1)	M	70	High	+	–	–
759	Ch (2)	M	37	Low	+	–	–
796	Ch (2)	M	61	Low	–	–	ND
798	Ch (2)	M	32	Low	+	–	ND
799	Ch (2)	M	54	Low	–	+	Exon 7
800	Ch (2)	F	14	Low	–	–	ND
801	Ch (2)	M	61	Low	+	–	ND
802	Ch (2)	M	44	Low	–	–	ND
804	Ch (2)	M	37	Low	–	–	–
807	Ch (2)	M	46	Low	–	–	ND
808	Ch (2)	M	45	Low	+	–	ND
810	Ch (2)	M	58	Low	–	–	ND
811	Ch (2)	M	66	Low	+	–	ND
814	Ch (2)	M	52	Low	+	–	ND
815	Ch (2)	M	?	Low	?	–	ND
816	Ch (2)	F	40	Low	+	–	ND
817	Ch (2)	M	48	Low	–	–	–
818	Ch (2)	M	60	Low	?	–	–
819	Ch (2)	M	59	Low	+	–	ND
820	Ch (2)	M	56	Low	+	–	–
821	Ch (2)	?	?	Low	?	–	ND
822	Ch (2)	M	53	Low	+	–	ND
823	Ch (2)	M	50	Low	+	–	Exon 6
824	Ch (2)	M	58	Low	+	–	ND
825	Ch (2)	M	67	Low	+	–	ND
826	Ch (2)	M	63	Low	+	–	ND
827	Ch (2)	M	47	Low	–	–	–
828	Ch (2)	M	56	Low	+	–	ND
829	Ch (2)	M	52	Low	–	–	–
830	Ch (2)	M	55	Low	–	–	ND
833	Ch (2)	M	53	Low	+	–	ND
834	Ch (2)	F	53	Low	+	–	ND
836	Ch (2)	?	?	Low	?	–	ND
837	Ch (2)	M	40	Low	+	–	ND
838	Ch (2)	M	61	Low	+	–	ND
839	Ch (2)	M	48	Low	+	–	ND
840	Ch (2)	M	52	Low	+	–	ND
841	Ch (2)	M	44	Low	–	–	–
842	Ch (2)	M	38	Low	+	–	ND
843	Ch (2)	M	52	Low	–	–	–
847	Ch (2)	?	?	Low	?	–	ND
848	Ch (2)	M	57	Low	–	–	ND
849	Ch (2)	M	45	Low	+	–	ND
850	Ch (2)	F	62	Low	+	–	ND
851	Ch (2)	?	?	Low	?	–	ND
852	Ch (2)	M	55	Low	–	–	ND
311	Ja (3)	M	67	Low	–	–	ND
312	Ja (3)	M	58	Low	–	–	ND
313	Ja (3)	M	64	Low	–	–	ND
314	Ja (3)	M	56	Low	–	–	ND
315	Ja (3)	M	59	Low	–	–	ND
316	Ja (3)	M	63	Low	–	–	ND
317	Ja (3)	M	68	Low	–	–	ND
318	Ja (3)	M	46	Low	–	–	ND
319	Ja (3)	M	54	Low	–	–	ND
320	Ja (3)	M	50	Low	+	–	ND
139	Ja (4)	F	?	Low	?	–	–
139	Ja (4)	F	?	Low	?	–	–
14,611	AN (7)	F	20	Low	+	–	–
14,612	AN (7)	F	16	Low	+	–	–
147	AN (7)	M	75	Low	–	–	–
148	AN (7)	M	69	Low	–	–	–
149	AN (7)	M	24	Low	+	–	–
149	AN (7)	M	26	Low	+	–	–
331	AN (7)	F	22	Low	+	–	–
508	AB (8)	M	?	High	+	–	ND
700	AB (8)	?	?	High	+	–	ND
701	AB (8)	?	?	High	+	–	ND
702	AB (8)	?	?	High	+	–	ND
704	AB (8)	?	?	High	+	–	ND
855	AB (8)	?	?	High	+	–	ND
856	AB (8)	?	?	High	+	–	ND
857	AB (8)	?	?	High	+	–	ND
858	AB (8)	?	?	High	+	–	ND
136	? (6)	M	01	?	–	–	–
140	? (6)	M	?	?	?	–	–
365	? (6)	F	43	?	–	–	–
366	? (6)	F	48	?	?	–	–
141	Ca (5)	M	45	Low	?	–	–
688	? (5)	M	31	?	?	–	–
659	Ca (5)	M	21	Low	?	–	Exon 7
348	Ca (5)	F	71	Low	?	–	Exon 7¶
349	Ca (5)	F	79	Low	?	–	ND
469	Ca (5)	M	49	Low	+	–	–
470	? (5)	F	29	?	?	–	–
150	? (4)	M	?	?	+	–	–
150	? (4)	M	?	?	+	–	–
138	? (4)	M	?	?	?	–	–
137	? (4)	F	?	?	?	–	–
509	Ca (4)	F	?	Low	?	–	Exons 6‖ and 8**
510	Ca (4)	M	75	Low	?	–	–
384	? (4)	M	?	?	?	–	–
354	Ca (4)	M	?	Low	?	–	Exon 7††
705	Ca (4)	M	50	Low	+	–	ND
703	Ja (4)	?	?	Low	+	–	ND
363	AA (9)	M	42	Low	+	–	–

ND, not done; ?, unknown; –, negative result.

*Ethnicity and source codes: Ch, Chinese; Ja, Japanese; AN, Alaskan Native; AB, African Black; Ca, Caucasian; AA, African American; 1, Shanghai Medical University, Shanghai, People's Republic of China (PRC); 2, Fourth Military Hospital, Xian, PRC; 3, Cancer Institute of Japan; 4, Philadelphia area hospitals; 5, National Disease Research Interchange (NDRI); 6, Liver Transplant Procurement and Distribution System (LTPADS); 7, Alaska Native Medical Center; 8, University of California, Irvine; 9, Ranchos Los Amigos Hospital, Downey, CA.

†HBV status was determined by testing for the presence of the HBV surface antigen; cases 139, 149, and 150 represent two independent tumors from the same individual.

‡Codon 237: ATG → ATA (Met → Ile); §Codon 249; ¶Codon 258: GAA → CAA (Gly → Gln); ‖Codon 213: CGA → CGG (Arg → Arg); **Codon 273: CGT → CAT (Arg → His); ††Codon 248: CGG → CAG (Arg → Gln).

7 (fragment 3), and exons 8 and 9 (fragment 4). The primers used and relative regions of the p53 locus amplified are summarized in Fig. 1. For the purpose of DGGE analysis a G+C-rich sequence (GC clamp) of 40 base pairs was added to the 5′ end of one primer of each set (8, 9). PCR amplification was performed with minor modifications of a previously reported protocol (9). Briefly, 500 ng of DNA was mixed with 50 pmol of each nucleotide primer and 37.5 nmol of each deoxyribonucleotide triphosphate in 100 μl of PCR buffer (67 mM Tris, pH 8.8/6.7 mM MgCl/16 mM ammonium sulfate/10 mM 2-mercaptoethanol/10% dimethyl sulfoxide) and 1.5 units of *Thermus aquaticus* (*taq*) DNA polymerase. The samples were overlaid with 50 μl of mineral oil and incubated at 94°C, 58°C, and 72°C for 30 sec in a Perkin–Elmer thermocycler for 40 cycles. The resulting amplification product (10 μl) was electrophoresed on a 1.5% agarose gel to verify amplification.

Codon 249 analysis. An aliquot of the exon 7 PCR product was digested with *Hae* III at 37°C for 2 hr; manufacturer-provided buffers and instructions were used. The digested DNA was electrophoresed on a 1.5% agarose gel for 2 hr. DNA was visualized by ethidium bromide staining, and the gel was photographed.

GC-clamp DGGE. Each PCR amplified product (≈15 μg) was analyzed on a denaturing gradient gel as described (8, 9). Briefly, an 8% polyacrylamide gel containing a linear gradient of DNA denaturants (100% denaturant is defined as 7 M urea/40% formamide) was prepared with a Hoefer Series SG gradient maker. Gradients of 60–80%, 40–70%, and 35–65% were used, respectively, for fragments 1, 2, and both 3 and 4. Appropriate gradient conditions were determined experimentally with perpendicular DGGE (10). Samples were loaded onto the gel and electrophoresed at 150 V at 60°C constant temperature for 11 hr for fragment 1, 8 hr for fragment 2, 6 hr for fragment 3, and 8 hr for fragment 4. The gel was then stained with ethidium bromide and photographed. Base changes were identified by the presence of one or more new bands or a shift in position of a band compared with nontumor tissue samples from the same individual.

Direct sequencing of PCR products. A subset of samples identified as containing a base change within an exon were sequenced to confirm the DGGE result and to identify the precise base change. Direct sequencing of the PCR-amplified product was performed by using a modification of previously described protocols (11). Briefly, the remaining PCR amplified product was run on a 1.5% preparative agarose gel to separate the amplified product from remaining primer. The fragment was recovered from the agarose by using a Costar 0.22-mm cellulose acetate centrifuge filter unit. The DNA was precipitated with ethanol and resuspended in 15 μl of the sample by using a United States Biochemical sequencing kit according to the manufacturer's instructions, except that 10 pmol of primer was used. Sequence analysis was on a 6% polyacrylamide sequencing gel.

Exon 5 Forward (3038) 5′ (GC) TTCCTCTTCCTGCAGTACTC - 3′
Exon 5 Reverse (3279) 5′ - CTGGGCAACCAGCCCTGTCGT - 3′

Exon 6 Forward (3259) 5′ (GC) ACGACAGGGCTGGTTGCCCA - 3′
Exon 6 Reverse (3446) 5′ - AGTTGCAAACCAGACCTCAG - 3′

Exon 7 Forward (3991) 5′ (GC) TCTCCTAGGTTGGCTCTGACTG - 3′
Exon 7 Reverse (4124) 5′ - GCAAGTGGCTCCTGACCTGGA - 3′

Exons 8 and 9 Forward (4437) 5′ - CCTATCCTGAGTAGTGGTAATC - 3′
Exons 8 and 9 Reverse (4768) 5′ (GC) CCCAAGACTTAGTACCTGAAG - 3′

(GC) = 5′ CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG - 3′

FIG. 1. p53 primer sequences.

RESULTS

A total of 107 tumors was examined for the codon 249 G-to-T mutation detected by *Hae* III digestion of the exon 7 PCR product (see Table 1). Tumors with mutations in this codon are not cleaved by *Hae* III. Only two of the 107 tumors demonstrated the previously described mutation (Fig. 2). One sample (number 799) was obtained from the Xian area of the People's Republic of China, an area with a relatively low AFB_1 exposure frequency (12), and the other (number 935) was from an area with endemic AFB_1 exposure (Shanghai). It is worth noting that in sample 799, the tumor appeared to be heterozygous for the codon 249 mutation. This overall frequency of codon 249 mutation is significantly lower than that observed in earlier studies ($\chi^2 = 38.79$, $P < 0.0001$). The frequency of 249 mutations in the Shanghai region is also significantly lower than in earlier reports ($\chi^2 = 4.39$, $P = 0.04$).

A subset of the tumors without codon 249 mutations from each of the ethnic groups and geographic locations was next analyzed for p53 mutations in exons 5–9 by DGGE. Exons 5–9 represent the most common locations of p53 mutations. These results are summarized in Table 1. Of the 51 tumors evaluated, 10 p53 DNA variants were observed (18%): 2 of the changes were observed in exon 6, 7 were observed in exon 7 (Fig. 3), and 1 case involved both an exon 6 and 8 change. However, the exon 6 change was also observed in the sample of uninvolved liver adjacent to the tumor. Sequencing of this change indicated that the change was an A-to-G transition in the third base position of codon 213, which does not result in an amino acid substitution. Four of the exon 7 changes have been sequenced (Fig. 4). Mutations that result in amino acid substitutions were observed in codons 237, 248,

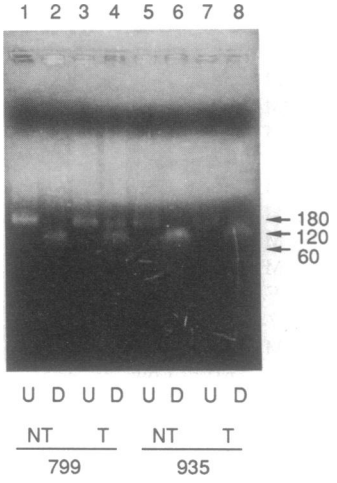

FIG. 2. PCR amplification/*Hae* III restriction digest results for the two samples 799 (lanes 1–4) and 935 (lanes 5–8) showing codon 249 mutations. For each sample, the first lane (lanes 1 and 5) shows an aliquot of amplified, undigested (U), uninvolved [nontumor (NT)] tissue. The second lane (lanes 2 and 6) shows the results of a *Hae* III restriction digest (D) from an aliquot of the same amplification. Neither sample showed an altered *Hae* III restriction site in uninvolved tissue. The next lane (lanes 3 and 7) for each sample shows amplified, undigested (U) tumor (T) DNA. The last lane (lanes 4 and 8) for each sample shows a *Hae* III restriction digest of an aliquot of amplified tumor DNA. Both samples show evidence of a mutation that removes the *Hae* III site. The presence of the digestion products also provides evidence that at least 50% of the wild-type sequence is present in the DNA sample amplified from the tumor. Sizes are shown in base pairs.

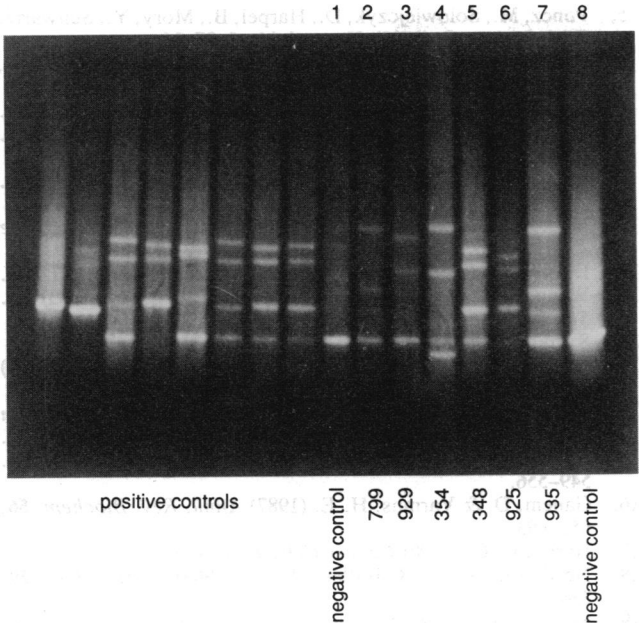

FIG. 3. PCR amplification/DGGE analysis results for a subset of samples with mutations in exon 7 of p53 DNA. Flanking the six tumor samples in lanes 2–7 are PCR products from samples containing only the wild-type p53 (negative control in lanes 1 and 8). Two alternative banding patterns are observed in lanes with p53 mutations. The first is a single homoduplex band of different mobility from the wild type. This indicates homozygosity within the sample for the p53 mutation. This pattern is observed only among the positive control samples. The second pattern consists of four bands, two of which are homoduplex products (the lower two bands) and two heteroduplex bands. Heteroduplex formation occurs as a result of the annealing of a wild-type DNA strand with the complementary mutant DNA strand in the last cycle of the PCR process in samples containing both wild-type and mutant p53 genes. Therefore, this pattern indicates heterogeneity of p53 constitution. Lanes 2–6 all show this pattern. This heterogeneity may be due to heterozygosity at the p53 locus or the contribution of wild-type p53 from surrounding nontumor tissue.

258, and 273. In three cases, a G-to-A transition was observed that would result in a substitution of isoleucine for methionine, glutamine for arginine, and histadine for arginine in codons 237, 248, and 273, respectively. In codon 258 a G-to-C change, which resulted in a substitution of glutamine for glycine, was observed.

DISCUSSION

Epidemiologic studies have identified a number of environmental factors that markedly increase risk of PHC. Foremost among these is chronic infection with the HBV (13, 14). Concordance of PHC incidence and environmental levels of the carcinogen AFB_1 led to the suggestion that AFB_1 exposure may also be an important risk factor (15–17). Two recent studies of p53 mutations in PHC have suggested a possible genetic basis for PHC and implied a mechanistic link to AFB_1 exposure (1, 2). Of 26 tumors examined, 11 were observed to have G-to-T changes in the p53 locus. More provocatively, all 11 tumors were observed to have mutations within codon 249 of the p53 locus. AFB_1 binds to G residues and commonly induces G-to-T mutations (3) and is a potent hepatocarcinogen in model systems of carcinogenesis (18). The tumors examined in these studies were obtained from regions where AFB_1 is a common food contaminant (19).

The current study finds a very different pattern of p53 DNA changes in PHC. First, only 2 in 107 tumors examined showed the codon 249 mutation. One of these tumors had a mutation pattern inconsistent with p53 gene inactivation.

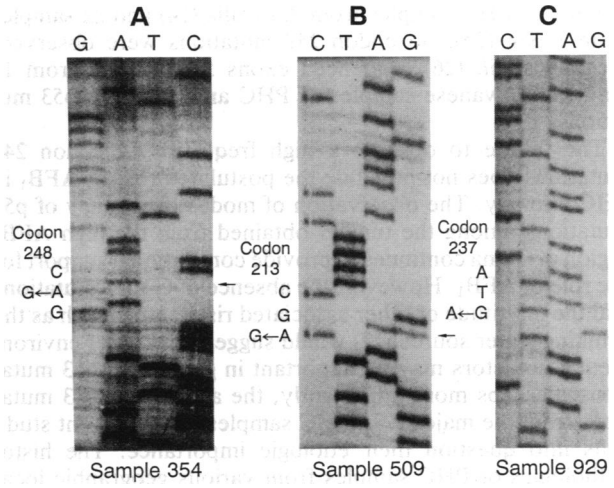

FIG. 4. Direct PCR sequencing results from three samples with p53 mutations. (*A*) Codon 248 G-to-A (arginine → glutamine) change observed in a Caucasian male of unknown HBV status. (*B*) G-to-A change in the third base of codon 213. This substitution does not result in an amino acid substitution and is found in both tumor and nontumor samples. (*C*) A-to-G change in codon 237. This mutation results in a methionine → isoleucine change and was observed in the tumor of a 26-year-old HBV-positive Chinese male.

Overall, the p53 mutation rate was also much lower than previously described, and a greater variety of mutations was identified. In the current study, 8 of 50 tumors examined by DGGE showed a mutation in exons 5–8 at the p53 locus. Finally, of the 5 samples sequenced, no G-to-T changes were observed. The substitution patterns observed here are more consistent with p53 mutation patterns observed in other cancers. It is important to note that the current sample differs from the previously reported results in two key aspects. First, while 21 samples were obtained from regions with high AFB_1 contamination (Shanghai, the People's Republic of China, and South Africa), the majority of samples were obtained from areas with low environmental levels of AFB_1. The ethnic composition of the current population was also more diverse than those in previous investigations. Among the samples obtained from regions with high environmental levels of AFB_1, only one codon 249 mutation was observed. However, the 12 tumors obtained from the Shanghai region of China examined by DGGE showed the highest frequency of p53 mutations. Of the 12 tumors, 3 showed mutations in exon 7.

Two of the samples were obtained from an Alaskan multicase family (4). A test was made that familial PHC may be due to germ-line p53 mutations in a manner similar to the Li–Fraumeni syndrome (20). No p53 mutations were observed in exons 5–8 in either sample. In three instances in addition to a sample from the original tumor, a sample from a recurrent tumor was also available. In no case did the original or the recurrent tumor show a p53 mutation.

It is unlikely that the observed lower p53 mutation rate is a consequence of low sensitivity of DGGE screening methodology. Theoretical and empiric studies of GC-clamp DGGE show a high mutation-detection efficiency (9, 21–23). More pragmatically, empiric trials conducted in a blinded fashion successfully identified 20 different p53 mutations observed in other tumor types (V.C.S., unpublished data). A subset of this panel is presented in Fig. 4 as positive controls. Moreover, both codon 249 mutations identified by *Hae* III digestion were independently identified by DGGE analysis.

The largely negative p53 mutation results observed in the current study are not without precedent. Three previous studies have not observed codon 249 mutations (24–26).

Among 16 PHC samples from Australia (24) and 22 samples from Japan (25), no codon 249 mutations were observed. Hosono *et al.* (26) sequenced exons 5 through 8 from 18 surgical Taiwanese samples of PHC and found no p53 mutations.

The failure to observe a high frequency of codon 249 mutations does not preclude the postulated role of AFB_1 in PHC etiology. The observation of modest frequency of p53 mutations among the tumors obtained from the high AFB_1 region of China continues to provide correlational support for the role of AFB_1. However, the absence of G-to-T mutations and the multitude of other associated risk factors such as the drinking water source (27) would suggest that other environmental cofactors may be important in generating p53 mutations. Perhaps more importantly, the absence of p53 mutations from the majority of PHC samples in the current study calls into question their etiologic importance. The histopathologies of PHC samples from various geographic locations have not been observed to differ. However, the observed difference in p53 mutations suggests that PHC has different underlying genetic etiologies in different regions of the world. Alternatively, the p53 mutations observed may not represent primary oncogenic effects and instead represent genetic changes due to tumor progression. AFB_1 exposure may facilitate the generation of these progressional changes.

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