p53 Tumor Suppressor Gene Mutations in Hepatocellular Carcinoma Patients in India

Sanjay Katiyar, M.Sc.[1]
Bipin C. Dash, Ph.D.[2]
Varsha Thakur, M.Sc.[2]
Raj C. Guptan, M.D.,[2]
Shiv K. Sarin, M.D.[2]
Bhudev C. Das, Ph.D.[1]

[1] Division of Molecular Oncology, Institute of Cytology and Preventive Oncology, Maulana Azad Medical College Campus, Bahadur Shah Zafar Marg, New Delhi, India.

[2] Department of Gastroentrology, G.B.Pant Hospital, New Delhi, India.

Current address for Sanjay Katiyar, M.Sc.: Department of Urology, University of Tennessee, F 215 Coleman, 956 Court Avenue, Memphis, TN 38163.

Address for reprints: Bhudev C. Das, Ph.D., Division of Molecular Oncology, Institute of Cytology and Preventive Oncology, Maulana Azad Medical College Campus, Bahadur Shah Zafar Marg, New Delhi 110002, India.

Received June 25, 1999; revisions received November 29, 1999; accepted November 29, 1999.

BACKGROUND. Specific mutations of the p53 tumor suppressor gene in hepatocellular carcinoma (HCC) have been reported from several parts of the world, but to the authors' knowledge to date the status of this gene has not been studied in HCC patients in India, where HCC is one of the major cancers and the frequency of chronic hepatitis B virus (HBV) as well as hepatitis C virus (HCV) infection and exposure to dietary aflatoxin B_1 is very high. The most frequent mutation of the p53 gene in HCC is an AGG^{Arg} to AGT^{Ser} missense mutation at codon 249 of exon 7.

METHODS. Liver biopsy specimens from 21 HCC patients and 10 healthy controls were obtained through surgery or by needle biopsy technique. Phenol-chloroform-extracted DNA specimens were employed for the detection of HBV infection and p53 gene mutations. Nucleotide mutations of exons 4–9 of the p53 gene were analyzed by polymerase chain reaction (PCR), single strand confirmation polymorphism, and direct sequencing. Third-generation sandwich enzyme-linked immunosorbent assay (ELISA) was used for the serologic detection of HBV and HCV infection.

RESULTS. Analysis of exons 4–9 of the p53 gene revealed only 3 mutations (3 of 21 specimens, 14.28%; 95% confidence interval, −0.7–29.3), 2 mutations at codon 249 showing G→T transversions, and 1 mutation (4.7%) at codon 250 with a C→T transition. The base substitutions at the third base of codon 249 resulted in a missense mutation leading to a change in amino acid from arginine to serine whereas at codon 250 it caused a change from proline to serine. Dot blot hybridization and PCR for HBV DNA from HCCs revealed 58.8% (10 of 17 specimens) and 90.47% (19 of 21 specimens), positivity, respectively. ELISA for hepatitis B virus surface antigen in serum showed a positivity of 71.42% (15 of 21 specimens), but there was only 40% positivity (8 of 20 specimens) for hepatitis B virus envelope antigen whereas 6 of 17 patients (35.29%) showed the presence of antibodies against hepatitis B virus envelope protein. No patient was found to be positive for the HCV antibody.

CONCLUSIONS. The very low frequency of p53 mutations and the extremely high frequency of HBV infection (> 90%) in HCC indicate that the mutations in the p53 gene frequently found in HCC reported from different endemic areas of the world may not play a direct role in the development of HCC in India. HBV infection and, possibly, exposure to the dietary aflatoxin B_1 appear to play major roles in the molecular pathogenesis of HCC in India. ***Cancer* 2000;88:1565–73.**
© 2000 American Cancer Society.

KEYWORDS: p53 mutation, hepatocellular carcinoma, hepatitis B virus, polymerase chain reaction (PCR), single strand conformation polymorphism (SSCP), aflatoxin B1, hepatitis B surface antigen (HbsAg).

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers in the world, particularly in Southeast Asia and Africa including Japan.[1–5] In India, human hepatocellular carcinoma is one of the

major cancers in both genders along with cervical carcinoma in women. Epidemiologic studies have established that chronic infection of hepatitis B virus (HBV) and to a certain extent hepatitis C virus (HCV) and exposure to dietary aflatoxin B_1 and intake of alcoholic beverages are important risk factors for the development of HCCs.[6–12] However, the precise molecular mechanisms of liver carcinogenesis still are not clearly understood.

Recently, specific mutations of p53 tumor suppressor gene have strongly been implicated in a variety of human cancers, and the majority of them is located in the highly conserved regions of the gene, which are involved in DNA binding and transcriptional activation.[13] Wild-type p53 gene acts as a tumor suppressor as it plays a central role in regulating transcriptional activation of crucial growth regulatory genes that control cell cycle progression and cell division. Mutations of the p53 gene abrogate its normal function leading to genomic instability and loss of growth control.[14] The loss of heterozygosity and abnormalities in structure and function of the p53 gene are frequent in HCCs. Specific base substitutions particularly G → T transversion at third position of codon 249 (AGG) of the p53 gene results in replacement of arginine with serine. This is the most common mutational "hot spot" found in HCCs in more than 50% of cases reported from different geographic regions, such as, China and southern Africa, where dietary aflatoxins are suspected to be the major liver specific carcinogens that cause p53 mutation.[5,13,15–18] Indeed, in vitro experiments have shown that aflatoxin B_1 can induce G:C to T:A transversion at codon 249.[19,20] Even the noncancerous liver tissues from these high aflatoxin exposed areas were found to harbor mutations in codon 249.[21] In contrast, absence or low aflatoxin exposures in countries like Japan, the United States, and Australia showed such mutational hot spots at codon 249 in only 0 to 26% of HCCs.[13,22–24] Recently, it also has been reported that HBV or HCV infection alone can induce the carcinogenic pathways causing p53 abnormalities other than codon 249.[18]

Hepatitis B virus infections are seen in 3–7% of the general population in India[25,26] in which dietary Aspergillus contamination is also very high.[27,28] Therefore, for a vast country such as India where both HBV infection and aflatoxin contamination of food are most prevalent, it is interesting to look for abnormalities of p53 gene in general and its codon 249 in particular because there is to our knowledge no information on the status of p53 gene mutation in HCC patients in India. Here, we report that the frequency of p53 mutation is considerably low, but the prevalence of hepatitis B virus infection is exclusively high in Indian patients with hepatocellular carcinoma.

MATERIALS AND METHODS

Patients and Tissue Specimens

Liver biopsy specimens from 21 hepatocellular carcinoma patients and 10 controls were obtained at surgery (15 patients) or by needle biopsy technique (6 patients) from Department of Gastroentrology, G.B. Pant Hospital in New Delhi. The patients included 19 males and 2 females, age 10–68 years, all belonging to high aflatoxin exposure regions of northern India. The control subjects were between the age of 29 and 65 years (male:female ratio, 5:5), and all had undergone abdominal surgery for unrelated causes (gallstone disease; 4 patients, intestinal obstruction; 3 patients, peptic ulcer; 3 patients). The 10 control patients were initially tested for liver disease but proved to have normal livers after histologic analysis.

No patient had undergone chemotherapy before tumor excision. Histologic tumor grading was done according to Edmondson and Steiner.[29] Tissue biopsies were stored in −70 °C until analysis. Informed consent was obtained from all the patients before biopsies were conducted.

Extraction of Genomic DNA

All HCC specimens included in the study were examined under a dissecting microscope to exclude normal tissue and to obtain only the tumor tissues for extraction of genomic DNA. High molecular weight genomic DNA was isolated from HCC and control liver tissues by standard proteinase K digestion and phenol-chloroform extraction as described previously[30]

Polymerase Chain Reaction for *p53* and HBV

DNA (100–200 ng) was amplified by polymerase chain reaction (PCR) by using DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). Oligonucleotide primer sequences used to amplify p53 gene exons 4–9 and HBV core gene (Table 1) were designed and synthesised in an ABI 381A DNA Synthesiser (Applied Biosystems, Foster City, CA) and purified by a reverse phase high-performance liquid chromatography (Schmadzu, Kyoto, Japan). Polymerase chain reaction amplification was performed in 25 μL reaction volume containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 125 μM of each dNTP (dATP, dCTP, dGTP, dTTP), 5 pmoles of each oligonucleotide primer, and 0.5 U Taq DNA polymerase (Perkin-Elmer Cetus). The temperature profile followed for amplification of p53 exons was initial denaturation at 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 seconds, 55 °C for 20 seconds, and 72 °C for 20 seconds, and a final

TABLE 1
Oligonucleotide Primer Sequences Used for PCR Amplification of Different Exons of p53 Gene and HBV DNA

Primer	Nucleotide position in genomic DNA	Amplified fragment (bp)	Primer sequences[a]
p53 exon 4	12041–12063 12279–12299	259	(+)5′-AAT GGA TGA TTT GAT GCT GTC CC-3′ (−)5′-CGT GCA AGT CAC AGA CTT GGC-3′
Exon 5	13055–13074 13219–13238	184	(+)5′-TAC TCC CCT GCC CTC AAC AA-3′ (−)5′-CAT CGC TAT CTG AGC AGC GC-3′
p53 exon 6	13320–13339 13413–13432	113	(+)5′-GTC TGG CCC CTC CTC AGC AT-3′ (−)5′-CTC AGG CGG CTC ATA GGG CA-3′
p53 exon 7	13992–14012 14096–14116	125	(+)5′-TCT CCT AGG TTG GCT CTG ACT-3′ (−)5′-TCC TGA CCT GGA GTC TTC CAG-3′
p53 exon 8	14452–14471 14519–14538	87	(+)5′-TGG TAA TCT ACT GGG ACG GA-3′ (−)5′-CGG AGA TTC TCT TCC TCT GT-3′
p53 exon 9	14666–14685 14750–14769	104	(+)5′-TTG CCT CTT TCC TAG CAC TG-3′ (−)5′-CCC AAG ACT TAG TAC CTG AA-3′
HBV	2269–2286 2415–2398	147	(+)5′-GGA GTG TGG ATT CGC ACT-3′ (−)5′-TGA GAT CTT CTG CGA CGC-3′

PCR: polymerase chain reaction; HBV: hepatitis B virus; bp: base pair.
[a] (+): sense primer; (−): antisense primer.

extended synthesis at 72 °C was performed for 5 minutes. For amplification of HBV DNA sequences, initial denaturation at 95 °C was for 5 minutes followed by 40 cycles, each consisting of denaturing at 95 °C for 30 seconds, annealing at 51 °C for 50 seconds and extension at 72 °C for 50 seconds, but a final synthesis at 72 °C was extended for 10 minutes. The amplified products were visualized on an ethidium bromide-stained 3% NuSieve agarose (FMC Bioproducts, Rockland, ME) gel under a ultraviolet light transilluminator.

*Hae*III Digestion of p53 Exon 7 Amplimers

For analysis of p53 gene mutation, particularly at codon 249, Hae III digestion of PCR products of exon 7 was performed. The 249th codon in the p53 gene is a mutation hot spot specific for HCCs. A mutational change either at the second or at the third nucleotide in this codon often results in abolition of restriction site for Hae III.[31]

After amplification of exon 7 in tumor DNA by PCR and electrophoresis of 125-base pair (bp) PCR products, the bands were excised from the gel and eluted by centrifugation or by diethyl amino-ethyl (DEAE) ion exchange membrane method. DNA was precipitated overnight at −20 °C by addition of one-tenth volume of 3 M sodium acetate (pH 5.0) and 2.5 volumes of chilled ethanol. After centrifugation, the pellet was resuspended in 10 μL tris-EDTA (TE) buffer and digested in 20 μL reaction volume containing 10 U of Hae III enzyme (Boehringer Mannheim, Mannheim, Germany). The resultant digests were electrophoresed in ethidium bromide-stained 3% NuSieve agarose (FMC Bioproducts) gels and observed for abolition of restriction site.

Single Strand Conformation Polymorphism

Single strand conformation polymorphism (SSCP) analysis was performed by radiolabeling the PCR products for an additional 15 cycles by using 1μCi of α^{32}P dCTP (Bhabha Atomic Research Centre, Trombay, Bombay, India) and cycling each at 95 °C for 30 seconds, 55 °C for 20 seconds, and 72 °C for 20 seconds. One microliter of the labeled product was diluted with 10 volumes of denaturing solution containing 95% formamide, 20 mM EDTA (pH 8.0), 0.05% xylene cyanol, and 0.05% bromophenol blue and heat-denatured for 5 minutes at 95 °C and chilled on ice for 5 minutes. Three microliters of this product was subjected to nondenaturing electrophoresis in a 6% polyacrylamide sequencing gel run in 0.5 × tris-borate-EDTA (TBE). The gel was run for 12 hours at 200 V in Base Ace sequencing gel apparatus (Stratagene GmbH, Heidelberg, Germany) at 17 ± 1 °C, dried, and exposed to X-ray film at −70 °C. Alterations in electrophoretic mobility shift in single strand DNA bands were analyzed in comparison to that of normal controls.

TABLE 2
Age, Gender, Histologic Grading, HBV DNA Positivity, and Its Serologic Profile and p53 Mutations of HCC Patients

Patient no.	Gender	Age (yrs)	Origin of specimen	Stage	HBV DNA		Assays in serum				p53 mutations
					Dot blot	PCR	HBsAg	HBeAg	Anti-HBe	HCV	
1	F	48	Surgery	I	−	+	+	+	−	−	−
2	M	10	Surgery	I	+	+	+	−	+	−	−
3	M	55	Surgery	I	−	+	−	−	−	−	−
4	M	44	Surgery	I	+	+	+	−	+	−	−
5	M	60	Surgery	II	−	−	+	+	−	−	−
6	F	45	Surgery	II	−	−	+	+	−	−	−
7	M	28	Surgery	II	−	+	−	+	−	−	−
8	M	28	Surgery	II	+	+	+	+	−	−	−
9	M	55	Surgery	III	+	+	+	+	+	−	+
10	M	40	Surgery	I	+	+	+	+	+	−	−
11	M	67	Surgery	III	+	+	+	−	+	−	−
12	M	54	Needle	I	−	+	+	−	−	−	−
13	M	45	Needle	I	+	+	+	−	+	−	−
14	M	40	Needle	I	ND	+	−	−	−	−	−
15	M	45	Surgery	III	−	+	−	−	ND	−	+
16	M	53	Surgery	I	+	+	+	−	−	−	+
17	M	55	Needle	I	ND	+	+	−	ND	−	−
18	M	68	Surgery	II	ND	+	−	ND	ND	−	−
19	M	52	Surgery	II	ND	+	−	−	ND	−	−
20	M	42	Needle	I	+	+	+	+	−	−	−
21	M	45	Needle	II	+	+	+	−	−	−	−
Total					10/17 (58.88)	19/21 (90.47)	15/21 (71.42)	8/20 (40)	6/17 (35.29)	0	3/21 (14.28) (95% CI, −0.7–29.3)

HBV: hepatitis B virus; HCC: hepatocellular carcinoma; PCR: polymerase chain reaction; HBsAg: hepatitis B virus surface antigen; HBeAg: hepatitis B envelope antigen; Anti-HBe: antibodies against hepatitis B virus envelope antigen; HCV: hepatitis C virus; F: female; M: male; ND: not done.

DNA Sequencing

DNA sequencing was performed on those PCR products that showed altered band mobility in SSCP analysis along with more than 50% of other HCC DNA samples. Fresh PCR products were prepared by PCR amplification of original tumor DNA and purified by using Qiagen PCR purification columns (Qiagen GmbH, Hilden, Germany). Nucleotide sequences were determined by using a modification of the Sanger et al. dideoxy chain termination method[32] with Sequenase version 2.0 (Amersham/United States Biochemicals, Cleveland, OH) and α^{32}P dATP (Bhabha Atomic Research Centre). For sequencing with Sequenase, the PCR products were first cloned in Promega Easy A-T Cloning System (Promega, Madison, WI). When the amount of template DNA was much less, we used double-stranded cycle sequencing method using Cyclist exo$^-$ Pfu sequencing kit (Stratagene GmbH).

Serologic Enzyme-Linked Immunosorbent Assay Tests

Serologic enzyme-linked immunosorbent assay (ELISA) was performed in serum samples collected from the HCC patients. Serologic tests for serum HBsAg, hepatitis B virus envelope antigen (HBeAg), and antibodies against hepatitis B virus envelope antigen (Anti-HBe) were performed using sandwich ELISA technique (Organon Teknika, Boxtel, The Netherlands) and anti-HCV by third generation sandwich ELISA (United Biomedical Inc., Hauppauge, NY) for detection of hepatitis B and hepatitis C virus infection, respectively.

RESULTS

The age, gender, histopathologic grading, and results of viral DNA positivity and serologic ELISA for 21 hepatocellular carcinoma patients are presented in Table 2. Histologic grading[29] done on the basis of nucleic acid atypia, chromatin content, and the size or multiplicity of the nucleus of tumor tissue biopsies identified 11 cases in Stage I, 7 to Stage II, and 3 to Stage III. Dot blot hybridization of genomic DNA extracted from hepatocellular carcinomas with HBV specific probe (3.2 kilobase pairs)[33] revealed 58.8% (10 of 17) whereas the same samples when subjected to PCR for HBV DNA revealed 90.47 % (19 of 21) positivity (Fig. 1). The PCR products also were reconfirmed by Southern blot hybridization procedure with certain modifications[34,35] by using HBV DNA probe (figure

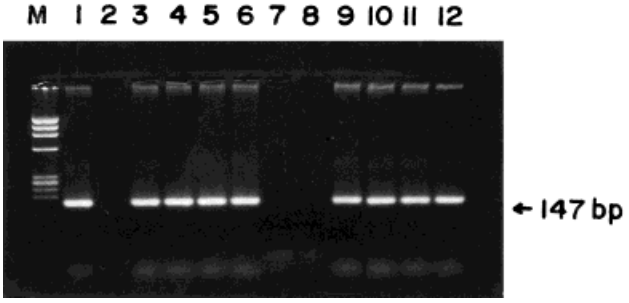

FIGURE 1. Polymerase chain reaction amplification of hepatitis B virus (HBV) DNA showing amplimer size of 147 base pair (bp) (arrow) is shown. Lane 1: positive control of HBV plasmid DNA; Lane 2: negative control (placental DNA); Lanes 3–12: represent results of amplification from hepatocellular carcinoma DNA samples of which Lanes 7 and 8 were negative for HBV DNA. M: Hae III digested ΦX174 DNA molecular weight marker.

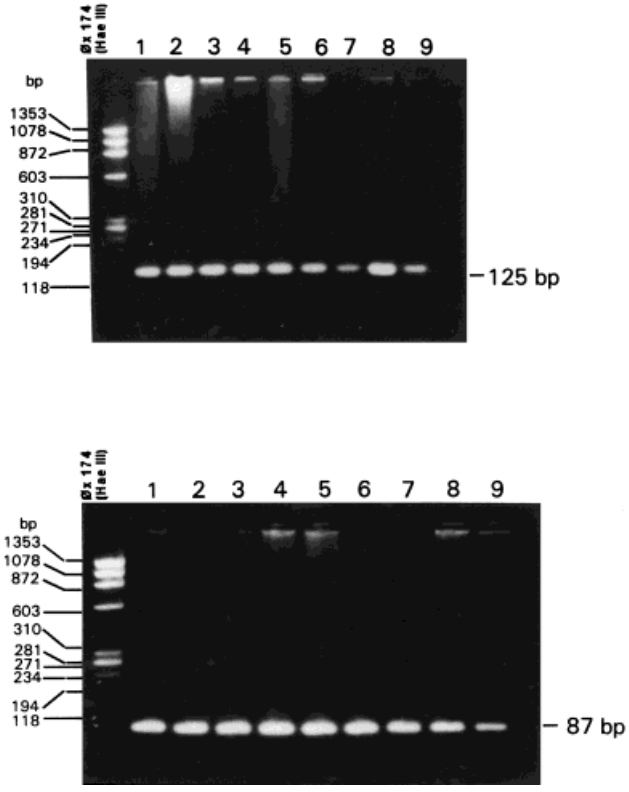

FIGURE 2. Polymerase chain reaction amplification of exons 7 and 8 of p53 gene in hepatocellular carcinoma (HCC) and control liver biopsy DNA samples showing (a) amplimer size of 125 base pair (bp) in exon 7 and (b) 87 bp in exon 8. In both the panels, Lanes 1 and 2 are control DNA samples whereas Lanes 3–9 are HCC DNA samples that show specific amplification of desired size of amplimers for exons 7 and 8 of the p53 gene. M: HaeIII digested specific ΦX174 DNA molecular weight marker.

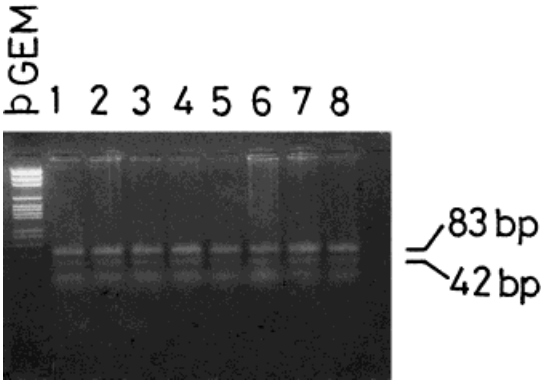

FIGURE 3: Hae III restriction enzyme digestions of p53 polymerase chain reaction products of p53 exon 7 amplimer (125 base pair [bp]) that show two distinct bands, 83 bp and 42 bp, respectively, indicating absence of a 249 codon mutation. pGEM: DNA molecular weight marker.

not shown). To our knowledge, this is a first PCR estimate of HBV frequency in HCC patients in India. None of the ten normal controls showed presence of HBV DNA by either dot blot or PCR. ELISA for hepatitis B virus surface antigen (HBsAg) in serum showed 71.42% (15 of 21) positivity, but there was only 40% (8 of 20) positivity for hepatitis B virus envelope antigen (HBeAg) whereas 6 out of 17 patients (35.29%) showed presence of Anti-HBe. No patient was found to be positive for HCV antibody.

PCR amplification of exons 4–9 of the p53 gene resulted in amplimers of sizes 259, 184, 113, 125, 87, and 104 bp, respectively, in all tumor biopsies. Figure 2a,b shows a representative PCR amplification of the exons 7 and 8, respectively. To check the mutation specifically at codon 249, Hae III restriction digestion of PCR products of exon 7 was performed. Only one sample showed absence of digestion indicating presence of p53 mutation at its codon 249 that also was confirmed by DNA sequencing. All other amplimers were completely digested with the Hae III restriction enzyme thereby producing the digestion products of 83 and 42 bp (Fig. 3).

Analyses of all the above p53 exons by SSCP revealed presence of only three mutations (14.28%, 3 of 21; 95% CI, −0.7–29.3) in exon 7 (Table 3) as indicated by a shift in electrophoretic mobility in single stranded DNA bands on 6% polyacrylamide nondenaturing gels (Fig. 4). In addition, the samples showing doubtful mobility shifts in SSCP also were sequenced. All of them except three showed altered DNA sequence when compared with the sequence obtained from DNA sequencing of normal liver tissue specimens. Interestingly, no mutation could be detected in exons 4–6 and 8, and 9 of the p53 gene. Nucleotide sequencing of the PCR products showing altered electro-

TABLE 3
Mutations of the p53 Gene as Revealed by PCR-SSCP and Sequencing in Hepatocellular Carcinomas in India

	Mutation detected at				
Patient no.	Exon	Codon	Nucleotide change	Amino acid change	Type of mutation
9	7	249	AGG→AGT	Arg→Ser	Transversion
15	7	249	AGG→AGT	Arg→Ser	Transversion
16	7	250	CCC→TCC	Pro→Ser	Transition

PCR: polymerase chain reaction; SSCP: single strand conformation polymorphism.

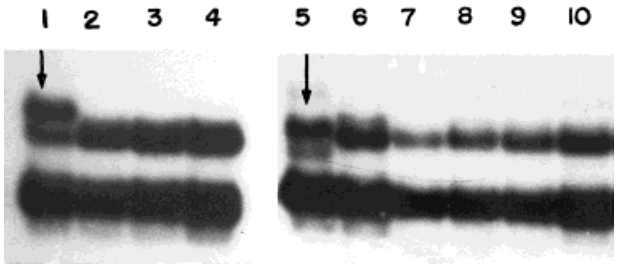

FIGURE 4: Polymerase chain reaction-single strand conformation polymorphism analysis of p53 gene mutation in hepatocellular carcinoma patients. PCR amplified 125-base pair fragments that encompass exon 7 of the p53 gene were electrophoresed on 6% polyacrylamide nondenaturing sequencing gels. Cases positive for mutation show different mobility shifts (arrows). Of the ten samples (Lanes 1–10), abnormal mobility shift was observed in Lanes 1 and 5.

phoretic mobility in SSCP along with normal controls (Fig. 5a) showed transversion of the third base at codon 249 from AGG to AGT (Fig. 5c) in two samples whereas at codon 250, it was a transition of CCC to TCC (Fig. 5b). These base substitutions at the third base (G→T) of the codon 249 resulted in a missense mutation leading to change of amino acid, arginine to serine, whereas at codon 250, it resulted in a change of proline to serine (see Table 3).

Of the two patients who showed mutations in codon 249, one (Patient 9) was shown to be positive for HBsAg as well as HBeAg by ELISA and also shown to be positive for HBV DNA by dot blot hybridization as well as PCR whereas the second patient (patient 15) was negative for HBsAg, HBeAg, and Anti-HBeAg but positive for HBV DNA by PCR. Both these patients were in late stage of cancer (Stage III). The lone case who showed mutation at codon 250 was found to be in Stage I and was positive for both HBsAg and HBV DNA.

DISCUSSION

p53 gene mutation is one of the most common events in human carcinogenesis, and approximately 90% of its mutations are of the missense type.[36] The profile of p53 gene mutations differs in different types and grades of liver carcinoma that have been associated with several other factors such as infection of HBV or HCV, exposure to aflatoxins, and genetic constitution or ethinicity of a population including certain cultural habits.[13,22] p53 mutations also have been reported to differ in HCCs from different geographic regions. It has been demonstrated that the most common mutation in HCCs is at codon 249, which is causally related to high aflatoxin B_1 exposure.[15,37] G:C to T:A transversions are the most common base substitutions observed in bacteria, in HCC patients and in in vitro experiments after exposure to aflatoxin B_1.[21,38] So far no analysis of p53 mutations in HCC patients in India has been reported. In the current study, analysis of 21 HCCs revealed a very low frequency (9.5%; 95% CI, −3.0–22.1) of p53 gene mutations at codon 249, the most common mutational hot spot in HCCs. A low frequency of p53 mutations involving codon 249 also have been reported in Germany,[23] Taiwan,[39] and Thailand,[40] but the rate of mutation is certainly higher than what has been observed in India. Interestingly, no mutation could be detected in any of the other highly conserved exons (4–9) except only in exon 7, which has been found to be mutated in only 3 patients (14.2%; 95% CI, −0.7–29.3) , a prevalence that is much lower than that reported from other low-prevalence countries.[15,16,24] This specific 249 codon mutation was also reconfirmed by the Hae III restriction enzyme digestion test,[31] which showed similar results. It is noteworthy because in 14 most common food commodities used in the states of India including the northern states from where the patients lived, the level of aflatoxin B_1 exceeded the Prevention of Food Adulteration limit of 30 $\mu g/Kg^{-}$.[127,28] This is in contrast to the observation that southern Africa and Qidong province in China where aflatoxin exposure is high, the rate of p53 gene mutation is also very high at the third base of codon 249. Several other authors[16,17] have achieved similar results. But in other parts of the world such as Australia, Japan, Germany, North Korea, The United States, and France where the dietary aflatoxin

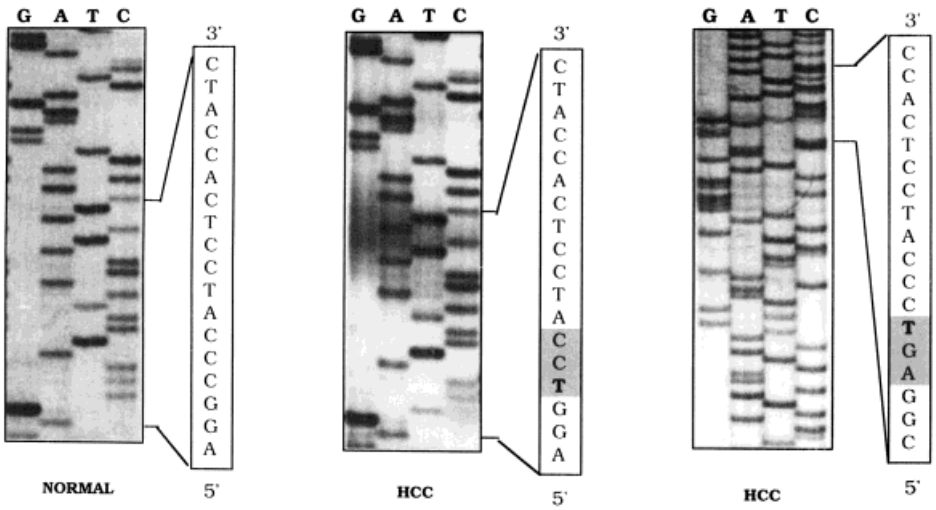

FIGURE 5. Direct DNA sequencing of polymerase chain reaction amplified fragments (125 base pair) of exon 7 of the p53 gene in hepatocellular carcinoma that showed mobility shift in single strand conformation polymorphism (lanes 1 and 7 in Fig. 2). A CCC to TCC transition was found at codon 250 (b) whereas a AGG to AGT transversion was observed in codon 249 (c). DNA of control patient (a) shows normal DNA sequence pattern of exon 7 of the p53 gene.

content is very low or nil, the mutation at codon 249 is also found to be rare or absent.[41] Although in places such as Beijing where exposure to aflatoxin is low, there is absence of mutation at codon 249, but a high rate of mutations (56%) at other codons was observed. However, this has not been observed in the current study that indicates that factors other than AFB or AFB-induced p53 mutations may be responsible for development of HCCs in India.

Although no particular etiologic agent has been identified for the development of hepatocellular carcinoma, infection and integration of HBV into the host cell genome[42] and partly also infection of HCV are considered to be the prime causative factors for HCCs in different parts of the world.[43] In the current study, although no patient was found to be infected with HCV, more than 90% of HCCs (19 out of 21) were positive for HBV DNA. The serologic detection of HBsAg, which is considered to be the definitive marker of an active viral replication and infectivity, also revealed more than 70% positivity (see Table 2). This is an extremely high prevalence of HBV ever reported for HCCs from other parts of the world.[44,45] The three patients who carried p53 mutations had been or were infected with HBV. These findings suggest that infection with HBV is the major etiologic agent, and possibly an interaction between HBV and aflatoxin B_1 could be responsible for the development of hepatocellular carcinoma in India. Furthermore, it has been suggested that HBV integration in the host cell genome may lead to interruption of growth regulatory genes and integration-mediated chromosome losses or specific allele losses, which may affect p53 gene function and viral transactivation.[12,46,47] It is also suggested that HBV-induced inflammatory process may influence selective 249 codon mutation.[48] Recently, it has been demonstrated that the hepatitis B virus transcriptional transactivating protein, HBX, which is essential for viral infection, is also implicated in liver carcinogenesis. HBX can bind to p53 protein[49–51] and inhibits sequence specific DNA binding and p53-mediated transcriptional transactivation activities. As p53 gets inactivated by binding with oncogene products of other DNA tumor viruses.[52] HBX protein also can bind with p53 to inactivate it, and this may facilitate hepatic damage by carcinogenic agents. It has been shown further that by activating the binding of the specific RNA polymerase II transcription factor, AP-1,[53,54] the hepatitis B virus protein HBX stimulates Ras-GTP complex and activates the Ras-Raf mitogen activated protein kinase signal transduction pathways, which may influence liver cell proliferation.[49]

Occurrence of a low frequency of p53 mutation at codon 249 in Indian patients may also be attributed to other factors such as ethinic variations in susceptibility to aflatoxin B_1. Aflatoxin B_1 is metabolized to its carcinogenic forms by a group of enzymes called cytochrome P_{450}, the level of which may vary in different populations.[55] In addition, there exists polymorphisms and ethinic variations in carcinogen detoxifying enzymes such as glutathione S-transferase mu 1 and epoxide hydrolase, which play an important role in detoxification of aflatoxins in humans.[56,57]

Because carcinogenesis is a multistep process and because the exact role of individual carcinogenic agent is not clear, the interaction of host cell factors along with viral and chemical carcinogens appears to play a key role in the development of hepatocellular carcinoma in humans. Furthermore, the use of PCR provides an effective complementation to serologic ELISA for a reliable estimate of HBV infection.

REFERENCES

1. Van Rensberg SJ, Cook-Mozaffari P, Van Schalkwyk DJ, Van Der Watt JJ, Vincent TJ, Purchase IF. Hepatocellular carcinoma and dietary aflatoxin in Mozambique and Transkei. Br J Cancer 1985;51:713–26.
2. Okuda K, Fujimoto I,Urano Y. Changing incidence of hepatocellular carcinoma in Japan. Cancer Res 1987;47:4967–72.
3. Di Bisceglie AM, Rustgi VK, Hoofnagle JH, Dusheiko GM, Lortze M. Hepatocellular carcinoma. Ann Intern Med 1988; 108:390–401.
4. Parkin DM, Laara E, Muir CS. Estimates of the worldwide frequency of sixteen major cancers in 1980. Int J Cancer 1988;41:184–97.
5. Murakami Y, Hayashi K, Hirohashi S, Sekiya T. Aberrations of the tumour suppressor p53 and retinoblastoma genes in human hepatocellular carcinoma. Cancer Res 1991;51: 5520–5.
6. Lutwick LI. Relation between aflatoxins and hepatitis-B virus and hepatocellular carcinoma. Lancet 1979;i:755–7.
7. Kew MC. The hepatitis-B virus and hepatocellular carcinoma. Semin Liver Dis 1981;1:59–67.
8. Yeh FS, Yu MC, Mo CC, Luo S, Ton MJ, Henderson BE. Hepatitis B virus, aflatoxins and hepatocellular carcinoma in southern Guangxi, China. Cancer Res 1989;49:2506–9.
9. Harris CC. Chemical and physical carcinogenesis advances and perspectives for the 1990s. Cancer Res 1991;51(Suppl): 5023s–44s.
10. Tabor E. Tumour suppressor genes, growth factor genes and oncogenes in hepatitis B virus-associated hepatocellular carcinoma. J Med Virol 1994;42:357–65.
11. Wands JR, Blum HE. Primary hepatocellular carcinoma. N Engl J Med 1991;325:729–31.
12. Soini Y, Chia SC, Bennett WP, Groopman JD, Wang JS, De Benedetti VMG, et al. An aflatoxin-associated mutational hotpsot at codon 249 in the p53 tumor suppressor gene occurs in hepatocellular carcinomas from Mexico. Carcinogenesis 1996;17:1007–12.
13. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumour suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994;55: 4855–78.
14. Lane DP. p53, gaurdian of the genome. Nature 1992;358: 15–6.
15. Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa. Nature 1991;350:429–31.
16. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in hepatocellular carcinomas. Nature 1991;350:427–8.
17. Oztruk M. p53 mutation in hepatocellular carcnoma after aflatoxin exposure. Lancet 1991;338:1356–9.
18. Teramoto T, Satonaka K, Kitazawa S, Fujimori T, Hayashi K, Maeda S. p53 gene abnormalities are closely related to hepatoviral infections and occur at a late stage of hepatocarcinogenesis. Cancer Res 1994;54:231–5.
19. Aguilar F, Hussain P, Cerutti P. Aflatoxin B_1 induces the transversions of G to T in codon 249 of the p53 tumour suppressor gene in hepatocytes. Proc Natl Acad Sci USA 1993;90:8586–90.
20. Foster PL, Eisenstadt E, Miller JH. Base substitution mutation induced by metabolically activated aflatoxin B_1. Proc Natl Acad Sci USA 1983;80:2695–8.
21. Aguliar F, Harris CC, Sun T, Hollstein M, Cerutti P. Geographic variation of p53 mutational profile in nonmalignant human liver. Science 1994;264:1317–9.
22. Oda T, Tsuda H, Scarpa A, Sakomoto M, Hirohashi S. p53 gene mutation spectrum in hepatocellular carcinoma. Cancer Res 1992;52:6358–64.
23. Kress S, Jahn UR, Bannasch P, Schwarz M. p53 mutations in hapatocellular carcinoma from Germany. Cancer Res 1992; 52:3220–3.
24. Kazachkov Y, Khaoustov V, Yoffe B, Solomon H, Klintmalm GBG, Tabor E. p53 abnormalities in hepatocellular carcinoma from United States patients: analysis of all 11 exons. Carcinogenesis 1996;17:2207–12.
25. Sarin SK, Chari S, Sundarum KR, Ahuja RK, Anand BS, Broor SL. Young v/s adult cirrhotic colon: a perspective comparitive analysis of the clinical profile, natural cause at survival. Gut 1988;29:101–7.
26. Sarin SK, Guptan RC, Banerjee K, Khandekar P. 1993 Prevelance of hepatitis B and C virus infection in patients with non-alcoholic liver disease in India. Ind J Gastroentrol 1988; 12A:107–10.
27. The Prevention Food Adulteration Act 1954 (370 of 1954 as amended up to act 70 of 1986). Lucknow, India: Estern Book Publishing Co., 69–69F.
28. Bilgrami KS. Scenario of aflatoxin contamination in India: surveillance, prevention and control of food contaminants. In: Toteja GH, Diwakar S, Saxena BN, editors. Proceedings of National Symposium. New Delhi: Indian Council of Medical Research, 1996:107–18.
29. Edmondson HA, Steiner PE. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. Cancer 1954; 7:462–503.
30. Das BC, Sharma JK, Gopalkrishna V, Das DK, Singh V, Gissmann L, et al. A high frequency of human papillomavirus DNA sequences in cervical carcinomas of Indian women as revealed by Southern blot hybridization and polymerase chain reaction. J Med Virol 1992;36:239–45.
31. Hayward NK, Walker GJ. Hepatocellular carcinoma mutation. Nature 1991;352:64.
32. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain termination inhbitors. Proc Natl Acad Sci USA 1977; 74:5433–67.
33. Lugassy C, Bernuau J, Theirs V Krosgaard K, Degott C, Wantzin P, et al. Sequences of hepatitis B virus DNA in the serum and liver of patients with acute benign and fulminant hepatitis. J Infect Dis 1987;155:64–9.
34. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 1975; 98:503–18.
35. Das BC, Sharma JK, Gopalkrishna V, Luthra UK. Analysis by polymerase chain reaction of the physical state of human papillomavirus type 16 in cervical preneoplastic and neoplastic lesions. J Gen Virol 1992;73:2327–36.
36. Harris CC. p53: at crossroads of molecular carcinogenesis and risk assessment. Science 1993;262:1980–1.
37. Fujimoto Y, Lori LH, Lin-di L, Wirth PJ, Thorgeirsson SS. Low frequency of p53 gene mutation in tumours induced by aflatoxin B_1 in nonhuman primates. Cancer Res 1992;52: 1044–6.
38. Vesey DA, Hayward NK, Cooksly WGE. p53 gene in hepatocellular carcinomas from Australia. Cancer Detect Prev 1992;18:123–30.
39. Hosono S, Lee CS, Chou MJ, Yang CS, Shih C. Molecular analysis of the p53 alleles in primary hepatocellular carcinomas and cell lines. Oncogene 1991;6:237–43.

40. Hollstein MC, Wild CP, Bleicher F, Chutimatawin S, Harris CC, Sivatanakul P, et al. p53 mutation and aflatoxin B1 exposure in hepatocellular carcinoma patients from Thailand. Int J Cancer 1993;53:51–5.
41. Bock LM, Ceruttin P. Aflatoxin B1 induces the transversions of G to T in codon 249 of the p53 tumour suppressor gene. Proc Natl Acad Sci USA 1998;94:8446–9.
42. Tiollais P, Pourcel C, Dejean A. The hepatitis B virus. Nature 1985;317:489–95.
43. Kiyosawa K, Sodayam T, Tanaka E, Gibo Y, Yoshikawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 1990;12:671–5.
44. Di Bisceglie, Simpsun LH, Lotze MT, Hoofnagle JH. Development of hepatocellular carcinoma among patients with chronic liver disesase due to hepatitis C viral infection. J Clin Gastroentrol 1994;19:222–6.
45. Edamoto Y, Tani M, Kurata T, Abe K. Hepatitis C and B virus infections in hepatocellular carcinoma: analysis of direct detection of viral genome in paraffin embedded tissues. Cancer 1996;77:1787–91.
46. Slagle B, Zhou YZ, Butel JS. Hepatitis B virus integration event in human chromosome commonly deleted in virus-positive hepatocellular carcinomas. Cancer Res 1991;51:285–93.
47. Rogler CE, Chisari FV. Cellular and molecular mechanisms of hepatocarcinogenesis. Semin Liver Dis 1992;12:265–78.
48. Scoresone KA, Zhou YZ, Butel JS, Slegle BL. p53 mutations cluster at codon 249 in hepatitis B virus-positive hepatocellular carcinoma. Cancer Res 1992;52:1635–8.
49. Benn J, Schneider RJ. Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. Proc Natl Acad Sci USA 1994;91: 10350–4.
50. Wang XW, Forrester K, Yeh H, Feitelson MA, Gu JR, Harris CC. Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. Proc Natl Acad Sci USA 1994;91:2230–4.
51. Ueda H, Ullrich SJ, Ganemi JD, Kappel CA, Ngo L, Feitelson MA, et al. Functional inactivation but not structural mutation of p53 causes liver cancer. Nat Genet 1995;9:41–7.
52. Neil JC, Cameron ER, Baxter EW. p53 and tumour viruses: catching the guardian off-guard. Trends Microbiol 1997;5: 115–20.
53. Natoli G, Avataggiati ML, Chirillo P, Costanzo A, Artini M, Balsano C, et al. Induction of the DNA-binding activity of c-jun/c-fos heterodimers by the hepatitis B virus transactivator X. Mol Cell Biol 1994;14:989–98.
54. Lucito R, Schneider RJ. Hepatitis B virus X protein activates transcription factor NF-kappa B without a requirement for protein kinase C. J Virol 1992;66:983–98.
55. Hall AJ, Wild CP. The toxicology of aflatoxins: human health. Veterinary and agricultural significance, In: Eaton DL, Groopman JD, editors. New York: Academic Press, 1994: 233–58.
56. Liu YH, Taylor J, Linko P, Lucier GW, Thompson CL. Glutathione S-tranferase mu in human lymphocyte and liver, role in modulating formation of carcinogen-derived DNA adducts. Carcinogenesis 1991;12:2269–75.
57. Mc Glynn KA, Rosvold EA, Lustbader ED, Hu Y, Clapper ML, Zhou T, et al. Susceptibility to hepatocellular carcinoma is associated with genetic variation in the enzymatic detoxification of aflatoxin B1. Proc Natl Acad Sci USA 1995; 92:2384–7.