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Advances in Brief

p53 Mutations Cluster at Codon 249 in Hepatitis B Virus-positive Hepatocellular Carcinomas from China[1]

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Abstract

DNA samples from 36 hepatocellular carcinoma (HCC) patients from China were screened for a specific mutation affecting codon 249 of the *p53* gene, recently identified as a hotspot mutation in some HCCs. We detected the tumor-specific *p53* codon 249 mutation in 21 (58%) of 36 HCCs examined. Thirteen patients with the specific codon 249 mutation had lost the remaining allele of *p53*, whereas the remaining eight patients appeared to have retained both copies of the gene. These results suggest that alterations of *p53* may be important events in the genesis of HCCs and that point mutation may precede allele loss.

Introduction

The development of HCC[3] is a multistep process, and the accumulation of multiple genetic changes is necessary for the emergence of a tumor. Risk factors such as chronic infection with HBV and exposure to dietary aflatoxins are clearly related to the high incidence of HCC in certain parts of the world (1–7), although the precise mechanism(s) involved remains unknown. Recent studies have suggested a role for the inactivation of tumor suppressor gene *p53* in the development of a subset of HCCs (8–11). The clustering of *p53* codon 249-specific point mutations in subsets of HCC patients from China (10, 12) and Africa (9, 12) is in contrast to the wide spectrum of *p53* point mutations observed in other types of human cancers (13, 14). However, the specific codon 249 mutation is lacking in some HCCs from China (12, 15) and from other parts of the world (12, 16). As part of our investigations of genetic changes in HBV-positive HCCs (8, 17, 18), we analyzed 36 Chinese HCC patients for the presence of the *p53* codon 249 mutation (Table 1). Matched normal and tumor samples from each patient were screened for the loss of a restriction site due to the mutation, as well as for the gain of an enzyme site. For final verification, several of the sample DNAs were sequenced to confirm the point mutation. The HCCs were further characterized for their *p53* allele status by RFLP analysis. Several insights can be gained by correlating *p53* allele loss with the presence of the codon 249 mutation, and the significance of these observations is discussed.

Materials and Methods

Tissue Samples and Preparation of DNA. HCC tissue and adjacent nontumorous liver tissue were obtained by surgical excision from patients in the Qidong Liver Cancer Institute, Jiangsu, People's Republic of China. The tissue samples were immediately placed in liquid nitrogen and were stored at −80°C until they were transported frozen to the United States. High-molecular-weight DNA was purified by digestion with proteinase K and extraction in phenol/chloroform as previously described (8).

Probe DNA. Plasmids containing the RFLP probes pHp53, YNZ22, and 144D6 were kindly provided by David H. Ledbetter (Institute for Molecular Genetics, Baylor College of Medicine) or were obtained from the American Type Culture Collection. Probe DNAs were released from plasmid vectors by restriction enzyme digestion and were isolated by gel electrophoresis in low-melting-temperature agarose gels (SeaPlaque; FMC Corp., Rockland, ME). The probes were radiolabeled within the gel by random priming using [^{32}P]deoxycytidine triphosphate (ICN Radiochemicals, Irvine, CA) and reagent kits from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Southern Blot Analysis. Ten μg of total genomic high-molecular-weight DNA were digested to completion with the appropriate restriction enzyme (depending on the probe to be used), and the fragments were separated on a 1% agarose gel. The DNA was then transferred electrophoretically to reusable nylon membranes according to the manufacturer's instructions (ZetaProbe; BioRad, Inc., Richmond, CA). Prehybridization and hybridization were performed as previously described (8). The membranes were exposed for autoradiography on Kodak XAR film (Eastman Kodak Co., Rochester, NY) using intensifying screens for 2–7 days.

PCR Analysis and Restriction Enzyme Digestion. Exon 7 of the *p53* gene was amplified from 2 μg of genomic DNA in a total volume of 100 μl containing 33 pmol of each primer, 200 μM of each deoxynucleotide triphosphate, 2.5 units of *Taq* polymerase, and 1× *Taq* polymerase buffer (Promega Corp., Madison, WI). DNAs were PCR-amplified using F3 and R3 primers (with *Eco*RI sites added to the 5′ end) (9) for 30 cycles of 2 min at 58°C, 3 min at 72°C, and 1 min at 94°C after an initial denaturation for 5 min at 95°C. The PCR products were extracted with phenol/chloroform, ethanol precipitated, and digested with *Hae*III, *Hin*fI, *Alw*nI, or *Rsa*I in a total volume of 20 μl. The DNA fragments were separated on a 20% polyacrylamide gel and were visualized by ethidium bromide staining.

Subcloning and Sequencing. The eluted PCR-amplified DNA was digested with *Eco*RI and ligated with *Eco*RI-digested Bluescript vector (Stratagene, La Jolla, CA) overnight at room temperature. Ligated DNA was used to transform XL1-Blue (Stratagene) competent cells, which were plated onto IPTG/X-gal ampicillin agar plates. After overnight incubation selected individual white colonies were expanded, and plasmid DNA was extracted. Plasmid DNA was purified using a Qiagen plasmid kit (Qiagen, Inc., Chatsworth, CA) prior to sequencing.

Sequencing double-stranded DNA templates, using the chain termination method, was performed using a Sequenase Version 1.0 kit (U.S. Biochemical Corp., Cleveland, OH), following the manufacturer's protocol. The T7 or T3 primer of the Bluescript vector was used to sequence PCR-amplified DNA from single clones.

Results and Discussion

***p53* Mutation at Codon 249.** Recent studies by others have identified a clustering of point mutations at codon 249 of the *p53* gene in 12 of 26 HCCs from southern Africa and China (9,

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; RFLP, restriction fragment-length polymorphism; PCR, polymerase chain reaction.

Table 1 *Characterization and pattern of allele loss of Chinese HBV-positive HCC patients*

Tumorous liver tissue and nearby nontumorous liver tissue were obtained from patients in the Qidong Liver Cancer Institute near Shanghai, China. Samples were stored at −70°C until they were transported to the United States.

Patient[a]	Sex[b]	Age (years)	No. of HBV integration sites[c]	Chromosome 17p markers[d] 144D6	YNZ22	pHp53	249 mutation[e]
This study							
89-3	M	48	3	—	○	○	−
89-4	M	52	4	●	○	—	+
89-5	F	49	[f]		●	●	−
89-7	M	42	5	●	●	●	+
89-8	M	38	5	●	—	—	−
89-9	M	38	[f]	●	●	●	−
89-10	M	43	4	○	●	●	
89-15	M	54	2	○	—	○	+
89-16	M	37	3	●	●	●	+
89-17	M	56	1	—	●	—	−
89-21	F	41	2	●	●	—	−
89-24	M	50	3	●	●	—	+
89-25	M	51	>6	○	○	○	−
89-26	M	38	5	○	—	○	+
89-30	M	48	2	●	—	—	−
89-33	M	40	2	●	●	●	+
89-34	M	37	4	○	—	—	+
Previous report[g]							
88-1	M	36	5	—	●	●	−
88-2	F	31	2	○	—	○	−
88-4	M	56	6	○	○	—	−
88-5	M	36	3	●	●	●	+
88-6	M	26	2	●	—	—	+
88-7	M	44	2	●	—	●	+
88-8	M	64	2	●	●	●	+
88-11	NA	NA	2	—	—	—[h]	−
88-12	NA	NA	2	○	○	○	−
88-14	M	48	2	○	—	○	+
88-15	M	45	2	—	○	—	+
88-16	M	33	2	○	○	○	+
88-17	F	50	2	—	●	—	+
88-18	M	55	1	●	●	●[i]	+
88-19	M	41	2	—	—	—[i]	−
87-25	F	32	2	○	○	—	+
87-26	M	37	1	—	—	—[i]	+
87-27	M	52	2	○	○	—	+
87-28	F	40	2	●	—	●	+

[a] Each patient was assigned a two-part number for the year in which the sample was collected followed by a patient number.
[b] M, male; F, female; NA, information not available.
[c] Hybridization analysis was performed on *Eco*RI-digested genomic DNA using the 3.2-kilobase genomic-length HBV probe (8).
[d] RFLP analysis using the chromosome 17p polymorphic markers 144D6, YNZ22, and pHp53 following procedures as described (8). —, normal tissue was homozygous for the marker (uninformative); ○, tumor tissue contained both alleles (heterozygous); ●, tumor-specific loss of one allele.
[e] PCR-restriction enzyme analysis. +, presence of 249 mutation; −, no mutation at codon 249.
[f] Unable to detect HBV-integrated sequences.
[g] Allele loss patterns previously described by Slagle *et al.* (8).
[h] Other chromosome 17p markers showed no tumor-specific allele loss.
[i] Other chromosome 17p markers showed tumor-specific allele loss.

10, 12). The mutation, a G→T transversion at the third base of codon 249, results in the loss of a *Hae*III site (GGCC→GTCC; Fig. 1*A*), providing a strategy for rapid screening of our 36 patients for the presence of this hotspot mutation. Genomic DNAs from matched normal and tumor samples were amplified by PCR using exon-7-specific oligonucleotide primers, the resulting 110-base pair amplified DNA was digested with *Hae*III, and the digestion products were analyzed on 20% polyacrylamide gels. Whereas DNA amplified from normal patient tissue yielded 75- and 35-base pair *Hae*III fragments (Fig. 1*A*, patients 89-7N, 89-15N, 89-21N, 89-24N, 89-25N), the 110-base pair DNA fragment amplified from matching tumor DNA frequently remained undigested with *Hae*III (*e.g.*, Fig. 1*A*, patients 89-7T, 89-15T, 89-24T). The amplified fragments could be digested with other restriction enzymes (*Alwn*I and *Rsa*I, data not shown). Thus, the loss of the *Hae*III restriction site revealed a somatic mutation not found in the corresponding nontumor DNA from the same patient. The tumor-specific loss of the *Hae*III site, indicating the presence of the codon 249 mutation, was detected in 21 (58%) of the 36 patients. The partial *Hae*III digestion observed in certain tumor samples (Fig. 1*A*, patients 89-15 and 89-24) was consistent with the presence of contaminating normal DNA (which would be sensitive to digestion). These results were reproducible in multiple independent PCR reactions of a specific DNA.

A similar approach was used to verify that the loss of the *Hae*III site was due to a specific mutation in the third position of codon 249. A G→T transversion mutation would result in the gain of a *Hinf*I site (Fig. 1*B*). Matched normal and tumor DNAs from all 36 patients were PCR amplified using the *p53* exon-7-specific oligonucleotide primers, and the amplified 110-base pair fragment was digested with *Hinf*I. All 21 tumor DNAs that had previously demonstrated a loss of the *Hae*III site showed a gain of a *Hinf*I site not present in the normal DNA from that patient (Fig. 1*B*, patients 89-7T, 89-15T, 89-24T, 89-26T, 89-32T, 89-33T). These results indicate that the loss of the *Hae*III restriction site was due to a G→T transversion. The lack of complete digestion with *Hinf*I in those tumor DNAs is likely due to reannealing of PCR-amplified mutant strands with wild-type strands amplified from contaminating normal DNA (or from a wild-type allele still present in those samples that had not shown chromosomal loss at the *p53* locus). Only when two amplified mutant DNA strands reanneal will the *Hinf*I restriction site be generated. Thus, the presence of any wild-type gene could have a great impact on the ability to detect a point mutation in *p53* in tumor DNA.

As a final approach to confirm the restriction digestion data and to identify the specific mutation, two sets of normal and tumor DNAs generated by PCR amplification of *p53* exon 7 were subcloned and sequenced by the dideoxy DNA method. Sequencing confirmed a G→T transversion at codon 249 in tumor samples that had lost the *Hae*III restriction site (Fig. 1*C*, patients 88-5 and 89-7).

Loss of Chromosome 17p Alleles in HCC. Previous studies from our laboratory identified frequent chromosome 17p allele loss in 19 HBV-positive Chinese patients with HCC (8). To determine if the chromosome 17 allele loss is a general feature of other HCCs from China, we analyzed matched normal and HCC tissue DNAs from an additional 17 patients using the same experimental procedure. Genomic DNA was isolated from matched tissues and was then digested with restriction enzymes appropriate for the detection of RFLPs using a panel of DNA probes specific for the short arm of chromosome 17 (17p). All 17 patients analyzed were heterozygous for one or more of the chromosome 17 markers. Tumor-specific loss of heterozygosity was detected in 12 (71%) of the 17 patient samples (Fig. 2*A*, patients 89-7, 89-8, and 89-24; Fig. 2*B*, patients 89-7, 89-21, and 89-24; Fig. 2*C*, patients 89-5, 89-9, and 89-10). The RFLP analysis included a *p53* complementary DNA probe, and 6 (60%) of the 10 patients heterozygous at the *p53* locus had a tumor-specific loss of one allele (Fig. 2*C*, patients 89-5, 89-9, and 89-10). Our previous study showed that patients with *p53* loss of heterozygosity also had loss of heterozygosity at the 144D6 and YNZ22 loci (8). Thus, examination of the allele loss patterns among all 17 patients (Table 1, top) provides indirect evidence for *p53* allele loss in an additional five patients (89-8, 89-17, 89-21, 89-24, and 89-30)

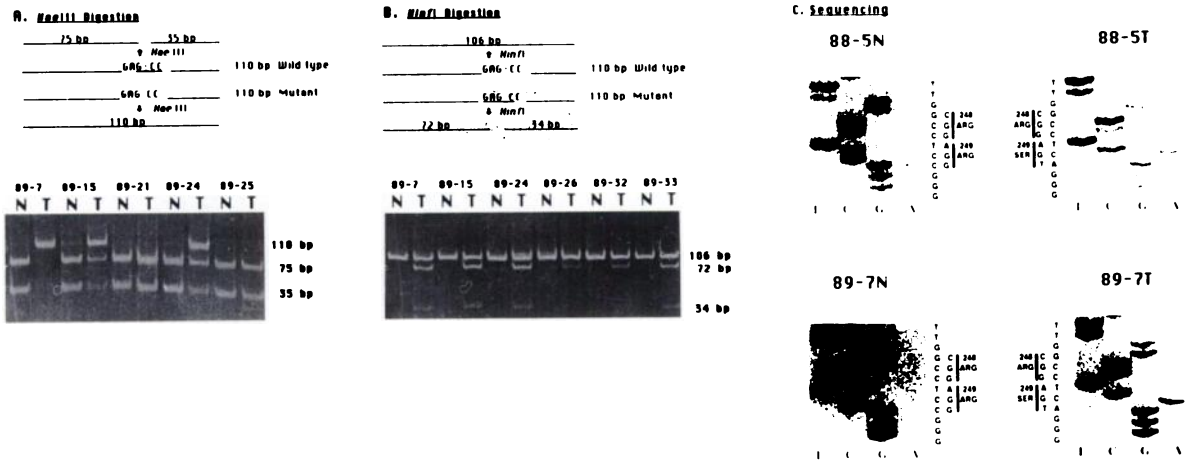

Fig. 1. Analysis of missense mutation in PCR-amplified exon 7 of *p53* from normal and tumor DNA. *A*, *Hae*III digestion (GG^CC). DNA samples containing the wild-type sequence yielded 75- and 35-base pair fragments. The loss of the *Hae*III site was observed in many tumor DNAs, resulting in an uncut 110-base pair DNA fragment. *B*, *Hinf*I digestion (G^ANTC). DNAs with the G→T point mutation at the third base of codon 249 were cut with this enzyme. The expected 72- and 34-base pair fragments were revealed. A *Hinf*I site at base 106 in both the wild-type and mutant DNAs results in a 4-base pair fragment that cannot be seen in the gel. *C*, sequencing autoradiograms of PCR-amplified exon 7 DNA of the *p53* gene in the region of codon 249. A G→T transversion (shown in the antisense as C→A) at codon 249 was verified in tumor samples 88-5T and 89-7T.

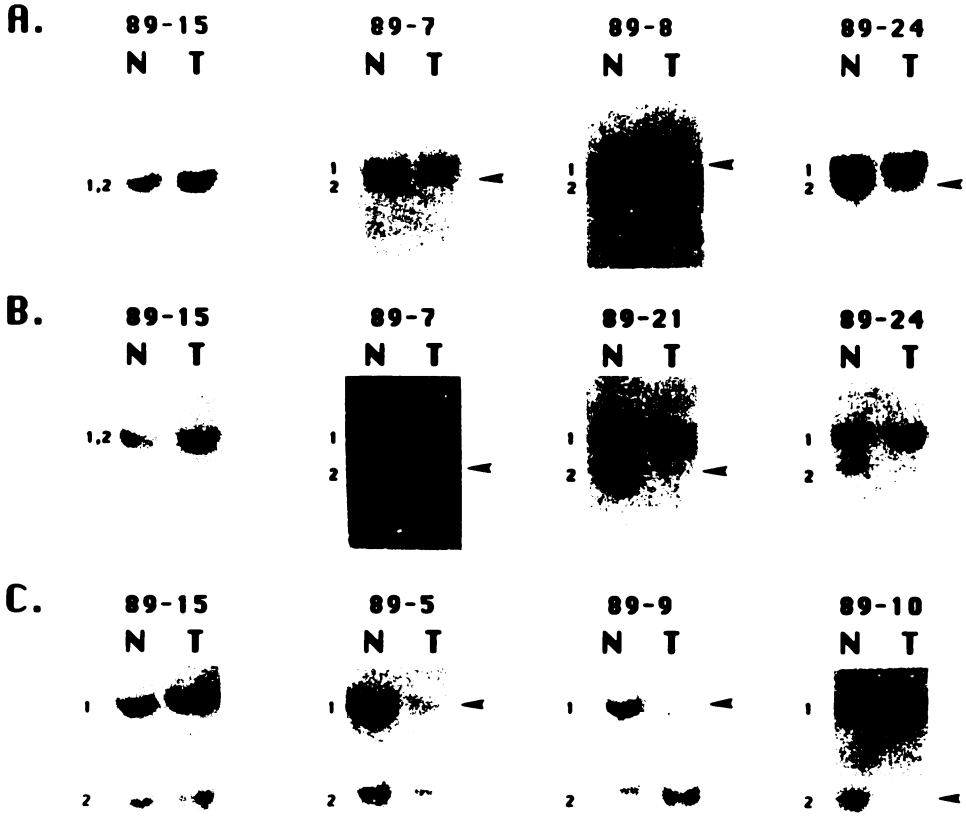

Fig. 2. RFLP analysis demonstrating tumor-specific loss of heterozygosity at loci on the short arm of chromosome 17. DNAs from hepatoma (*T*) and matched normal (*N*) tissues were digested with appropriate restriction enzymes and were analyzed by Southern hybridization (8) using the probes 144D6 (*A*), YNZ22 (*B*), and pHp53 (*C*). The *numbers* (*1* and *2*) *at the left of each panel* refer to the larger and smaller restriction fragments, respectively, containing the alleles recognized by a given probe (different pairs of alleles are present in individual patients recognized by the variable number of tandem repeats polymorphic markers 144D6 and YNZ22). *Arrowheads*, specific allele loss. The patients were identified by a two-part code as described in Table 1. The faint hybridization signal from the missing allele observed in some tumor DNAs (*e.g.*, Fig. 1*A*, 89-24; Fig. 1*C*, 89-5) is presumably due to DNA from nontumorous tissue present in tumor samples.

who lost distal 17p markers but were uninformative at the *p53* locus.

Our results suggest that a two-step inactivation of the *p53* gene occurs frequently in this sample population. A total of 22 (61%) of the 36 tumors showed either direct (actual *p53* allele loss) or indirect (loss of another 17p marker but not informative at the *p53* locus) evidence for the loss of one copy of *p53*. Thirteen (59%) of the 22 patients contained the specific *p53* codon 249 point mutation. Although the remaining nine patients lacked the 249 mutation, our analysis was confined to exon 7, and the remaining *p53* alleles in those tumors are candidates for point mutations elsewhere in the gene.

Additional information may be derived from a comparison of allele loss and the codon 249 mutation data. Of the 21 HCC patients with the codon 249 mutation, 13 had evidence of *p53* allele loss, whereas the remaining 8 patients appeared to retain both copies of *p53*. This suggests that the first step in *p53* inactivation is a point mutation in one allele, with a subsequent loss of the wild-type allele. This pattern differs from that in colon cancer in which *p53* mutations were infrequently observed in tumors that contained both copies of chromosome 17p (19). It is possible that there is not a strong selection for the loss of a wild-type *p53* allele during the development of HCC. In addition, the effect of the codon 249 mutation is unknown, and

it may be possible that there is some growth advantage to cells that contain both this particular *p53* alteration and a wild-type allele. Alternatively, the remaining *p53* allele in those eight patients with the codon 249 mutation may not be wild type; our limited analysis of exon 7 would not have detected mutations elsewhere in the *p53* gene. Because our tumor samples were obtained from patients undergoing surgery for advanced disease, we are not able to associate *p53* alterations with specific stages in the development of HCC.

A wide spectrum of *p53* mutations has been detected in other human cancers (13, 14, 20), and the clustering of identical point mutations appears unique to HCC. It has been suggested that the potent liver carcinogen aflatoxin B_1 may be the causative agent for the frequent G→T transversions observed in HCCs (9, 10, 12, 16). Because not all HCCs from aflatoxin-rich regions contain codon 249 mutations (9, 10, 12, 15), other carcinogens that can generate G→T transversions (21) should also be considered potential cofactors responsible for the large percentage of codon 249 mutations in HCCs from this region of China. We have no evidence of an obligatory hepatocyte-specific selection of a particular form of mutant *p53* required for liver tumorigenesis, although the observed preponderance of codon 249 mutations indicates some type of selective process.

Whether HBV is involved in the chromosome losses reported here is unknown. Clearly, allele losses in HCC can occur independently of HBV integration (22, 23); however, given the mutagenic nature of HBV integration events, we suggest that viral integration may mediate specific allele losses in subsets of HCCs, some of which may affect the *p53* gene (8). In addition, the HBV-induced inflammatory process may result indirectly in chromosome loss or in the selection for cells containing the *p53* codon 249 mutation.

The development of HCC is a multistep process in which many genetic changes are required. The interactions of host factors with viral and chemical agents seem to be key components in the formation of these tumors. Although the exact contribution of each agent is unknown, the frequent *p53* allele loss and the presence of the codon 249 mutation suggest that alterations in *p53* may be important events in the genesis of HCCs.

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