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Abnormal structure and expression of p53 gene in human hepatocellular carcinoma

(hepatocarcinogenesis/recessive oncogene/gene expression)

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ABSTRACT **There is little information regarding the molecular mechanisms of hepatocarcinogenesis. We studied the p53 gene at the DNA, RNA, and protein level in seven human hepatocellular carcinoma (HCC)-derived cell lines; six of seven showed p53 abnormalities. By Southern blotting, the p53 gene was found to be partially deleted in Hep 3B and rearranged in SK-HEP-1 cells. Transcripts of the p53 gene were undetectable in Hep 3B as well as in FOCUS cells that had no apparent deletion or rearrangement of the p53 gene. Immunoprecipitation after [^{35}S]methionine labeling of HCC cells demonstrated that p53 protein was absent in Hep 3B and FOCUS and reduced in concentration in PLC/PRF/5 cells. p53 synthesized by Mahlavu cells showed a slower migration on SDS/polyacrylamide gels suggesting it was an abnormal protein. In Huh7 cells, p53 protein had a prolonged half-life leading to its accumulation in the nuclei; increased levels of p53 protein were also found by immunoblotting. The p53 gene and its expression appeared to be unaltered in the hepatoblastoma-derived Hep G2 cell line. We found that the loss of p53 expression did not occur as a late *in vitro* event in the FOCUS cell line because p53 protein was also nondetectable at an early passage. We conclude that the loss of p53 expression or the presence of abnormal forms of the protein are frequently associated with HCC cell lines. These observations suggest that alterations in p53 may be important events in the transformation of hepatocytes to the malignant phenotype.**

Human hepatocellular carcinoma (HCC) is one of the most frequent tumors worldwide. Although HCC is relatively rare in North America and Europe, it ranks as one of the four most prevalent malignant diseases of adults in China, Taiwan, Korea, and sub-Saharan Africa (1). Infection with hepatitis B virus (HBV) as well as alcoholic cirrhosis and other factors associated with chronic inflammatory and hepatic regenerative changes are found to be important risk factors for hepatocarcinogenesis (1–3). However, the molecular mechanisms involved in malignant transformation of hepatocytes remain largely unknown. Because of strong epidemiological evidence showing a close correlation between the prevalence of hepatitis B surface antigen chronic carriers and the incidence of HCC, the role of HBV in hepatocarcinogenesis has been extensively studied (4). However, the HBV genome is unlikely to carry oncogenic sequences, and there is little evidence to suggest that integration of viral DNA into hepatocyte DNA activates cellular protooncogenes (3). Indeed, no specific association between activation of any known oncogene and HCC has yet been found.

The role of recessive oncogenes in HCC has not been studied in detail. T'ang *et al.* (5) have analyzed the genomic structure of the retinoblastoma gene in HCC; no major deletions or rearrangements were found in these tumors. Recent studies have shown that p53, like the product of the retinoblastoma gene, may act as a tumor suppressor (6, 7). In addition, the p53 gene has been reported to be a frequent target for genetic abnormalities in leukemias, lymphomas (8, 9), osteogenic sarcomas (10) as well as colorectal (11) and lung tumors (12). In most tumor cells studied, p53 gene abnormalities lead to loss of the p53 translational product or to the expression of mutant forms of the protein (8–12). Recent studies have also shown that some mutant p53 proteins have transforming activity, whereas the wild-type p53 does not possess this activity (6, 7, 13–17). Because the state of the p53 gene has not been previously studied in HCC, we explored its structure and expression in seven HCC-derived cell lines.

MATERIAL AND METHODS

Cell Lines. Human hepatoma cell lines Hep 3B (18), Hep G2 (18), Huh7 (19), Mahlavu (20), PLC/PRF/5 (21), SK-HEP-1 (22) and early (6th passage) as well as late (several hundredth) passage of FOCUS (23) were grown in Eagle's minimal essential medium containing 10% fetal calf serum and 1% nonessential amino acids.

Immunoblotting. Cell and tissue extracts were prepared as described (24) and samples adjusted to equal protein concentrations (100–200 μg) were separated by SDS/PAGE, electrotransferred to nitrocellulose paper, and analyzed by using the ^{125}I-labeled Pab122 antibody (25).

Indirect Immunofluorescence. Cells grown on cover slips under standard culture conditions were used for indirect immunostaining with monoclonal antibody (mAb) Pab122 by using a described protocol with minor modifications (26). Briefly, cells were fixed in 4% (vol/vol) formaldehyde and incubated with 1% bovine serum albumin (BSA)/10 mM phosphate/140 mM NaCl, pH 7.2. Cells were then incubated with mAb Pab122 (1:1000 dilution of ascitic fluid in BSA), washed in 10 mM phosphate/140 mM NaCl, pH 7.2 (PBS), and incubated with rhodamine-conjugated sheep anti-mouse IgG antibodies (Cappel Laboratories). After the final wash, cover slips were mounted on glass slides using Aqua-mount (Lerner Laboratories, New Haven, CT) and examined under the fluorescence microscope.

Immunoprecipitation. Subconfluent cells were cultured for 1 hr in methionine-free medium containing 10% dialyzed fetal calf serum. Cells were labeled with [^{35}S]methionine at 200

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; mAb, monoclonal antibody; HCC, human hepatocellular carcinoma; HBV, hepatitis B virus.

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μCi/ml (Amersham, specific activity >1000 Ci/mmol; 1 Ci = 37 GBq) for 4 hr in 10-cm culture dishes. Cells were then washed in PBS and extracted in 1 ml of lysis buffer (50 mM Tris·HCl, pH 7.4/0.25 M NaCl/0.1% (vol/vol) Triton X-100/5 mM EDTA/50 mM NaF). All solutions contained a mixture of protease inhibitors (phenylmethylsulfonyl fluoride at 50 mg/liter, soybean trypsin inhibitor at 10 mg/liter, aprotinin at 1 mg/liter). Immunoprecipitation experiments were performed following a protocol described by Giordano *et al.* (27). Briefly, samples were precleared with 40 μl of 50% (vol/vol) protein A-Sepharose suspension (Pharmacia) for 1–16 hr at 4°C. Two microliters of mAb Pab122 ascitis were added. After a 1-hr incubation on wet ice and a 5-min centrifugation at 13,000 × *g*, supernatants were added to Eppendorf tubes containing 40 μl of protein A-Sepharose beads. Antigen–antibody complexes were captured by a 60-min incubation at room temperature, and the beads were pelleted by centrifugation. The beads were washed 4 times in lysis buffer. The immunoprecipitates were directly resuspended in SDS/PAGE sample buffer (28), heated at 75°C for 5 min, and subjected to SDS/PAGE using 7.5% or 10% polyacrylamide slab gels. After electrophoretic protein separation, gels were processed for fluorography with Amplify solution (Amersham), according to the manufacturer's instructions. Autoradiography was done at −70°C.

Southern and RNA Blot Analysis. RNA was isolated from cells by the guanidinium thiocyanate extraction procedure (29), and 20-μg aliquots of each preparation were subjected to electrophoresis under denaturing conditions (30). DNA was isolated using the proteinase K/phenol extraction protocol as described (30). DNA digested with *Eco*RI, *Bam*HI, *Hin*dIII, or *Xba* I was separated by electrophoresis on agarose gels. Both RNA and DNA were transferred to Nytran filters (New England Nuclear) and hybridized to ^{32}P probes, as described (30). Randomly primed ^{32}P-labeled cDNA probes (31) were prepared from the plasmid php53B (32) by purifying a 2.2 kilobase pair (kbp) human p53 cDNA insert.

RESULTS

Genomic Analysis. DNA from hepatoma cell lines were digested with *Xba* I, *Hin*dIII, *Bam*HI, or *Eco*RI restriction enzymes and hybridized with a p53 cDNA probe that covers the 11 exons of the p53 gene (32, 33). As illustrated in Fig. 1, abnormalities of the p53 gene restriction patterns were detected in Hep 3B and SK-HEP-1 cells but not in other HCC cell lines. After digestion with *Xba* I, a single hybridizing band (≈13 kbp) was detected in Huh7, Hep G2, Mahlavu, FOCUS, and PLC/PRF/5 cells (Fig. 1*A*). In Hep 3B cells, a shorter fragment (6 kbp) was detected, suggesting a large deletion (7 kbp). In SK-HEP-1 cells, a second shorter band (9.5 kbp) was observed in addition to the normal-sized fragment, suggesting a partial deletion or a rearrangement in at least one allele. Using the restriction enzyme *Hin*dIII, two fragments migrating as 7 kbp and 2.5 kbp bands were seen in Huh7, Hep G2, Mahlavu, FOCUS, and PLC/PRF/5 cells (Fig. 1*B*). In Hep 3B cells, only the 2.5-kbp fragment was detected; the larger 7-kbp fragment was absent. These results confirm that a large deletion of p53 gene occurred in Hep 3B cells. DNA from SK-HEP-1 cells showed a complex pattern of restriction fragments with this enzyme as well. In addition to the expected 7-kbp and 2.5-kbp fragments, another fragment (8.5 kbp) was seen, suggesting a structural rearrangement or a partial deletion involving at least one of the alleles. Southern blots with DNA digested with *Eco*RI and *Bam*HI also showed abnormalities in Hep 3B and SK-HEP-1 cell lines (data not shown).

p53 Transcripts. RNAs from seven hepatoma cell lines were analyzed by RNA blotting. As seen in Fig. 2, Hep G2, SK-HEP-1, Mahlavu, and Huh7 cells showed 2.5-kbp transcripts. Transcripts of p53 were greatly reduced in PLC/PRF/5 cells and were not detectable in Hep 3B and FOCUS cells. Finally, hybridization of the same blot with a β-actin probe indicated that changes in the levels of p53 transcripts were not due to sample size variation or RNA degradation (Fig. 2).

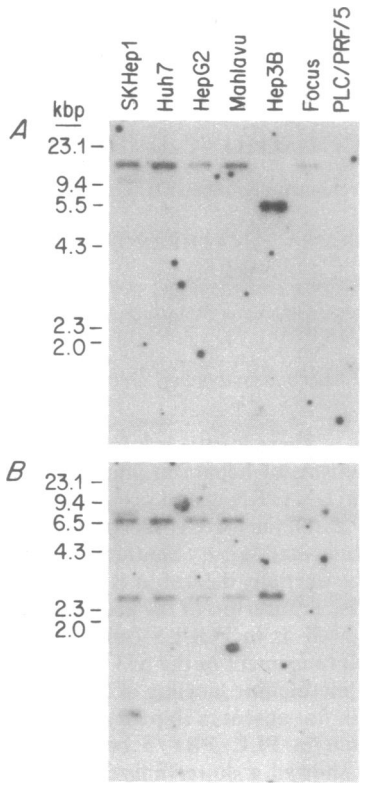

FIG. 1. Southern blot analysis of p53 gene in HCC cell lines after digestion with *Xba* I (*A*) or *Hin*dIII (*B*).

p53 Protein Levels in Liver and Hepatoma Cell Lines. p53 protein expression was analyzed using several techniques. mAb Pab122, which is directed to a conserved epitope near the carboxyl end of p53 (25, 34) was used for these studies. We first studied p53 expression by immunoprecipitation after [^{35}S]methionine labeling. Using this sensitive technique, we found that p53 was present in Huh7, Hep G2, SK-HEP-1, and

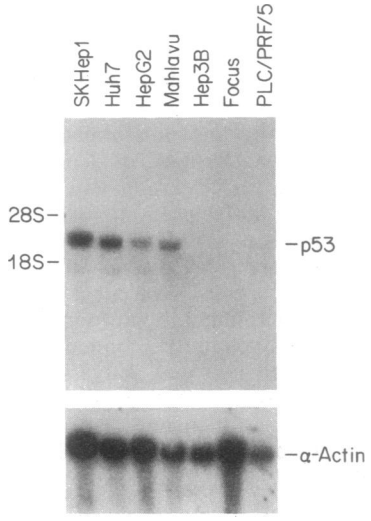

FIG. 2. RNA blot analysis of p53 transcripts in HCC cell lines.

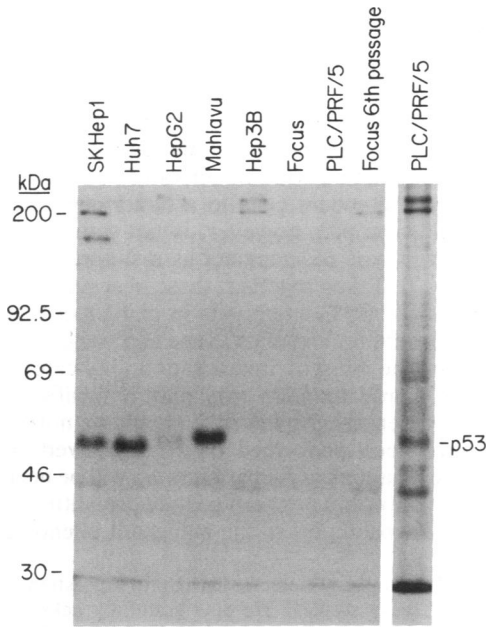

FIG. 3. SDS/PAGE analysis of p53 immunoprecipitates from HCC cell lines. In PLC/PRF/5 cells, p53 is detectable only after extended exposure (far right lane).

Mahlavu cells. p53 protein from Mahlavu cells migrated more slowly than the others; this finding suggests that Mahlavu cells express a mutant form (Fig. 3). p53 protein was found at greatly reduced levels in PLC/PRF/5 cells; it was detectable only after extended autoradiography exposure (4 days). p53 protein was undetectable in Hep 3B and FOCUS cells, (Fig. 3) even after 1 week of exposure (data not shown). We also tested an early passage (6th) of FOCUS cells to determine whether the loss of p53 expression occurred after the establishment of this cell line. As shown in Fig. 3, p53 protein was also undetectable at the 6th passage, suggesting that the loss of p53 expression probably occurred before the establishment of this cell line.

We also studied levels of p53 by immunoblotting. Western immunoblots of normal liver and hepatoma cell line extracts are shown in Fig. 4*A*. The presence of trace amounts of p53 in normal liver was demonstrated (Fig. 4*A*). As compared to normal liver, Huh7 cells expressed high levels of p53 protein. Low but detectable levels of a more slowly migrating p53 protein were also found in Mahlavu cells. p53 was undetectable in other HCC cell lines using this technique. By indirect immunofluorescence we could detect the presence of p53 in the nuclei of Huh7 cells (data not shown). To test whether increased levels of p53 in Huh7 cells were due to an increased half-life, pulse-chase experiments were performed. Huh7 and Hep G2 cells were metabolically labeled for 60 min with [^{35}S]methionine and then cultured in the presence of 1 mM unlabeled methionine. After 0, 30, 60, and 240 min of chase, cells were processed as described. As shown in Fig. 4*B*, p53 protein half-life was short in Hep G2 cells (<30 min). In Huh7 cells, p53 protein half-life was markedly increased (>240 min). It is noteworthy that a 70-kDa family of proteins coprecipitated with p53 in Huh7 cells. These proteins probably represent heat shock proteins that have been shown to form complexes with mutant p53 proteins (16).

DISCUSSION

Our studies emphasize that abnormal p53 gene structure and expression is a frequent event associated with HCC. Several types of abnormalities were observed. At the genomic level the p53 gene was found to be abnormal in two cell lines. In Hep 3B cells, a major portion of the p53 gene is deleted. This deletion is accompanied by the absence of p53 transcripts and p53 protein in this cell line. In SK-HEP-1 cells, one of the p53 alleles appeared to be intact by restriction enzyme analysis; the presence of another abnormally sized fragment of p53 gene suggests that there is at least one rearranged or partially deleted allele. However, these genomic changes did not overtly affect the expression of p53 protein because we found normal-sized transcript and protein in this cell line. In FOCUS cells, the p53 gene appeared unaltered by Southern analysis; however, both p53 transcripts and protein were not detectable. It is noteworthy that in FOCUS cells an absence of p53 expression was seen as early as the sixth passage. This observation suggests that the expression of p53 was lost before the establishment of this cell line. The absence of transcripts of an "apparently normal" p53 gene in some lung cancer cell lines has been reported (12). This phenomenon

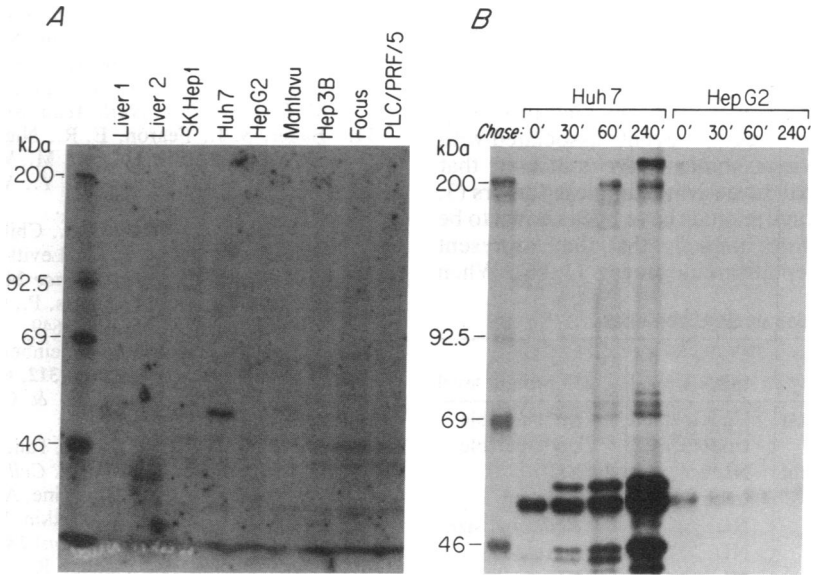

FIG. 4. (*A*) Immunoblot analysis of p53 protein levels in normal liver and HCC cell lines. In liver 1, p53 protein levels are low but detectable. An ≈40-kDa breakdown product is seen with extracts from liver 2. (*B*) Pulse-chase analysis of p53 protein in Huh7 and Hep G2 cells.

may be due, in part, to the lack of transcription or to instability of p53 transcripts.

Previous studies on p53 protein indicate that the mutant forms may be distinguished from the wild type by abnormal migration on polyacrylamide gels, by an increased half-life, which leads to accumulation of the mutant protein in the nucleus, and/or by complex formation with heat shock proteins (16, 35–37). Indeed, a single amino acid change in p53 may cause an abnormal migration on SDS/polyacrylamide gels (35) or an increased half-life (16). In this regard, we observed that p53 proteins expressed in two HCC cell lines appeared abnormal. The one synthesized by Mahlavu cells migrated more slowly than the wild-type protein (Fig. 3). In Huh7 cells, the size of p53 appeared normal, but its levels were increased. Immunoblotting allowed us to estimate steady-state levels of p53 protein in HCC cell lines as compared to normal liver. The p53 concentration in Huh7 cells was higher than that found in normal liver and other HCC cell lines. High levels of p53 protein found in this cell line are probably due to an increased stability because we found that the half-life of the protein is markedly prolonged. This increased half-life may result from complexation of a mutant p53 with the 70-kDa heat shock protein family (16). From these observations, we conclude that p53 proteins expressed in Mahlavu and Huh7 cells are probably abnormal, and this may be due, in part, to minor mutations in the coding region of the p53 gene. It is noteworthy that such mutations have been described in other tumor cells (11, 12).

There are no detectable HBV sequences in Huh7, Hep G2, SK-HEP-1, and Mahlavu cell lines (data not shown). Three of the HCC cell lines studied—namely, FOCUS, Hep 3B, and PLC/PRF/5 have previously been found to contain integrated HBV sequences (23, 38–40). It is important to note that in these cell lines p53 expression was either undetectable (Hep 3B and FOCUS) or greatly reduced (PLC/PRF/5). The p53 gene has been localized to the short arm of chromosome 17, and HBV integration into chromosome 17 has been demonstrated in three HCCs (41–43). This association suggests that an abnormal structure and expression of p53 gene in some HCC cell lines may be related to HBV DNA integration. However, we found no gross genomic alterations of the p53 gene in FOCUS and PLC/PRF/5 cells. Hep 3B was the cell line where a major genomic alteration was demonstrated. Previous studies have shown that HBV sequences in Hep 3B cells were integrated into chromosome 12 and not chromosome 17 (40). It would be unlikely, therefore, that HBV integration played a direct role in aberrant p53 gene expression in these cell lines.

In this report, we demonstrate that p53 gene structure and expression is altered in six of seven HCC cell lines (Table 1). Thus, p53 abnormalities are frequent events associated with HCC development. Previous studies have indicated that certain protooncogenes may be activated in these tumors (3, 44–46). However, such abnormalities have been shown to be rare events. It is, therefore, unlikely that they represent general mechanisms of hepatocarcinogenesis (3, 46). When we compare the present results with previously described oncogenic changes, it appears that abnormal structure and expression of the p53 gene may be one of the most frequent genetic events associated with hepatocyte transformation.

Table 1. Abnormalities of p53 in HCC cell lines

Cell line	Type of mutation	mRNA level	p53 protein level
Hep 3B	Partial deletion	Undetectable	Undetectable
FOCUS	ND	Undetectable	Undetectable
SK-HEP-1	Rearrangement	NL*	NL†
PLC/PRF/5	ND	trace	Reduced
Mahlavu	ND	NL	Abnormal size
Huh7	ND	NL	Increased
Hep G2	ND	NL	NL

ND, Not detectable by Southern blot analysis; NL, normal; NL*, normal by RNA blot; NL†, normal by immunochemical studies.

It has been proposed that p53 protein may play a direct role in malignancy. Overexpression of mutant p53 proteins when assayed in conjunction with an activated *ras* gene, leads to transformation of rodent cells to a tumorigenic phenotype (13–15). Wild-type p53, however, is not transforming (17); moreover, wild-type p53 may act as a suppressor of transformation (6, 7). Taken together, these observations point to the significance of p53 alterations in malignant transformation. The frequent finding of p53 gene abnormalities in HCC, as shown in the present investigation, indicates that the inactivation of p53 function may play a significant role in hepatocarcinogenesis. However, it should be noted that our studies have been performed on HCC-derived cell lines. Further investigations of primary tumors will be necessary to define the relationship between p53 abnormalities and transformation of hepatocytes to the malignant phenotype.

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