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Cooperation of tumor-derived HBx mutants and p53-249^{ser} mutant in regulating cell proliferation, anchorage-independent growth and aneuploidy in a telomerase-immortalized normal human hepatocyte-derived cell line

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Abstract

Hepatocellular carcinoma (HCC) is a common cancer and hepatitis B virus (HBV) is a major etiological agent. Convincing epidemiological and experimental evidence also links HCC to aflatoxin, a naturally occurring mycotoxin that produces a signature p53-249^{ser} mutation. Recently, we have reported that tumor-derived HBx variants encoded by HBV exhibited attenuated transactivation and pro-apoptotic functions, but retained their ability to block p53-mediated apoptosis. These results indicate that mutations in HBx may contribute to the development of HCC. In this study, we determined whether tumor-derived HBx mutants along, or in cooperation with p53-249^{ser}, could alter cell proliferation and chromosome stability of normal human hepatocytes. To test this hypothesis, we established a telomerase immortalized normal human hepatocyte line HHT4 that exhibited a near diploid karyotype and expressed many hepatocyte-specific genes. We found that over-expression one of the tumor-derived HBx mutants, CT, significantly increased colony forming efficiency (CFE) while its corresponding wild-type allele CNT significantly decreased CFE in HHT4 cells. p53-249^{ser} rescued CNT-mediated inhibition of colony formation. While HHT4 cells lacked an anchorage independent growth capability as they did not form any colonies in soft agar, the CT-expressing HHT4 cells could form colonies, which could be significantly enhanced by p53-249^{ser}. Induction of aneuploidy could be observed in HHT4 cells expressing CT but additional recurring chromosome abnormalities could only be detected in cells coexpressing CT and p53-249^{ser}. Our results are consistent with the hypothesis that certain mutations in HBx and p53 at codon 249 may cooperate in contributing to liver carcinogenesis.

Keywords

p53; HBx; cell proliferation; anchorage-independent growth

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INTRODUCTION

Primary liver cancer is one of the most common and deadly cancers in the world. Based on epidemiological data, chronic HBV infection is a major etiological factor associated with the development of hepatocellular carcinoma (HCC), the major type of primary liver cancers (1). Epidemiological studies also indicate that dietary aflatoxin B1 (AFB1) intake contributes to the development of HCC, which is associated with a high frequency of a p53 mutation at codon 249 (2;3). Convincing evidence indicate that both HBV and AFB1 have a significant synergistic effect in HCC development (4). However, the molecular basis of the association of HBV infection with liver carcinogenesis remains unclear. Recent studies by many groups including ours indicate that HBx, a 17-Kd protein encoded by HBV, may act as an oncogene during liver carcinogenesis (5). In HBx transgenic mice, liver tumors were observed with a higher frequency compared to those in wild-type mice (6;7). In *in vitro* studies, HBx can induce transformation in NIH3T3 cells in cooperation with oncogenes such as ras (8), promote abnormal cell proliferation and abnormal mitosis (9–14). Functional studies also show that HBx binds directly to p53 and inhibits p53-mediated apoptosis (15). Expression of HBx has been shown in both pre-malignant and HCC samples (16). Recently, the availability of several naturally occurring tumor-derived HBx mutants provides a good tool to investigate the role of HBx in liver carcinogenesis (17–19). These tumor-derived HBx mutants mainly contain several point mutations distributed in different domains of HBx. These tumor-derived HBx mutants retain the ability to bind to p53 and block p53-mediated apoptosis, as compared with their corresponding wild-type variants. However, they have attenuated activity of NFκB and loss of inhibition of colony formation when overexpressed compared to wild-type HBx (17;20). These results support the hypothesis that HBx mutants may provide an advantage for tumor cells to grow in the host environment during liver carcinogenesis.

Previous *in vitro* studies related to HBV-associated liver carcinogenesis were mainly involved in HCC-derived cell lines or non-liver cell lines (8;21). This is largely due to the unavailability of a good normal liver-derived cell culture system. Data obtained in non-liver cells are often difficult to be interpreted because HBV carcinogenicity is hepatotropic. Normal human liver-derived cell lines were established previously in our laboratory by using a SV40 T immortalization protocol and these cell lines have been utilized in studies of carcinogen metabolism, mutagenesis and neoplastic transformation (22;23). However, similar to tumor-derived cell lines, T antigen immortalized cells are genetically unstable and aneuploid, and lose certain phenotypic traits including the loss of most cytochrome P450 (CYP) gene expression. Recent studies indicate that the human telomerase reverse transcriptase gene (hTERT) can immortalize human epithelial cells efficiently and that the immortalized cells remain mostly diploid and demonstrate a differentiated phenotype (24;25).

In this study, we established an hTERT-immortalized human hepatocyte cell line, HHT4. Using this cell model, we addressed the following questions: (1) whether tumor-derived HBx mutants induce neoplastic transformation of hTERT-immortalized human hepatocytes HHT4; and (2) whether tumor-derived HBx mutants and p53-249^{ser} cooperate in the neoplastic transformation of HHT4 cells. Our results indicate that tumor-derived HBx mutants, but not their corresponding wild-type variants, can enhance cell proliferation and chromosome instability by cooperating with mutant p53.

MATERIALS AND METHODS

Plasmids and retrovirus production

The pCL-hTERT construct was produced by sub-cloning the human telomerase reverse transcriptase (hTERT) cDNA as an EcoR I fragment from pGRN145 (Geron Corporation, Menlo Park, CA) into the pCLXSN retroviral vector (26). pCLXSN is a derivative of the pLXSN retroviral vector containing a human cytomegalovirus immediate early promoter upstream of the multiple cloning site which allows more robust, long-term expression of the gene of interest. Various tumor-derived HBx mutant fragments (CT, CNT, MT, MNT) were amplified by polymerase-chain reaction (PCR) as previously described (20). These fragments were inserted into the HindIII and ClaI sites of a retroviral expression vector, pLHC (Invitrogen). The p53-249^{ser} mutant fragment was cloned into the BamHI and SnaBI sites of a retroviral vector pBABE (kindly provided by Dr. Soreano). To produce hTERT-encoded retrovirus, 80% confluent Phoenix-A producer cells were transfected with pCL-hTERT construct using LipofectAMINE PLUS Reagent (Invitrogen, Carlsbad, CA) in serum-free DMEM medium. The medium was replaced 24 hours after transfection with either fresh LCM+10% chemically-denatured serum (CDS) (for primary hepatocytes) or DMEM (for all other cell types). Virus was collected as a raw supernatant 24–48 hours later, centrifuged, and filtered through 0.45- μ m, low protein-binding filters. For HBx and p53 mutant retrovirus production, amphopack 293 cells were transfected with pLHC-CT, pLHC-CNT, pLHC-MT, pLHC-MNT, or pLHC-p53-249^{ser} and viral supernatant collected 48 hours later. The titer of viral supernatant were quantified and stored at -80°C for infection of HHT4 cells. The NIH 3T3 cell colony formation assay was used to determine viral titer.

Liver cell immortalization, culture, characterization and transfection

Freshly isolated normal human liver epithelial cells (hepatocytes enriched population) were obtained from organ donors who died of trauma at the University of Pittsburgh Liver Transplantation Center or from surgical resection at the University of Maryland. Normal human liver epithelial cells were obtained by a collagenase/dispase perfusion technique described previously (27) and plated onto flasks that were coated with BFN mix (10 μ g/ml of bovine plasma fibronectin, Calbiochem; 0.1 mg/ml of BSA; collagen I (VitrogenTM, Cohesion, Palo Alto, CA); in LHC basal medium, (Invitrogen, Carlsbad, CA) at a cell density of 1×10^6 cells per 100mm plate. Cells were incubated overnight in HBM/HCM media (Clonetics, San Diego, CA) plus 10% fetal bovine serum.

Cells were switched to HBM/HCM media containing pCL-hTERT virus in the presence of 4 mg/ml of polybrene (Sigma, St. Louis, MO) for five hours. CDS was added at the final concentration of 10%. Cells were incubated overnight and fresh HBM/HCM media plus 10% CDS with or without 35 μ g/ml of G418 were added. Fresh media were changed every 3–4 days. G418 was only used for the first two weeks of cell culture. Cells without any virus showed minimum signs of cell proliferation and died, usually within four weeks. In viral-infected cells, healthy colonies with epithelial cell morphology were visible in about two weeks. After four weeks of culture, healthy colonies were isolated using cloning cylinders into plated into 12-well plates. Cells were maintained in culture with less than 80% confluency, and were passaged every week with 1:4 split ratio; using EPET solution (Invitrogen, Carlsbad, CA) followed by SBTI solution (Invitrogen, Carlsbad, CA). From two batches of hepatocytes, two independent clones (i.e., HHT3 and HHT4) were successfully expanded and continuously passaged. These cells were cultured routinely in HBM/HCM plus 3% CDS and further characterized.

cDNA microarrays and telomerase activity analyses

Total RNA samples were isolated from log-phase growing HHT3 and HHT4 cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The detailed microarray platform, hybridization, quality control, data acquisition and data filtering were performed essentially as previously described (28). Telomerase activity assays were performed on the cells using a real-time PCR based assay (29). This assay utilizes the TRAPeze® XL Telomerase detection kit (Intergen, Purchase, NY) modified for fluorescence detection from a probe as the telomerase performs its telomere lengthening function with a Sequence Detection System 7700 instrument (Applied Biosystems, Foster City, CA). Each assay was performed using 1.5µg of total cellular protein, in triplicate, with a heat-inactivated sample used as a negative control. NCI-H1299 cells were used as the quantitation standard for the assay. All values were reported as the cell equivalent activity of NCI-H1299 cells. The detection limit of the assay was 5 NCI-H1299 cell equivalents.

Colony formation and soft agar assays

HHT4 cells were infected with retroviruses containing HBx mutants (MOI=1) and selected by hygromycin (50µg/ml). Drug-resistant colonies appearing 3 weeks later were fixed, stained and counted. Cells from five clonally derived CT lines (10,000 cells per 10cm plate) were mixed with 0.5% agar and seeded on 0.8% agar at bottom layer. Colony formation was monitored under the microscope. Three weeks later, the plates were fixed, stained and colonies were counted. To recover colonies from soft agar cultures, colonies were randomly chosen and picked up under the microscope using 200µl pipette tips. They were then incubated with 50µl E-PET for 5 minutes and cultured with HCM with 10% CDS in a 37 °C incubator supplemented with 5% CO₂. Each cell line was tested in triplicate.

Western blotting and RT-PCR

Proteins from total cell extracts were fractionated on 16% SDS-PAGE gels, followed by transfer and blocking in 5% non-fat dry milk. Blots were probed with anti-M2 antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibody. Antibody-antigen complexes were detected by enhanced chemiluminescence (ECL) (GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol.

Metaphase preparation and conventional G-banding analysis

Metaphase preparations were obtained for cytogenetic studies using standard chromosome harvest techniques for both the HHT3 and HHT4 cell lines (30). Chromosomal G-banding was performed using a trypsin pretreatment followed by Giemsa staining protocol (30). For each cell line, 20 metaphases were analyzed. Chromosomes were identified and classified according to the nomenclature proposed by the International System for Human Cytogenetic Nomenclature (ISCN) (31).

Spectral karyotyping (SKY) and FISH analyses

SKY was performed as previously described (32). In brief, twenty-four human chromosome-specific DNA libraries were generated by bivariate, high-resolution flow sorting and amplified using degenerate oligonucleotide primed PCR. DNA labeling was performed by directly incorporating haptenized or fluorochrome-conjugated dUTPs. The differentially labeled probe sets were combined and precipitated in the presence of an excess of unlabeled human Cot-1 DNA (Invitrogen, Carlsbad, CA). Hybridization and post-hybridization washes were completed as previously described (32). Spectral images were acquired with a SD200 SpectraCube system (Applied Spectral Imaging, Vista, CA) coupled via a c-mount adapter to a Leica DMRBE microscope followed by spectrabased classification. FISH analysis using whole chromosome painting probes for chromosomes 1 and 16, and subtelomeric probes for

1p and 16q was performed in separate experiments, using commercially available probes (Vysis, Inc.) according to standard laboratory protocols.

RESULTS

Establishment of hTERT-immortalized human hepatocyte-derived cell lines

We attempted to establish immortalized human liver cell lines from freshly-isolated primary normal hepatocytes by expressing a human telomerase reverse transcriptase gene (hTERT) as this gene could effectively immortalize normal human fibroblasts and epithelial cells. A modified retroviral vector (pCLXSN) (26) was used as it contained a human cytomegalovirus immediate early promoter (CMV) upstream of the multiple cloning site which allows more robust, long-term expression of hTERT. Two batches of donor hepatocytes were used for infection and selection under G418. Among many colonies that initially survived G418 selection, only two independent clones were capable of expanding into mass cultures (data not shown). These clones were designated as HHT3 and HHT4, respectively. These cells were passaged for more than 12 months in a serum-free LCM medium. They displayed epithelial morphology reassembly of primary cultured hepatocytes (Fig 1A), expressed telomerase activity as determined by the TRAP assay (Fig 1B) and retained some of the hepatocytes markers, i.e., immunopositivity for cytokeratin 14 and 18 but were unable to grow in soft agar and were not tumorigenic in athymic nude mice (data not shown).

Further characterization of HHT3 and HHT4 cells revealed that these cells appeared to have normal cellular features. For example, they showed the same feature of contact inhibition as immortalized normal human fibroblasts (Fig 2A). Electron microscope analysis indicated that HHT4 cells shared features found in primary hepatocytes such as abundant glycogen granules in the cytoplasm (Fig 2A, b-c). Both HHT3 and HHT4 were diploid/near diploid as determined by G-band metaphase analysis. In 20 metaphases of HHT3 cells examined, we observed a non-mosaic abnormal male karyotype with extra material of unknown origin attached to 16q. Twenty four color fluorescence *in situ* hybridization (FISH) analysis by spectral karyotyping (SKY) showed that the additional material on the abnormal chromosome 16 was derived from chromosome 16 (data not shown). Subsequent FISH analysis with chromosome 16 whole chromosome painting probe confirmed the above SKY finding. FISH analysis with a 16q subtelomere probe showed no signal on the abnormal 16q. Information from these FISH studies as well as the G-band analysis permitted us to reinterpret the abnormal chromosome 16 to have an inverted duplication of the long arm from band q22 to q23 with accompanying loss of 16q24 (Fig 2A, d-f). All 20 metaphases of HHT4 cells examined had the following clonal chromosomal abnormalities: a structural rearrangement of 1p, loss of chromosome 6 (monosomy 6) and an apparent terminal deletion of 9p. SKY analysis of HHT4 cells showed that the rearranged 1p consisted entirely of chromosome 1 material. FISH analysis with whole chromosome 1 painting probes confirmed the SKY results, and FISH analysis with a 1p subtelomere probe showed absence of signal on the structurally abnormal chromosome 1. These tests suggested that the abnormality on chromosome 1 is an inverted duplication of the short arm from band p13 to p34.3 with accompanying loss of the material from p35 to the p terminus (Fig 2A, g-i). In summary, we concluded that HHT3 had a karyotype of 46, XY, der(16)dup(16)(q23 q22)del(16)(q24) and HHT4 had a karyotype of 45, XY, der(1)dup(1)(p34.3 p13)del(1)(p35), -6, del(9)(p21). HHT3 and HHT4 had few apparent structural and/or numerical chromosomal abnormalities which may represent a random event during immortalization with hTERT.

To further determine whether HHT cells express hepatocyte-specific genes, we performed cDNA microarray analysis by comparing HHT3 and HHT4 to an hTERT-immortalized

normal human fibroblast cell line (hTERT-NHF). A total of 201 genes had a ≥ 2 -fold increased expression in HHT3 or HHT4 cells as compared to hTERT-NHF (data not shown). Among them, 28 (14%) (Fig 2B) were known genes specific for hepatocytes (33). For example, the most abundantly expressed gene was tissue factor pathway inhibitor 2 (TFPI2) followed by alcohol dehydrogenase 1B (ADH1B), apolipoprotein L (APOL), glutathione S-transferase (GST), aldo-keto reductase (AKR) family, P450 cytochrome genes, including CYP7B1, CYP1B1, CYP3A7, CYPB5, CYP2CB and CYP2C18, and epithelial-specific markers such as fibronectin (Fig 2B). Thus, there is an enrichment of hepatocyte specific gene expression associated with HHT3 and HHT4 cells.

Expression of tumor-derived HBx and p53-249^{ser} mutants in HHT4 cells

To improve success in obtaining clones that stably express HBx recombinant DNA, we constructed retroviral vectors encoding various tumor-derived HBx mutants and their corresponding variants from the same individuals, which were previously described as CT, CNT, MT and MNT, respectively (20). Specifically, CNT encodes a wild-type HBx gene isolated from non tumor tissue in patient C and CT is the corresponding HBx mutant isolated from tumor tissue of the same patient. Similarly, MNT is a HBx gene isolated from non-tumor tissue in patient M and MT is the corresponding HBx mutant isolated from tumor tissue of the same patient. We also constructed a retroviral vector encoding a p53-249^{ser} gene, a signature mutant allele frequently occurring in HCC from areas where AFB1 food contamination and HBV chronic infection were prevalent (4). HHT4 cells were infected individually with equal amounts of retroviruses (MOI=1) encoding various mutants or a control virus (vector) followed by a selection with hygromycin. Drug-resistant colonies appearing 3 weeks later were fixed, stained and counted. Reproducibly, one tumor-derived HBx mutant CT significantly increased colony-forming efficiency, while its variant CNT, which was derived from the same patient, significantly decreased colony-forming efficiency when compared to a vector control virus (Fig 3A-B). While the MT mutant had a minimum activity, the corresponding MNT also appeared to inhibit HHT4 cell colony formation although the difference was not significant. Consistent with previous published reports (34;35), p53-249^{ser} significantly induced HHT4 cell colony formation (Fig 3A-B). These results indicate that a mutation in HBx can enhance colony formation in immortalized HHT4 cells. Interestingly, the CNT-mediated growth inhibitory activity in HHT4 cells could be reversed by expression of p53-249^{ser} (Fig 3C). Co-expression of p53-249^{ser} further enhanced CT-mediated colony-forming efficiency (Fig 3C). However, when we monitored cell cycle progression of HHT4 cells expressing HBx mutants and/or p53-249^{ser} mutant as determined by bromodeoxyuridine (BrdU) pulse labeling followed by flow cytometric analysis, we found minimum cell cycle perturbation or change in sub-G1 population indicative of apoptosis (data not shown). It appears there is no strong effect of HBx mutants or p53-249^{ser} mutant on cell cycle transition or apoptosis. Nevertheless, the p53-249^{ser} mutant appeared to act cooperatively with the tumor-derived HBx mutant CT in promoting HHT4 cell colony formation.

In vitro cellular transformation of HHT4 cells by CT and p53-249^{ser}

An ability to grow in soft agar is one of the hallmarks of *in vitro* cellular transformation of normal human cells. The soft agar growth potential of HHT4 cells expressing various HBx mutants was monitored. Colonies arising from the vector control, CT, MT, or MNT-infected HHT4 cells were isolated and attempts were made to expand them into mass cultures. No viable surviving colonies could be isolated from CNT-expressing cells thus were not included for further studies. Similarly, no viable survival colonies from p53-249^{ser} virus alone infected cultures could be expanded into a mass culture. Thus, we were unable to assess clonally derived HHT4 cells stably expressing only p53-249^{ser} mutant. The clonally derived cells expressing CT, MT or MNT were then infected with a control retrovirus or

p53-249^{ser} virus, and subsequently mixed with 0.5% agar and seeded on 0.8% agar at bottom layer. After 3 weeks, the plates were fixed, stained and the number of viable colonies was counted. Representative colonies growing on soft agar expressing CT alone or co-expressed with p53-249^{ser} are shown in Fig 4A, and quantitative histograms are shown in Fig 4B. While no colony growth was observed in the parental cell line or 3 clones derived from the vector-infected HHT4 cells, expression of the CT, MT and MNT mutants resulted in an induction of soft agar colony formation with a much more pronounced effect by the CT mutant. About 2 in 10,000 seeded MT or MNT-expressing cells could form colonies in soft agar. In contrast, 3 of 5 clones of CT-expressing cells formed colonies in soft agar efficiently. Interestingly, infection of these cells with p53-249^{ser} appeared to improve their ability to grow in soft agar, with more pronounced effects observed in CT-expressing cells but minimum effects in MT or MNT-expressing cells (Fig 4B). Expression of p53-249^{ser} alone had a minimum effect on soft agar growth. Expression of HBx in HHT4 cells infected with retroviral vectors encoding various HBx variants was verified by western blotting analysis (Fig 5A). In addition, expression of HBx in CT stably transduced clones, i.e., CT1-1, CT2-2, CT2-4 and CT2-5, was verified by RT-PCR analysis (Fig 5B).

It appeared that G-banding and 24-color FISH analyses including two additional p53-249^{ser}-expressing CT2-4 derivative clones independently isolated from soft agar culture, i.e., CT2-4-1, CT2-4-3, indicated that additional recurring chromosome abnormalities were acquired after an introduction of HBx and p53 mutants (Table 1). While additional persistent abnormal chromosome abnormality was not visible in CT2-4 cells as compared to parental HHT4 cells, translocation involved in chromosome 15 (M2) and deletion in chromosome 9 (M3) were frequent events in 3 CT2-4 derivatives expressing p53-249^{ser} (Table 1). An induction of aneuploidy could be observed in HHT4 cells expressing CT and p53-249^{ser} mutants. We also performed karyotype analyses in HHT4 cells stably expressing only p53-249^{ser} as a control. Since single colony-derived cells could not be expanded into mass culture, we combined all survival colonies infected with p53-249^{ser} mutant virus for karyotype analyses. Interestingly, we found that p53-249^{ser} expressing cells had recurrent loss of chromosomes 4 and 13. These changes appear unique to p53-249^{ser} since they were absent in CT2-4 derivatives (Table 1).

Karyotype analysis of HHT4 cells expressing CT and p53-249^{ser}

G-band karyotype analysis was performed on CT2-4 as well as CT2-4-1, CT2-4-2, and CT2-4-3, which are derivative clones of CT2-4 expressing p53-249^{ser} that were recovered from soft agar culture. While HHT4 cells had a mean chromosome count of 45, CT2-4 and CT2-4-2 cells had a mean chromosome count of 44 and 43, respectively, with an increase in the percent of aneuploid cells, as determined by metaphase counting analysis (Fig 6A-B). Twenty-four color FISH assisted in further characterization of structural abnormalities (Fig 6C). All cell lines were lacking a normal copy of chromosome 1 which was replaced by a complex structurally abnormal chromosome 1 with duplication and deletion of material. Furthermore, all cell lines were lacking chromosome 6. CT2-4-3 was identical to the parental HHT4 cell line. CT2-4-1 and CT2-4-2 had an additional anomaly comprised of a rearrangement between chromosome 15 and 20. Therefore, additional recurring chromosomal abnormalities were acquired in CT2-4-1 and CT2-4-2 after the introduction of a p53 mutant to CT2-4 (Table 1). The presence of p53-249^{ser} mutant in CT-expressing cells was confirmed using a diagnostic restriction enzyme digestion approach based on RT-PCR and *Hae*III digestion as previously described (data not shown) (36).

DISCUSSION

In this study, we have shown that by using a newly established telomerase-immortalized human hepatocyte-derived cell line (HHT4), the tumor-derived HBx mutant CT significantly

increased colony formation of HHT4 cells, while the non-tumor-derived variant (CNT) derived from the same carrier patient significantly decreased colony formation. Coexpression of p53-249^{ser} rescued CNT-induced colony formation inhibition. Expression of p53-249^{ser} enhanced soft agar growth in HHT4 cells expressing CT, while cells expressing CT alone grew poorly in soft agar. The cloned CT cells coexpressing p53-249^{ser} were aneuploid and showed additional abnormal changes in chromosome 15. However, HHT4 cells expressing p53-249^{ser} alone have lost one copy of chromosome 4 and one copy of chromosome 13 and these changes appear unique to p53 mutant alone (Table 1). Our results are consistent with the hypothesis that CT or p53-249^{ser} mutant alone could induce random chromosome changes but a right combination of chromosome changes when acting together may provide a favorable condition and promote neoplastic transformation.

The newly established telomerase-immortalized human hepatocyte-derived cell line HHT4 has proven to be a good *ex vivo* model to study liver carcinogenesis. Previous studies related to HBV-associated liver carcinogenesis were mainly involved in liver tumor-derived cell lines or non-liver cell lines (8;17;20;21). These cell lines could not truly reflect the normal process in liver carcinogenesis. This is largely due to the unavailability of a good normal liver-derived cell culture system. We have shown that this newly established cell line HHT4 is near diploid, expresses hepatocyte-specific genes, does not grow in soft agar and is not tumorigenic in nude mice.

The observation of suppression of colony formation by wild-type HBx in HHT 4 cells is consistent with previous reports (17;20;21). It has been reported that overexpression of wild-type HBx can induce or enhance apoptosis in a p53-dependent or p53-independent manner (17;20). Furthermore, HBx expression can induce a late G1 cell cycle block prior to their counterselection by apoptosis (17).

In our study, we also found that one tumor-derived HBx mutant CT increased colony formation in HHT4 cells, which is different from previous studies. The tumor-derived HBx mutant CT contains point mutations in multiple domains of HBx. One possibility is due to the *ex vivo* model used in this study. As mentioned before, the HHT4 cell line may more accurately reflect the natural process of human liver carcinogenesis. Previous studies from our group and others have shown that these tumor-derived HBx mutants exhibit attenuated transactivation and pro-apoptotic functions, but retained their ability to block p53-mediated apoptosis (17;20;37). Our result further supports the idea that mutations in HBx may contribute to the development of hepatocellular carcinoma.

The observation of a cooperative effect of tumor-derived HBx mutants and p53 249^{ser} mutant in both colony formation and growth in soft agar supports the following hypothesis. The mutation in HBx may provide a growth advantage to those liver cells with mutation in p53. Epidemiological studies as well as molecular studies have shown that there is a high correlation between HBx mutations and p53 mutation at codon 249 (38–41). However, these cells failed to produce any tumor when 2×10^6 cells of these clones were injected subcutaneously into either nude mice or NOD/SCID mice (data not shown). Taken together, these results indicate that HBx mutants and p53-249^{ser} mutant are weak oncogenes but forced expression of these genes can improve cell viability and anchorage-independent growth. Additional steps may be needed for the tumorigenic conversion of HHT4 cells.

An earlier study by Ponchel and colleagues indicates that p53-249^{ser} mutant increases cell survival and mitotic activity in a p53-deficient hepatoma cell line, Hep3B (35). Interestingly, when we monitored cell cycle progression of HHT4 cells expressing HBx mutants and/or p53-249^{ser} mutant as determined by BrdU pulse labeling followed by flow cytometric analysis, we found minimum cell cycle perturbation or change in sub-G1 population as an

indicator of apoptosis (data not shown). It appears there is no strong effect of HBx mutants or p53-249^{ser} mutant on cell cycle transition or apoptosis in hTERT-immortalized normal hepatocyte derived cells. In contrast, single colonies derived from p53-249^{ser} transduced HHT4 cells always fail to propagate into a mass culture, regardless of repeated attempts. The reason for failure to expand p53-249^{ser} mutant expressing HHT4 cells is unclear, but it suggests that cell-cell contact may be critical in supporting cell growth in p53-249^{ser} expressing HHT4 cells. It also suggests that the effect of HBx and/or p53-249^{ser} mutant on cell growth is complex and that the carcinogenic activity of these genes must be subtle and it may take several cell divisions to reach full effect. We speculate that cell type differences (i.e., normal liver cells vs HCC cells) may contribute to the observed discrepancy.

It is interesting to note that hot-spot mutations in HBx at codons 130 and 131, corresponding to lysine to methionine and valine to threonine substitutions, respectively, are prevalent in HCC cases from Qidong, China, where p53-249^{ser} mutation and the HBV genotype C are common (42). In contrast, the CT mutant, derived from an individual with HBV genotype A from an area where p53-249^{ser} mutations are not common, contains multiple amino acid substitutions including those at codons 130 and 131 from lysine to methionine and valine to threonine, respectively. The biological difference among these various mutants is unclear at the present time and thus it is cautionary when interpreting the data presented. Future studies are needed to explore the oncogenicity of these mutations.

In summary, our study indicates that certain tumor-derived HBx mutants, not their wild-type variants, may contribute to liver carcinogenesis by cooperating with a hot-spot p53 mutant, i.e., p53-249^{ser} commonly found to be associated with chronic HBV carriers. The finding of a tumor-derived HBx mutant CT in promoting cellular transformation of normal human hepatocytes is important as it may provide a rationale to develop a diagnostic tool by detecting this allele for liver carcinogenesis. It will be interesting to determine molecular profiles of these tumor-derived HBx mutants in telomerase-immortalized human hepatocytes, similar to those published previously (43–46).

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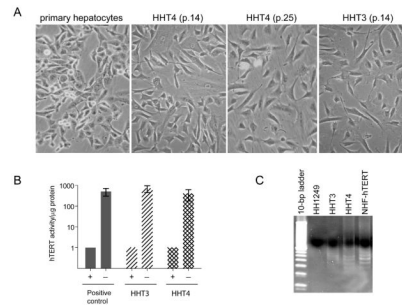


Figure 1. Establishment of hTERT-immortalized human hepatocyte-derived cell lines. (A) Morphological characteristics of primary cultured human hepatocytes and their hTERT-immortalized derivatives HHT3 and HHT4 at different passages. (B) Telomerase activities of HHT3 and HHT4 cells determined by the telomeric repeat amplification protocol (TRAP) assay. (C) Representative amplification products, as analyzed by electrophoresis in a non-denaturing 10% polyacrylamide gel, from (B) are shown. The primary human hepatocytes (HH1249) are included as a negative control, and hTERT-immortalized NHF cells (NHF-hTERT) as a positive control.

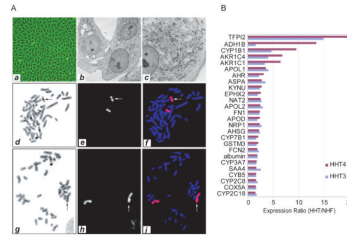


Figure 2. Characterization of HHT3 and HHT4 cells. (A) Representative phase-contrast cell image (a) and electron micrographs (b, c) of HHT4 cells are shown. Representative images of a mitotic HHT3 cell (d-f) and a mitotic HHT4 cell (g-i) analyzed by fluorescence in situ hybridization (FISH) with a whole chromosome 16 painting probe or whole chromosome 1 painting probe, respectively. (B) Expression profiles of hepatocyte-specific genes in HHT3 and HHT4 cells analyzed by cDNA microarray. The expression ratios of HHT3 or HHT4 cells over hTERT-immortalized normal human fibroblasts (NHF) are shown. Hepatocyte-specific genes were selected based on a SAGE transcript profile of freshly isolated and cultured normal primary human hepatocytes (33).

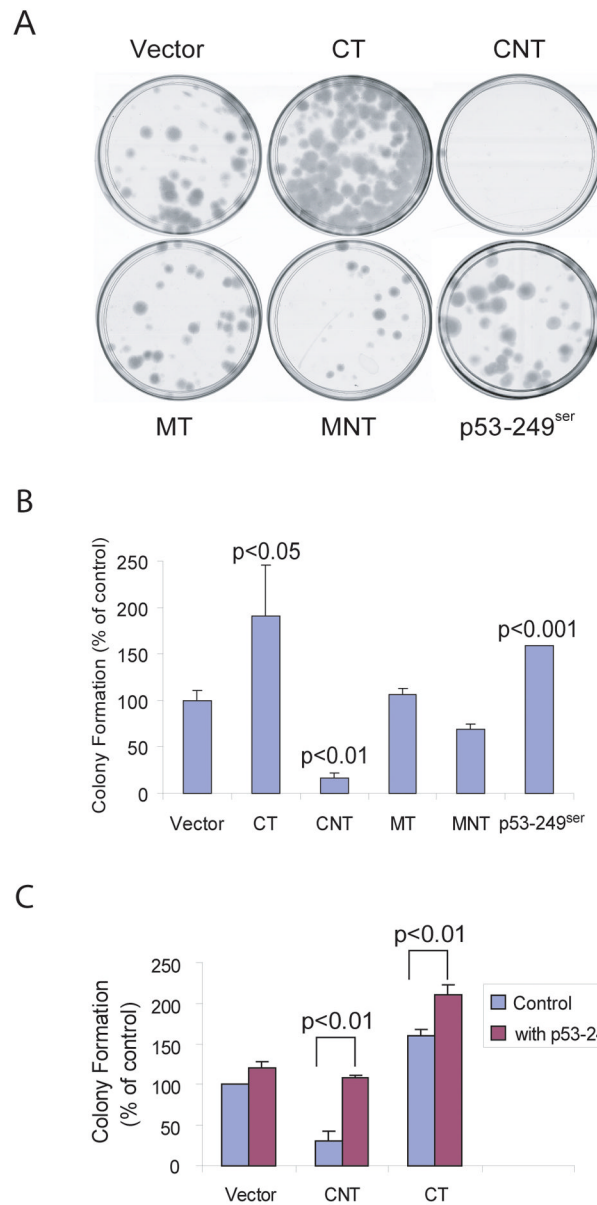


Figure 3. Effect of p53-249ser mutant or various tumor-derived HBx mutants on HHT4 cell growths. (A) Representative cultured plates with HHT4 cell colonies expressing various recombinant genes. (B) Colony formation efficiency of HHT4 cells expressing CT, CNT, MT, MNT or p53-259ser mutants. (C) Effect of p53-249ser on colony formation of HHT4 cells expressing CNT or CT.

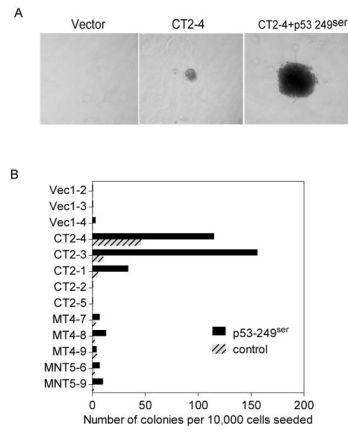


Figure 4. Anchorage-independent growth of HHT4 cells stably expressing CT, MT or MNT with or without expression of p53-249ser mutant. (A) Representative colonies of a CT stably expressing HHT4 clone CT2-4 in soft agar or coexpressing p53-249ser. (B) Six CT-transduced clones, 3 MT-transduced clones and 2 MNT-transduced clones from HHT4 cells alone with 3 vector control clones were evaluated for colony formation in soft agar.

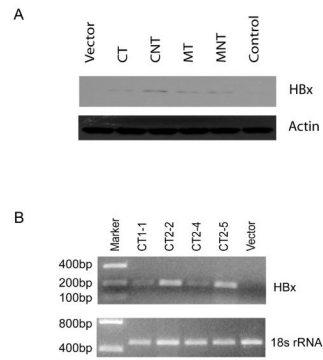


Figure 5. Expression of HBx in HHT4-derived clones stably transducing various HBx mutants analyzed by western blot (A) or qRT-PCR (B).

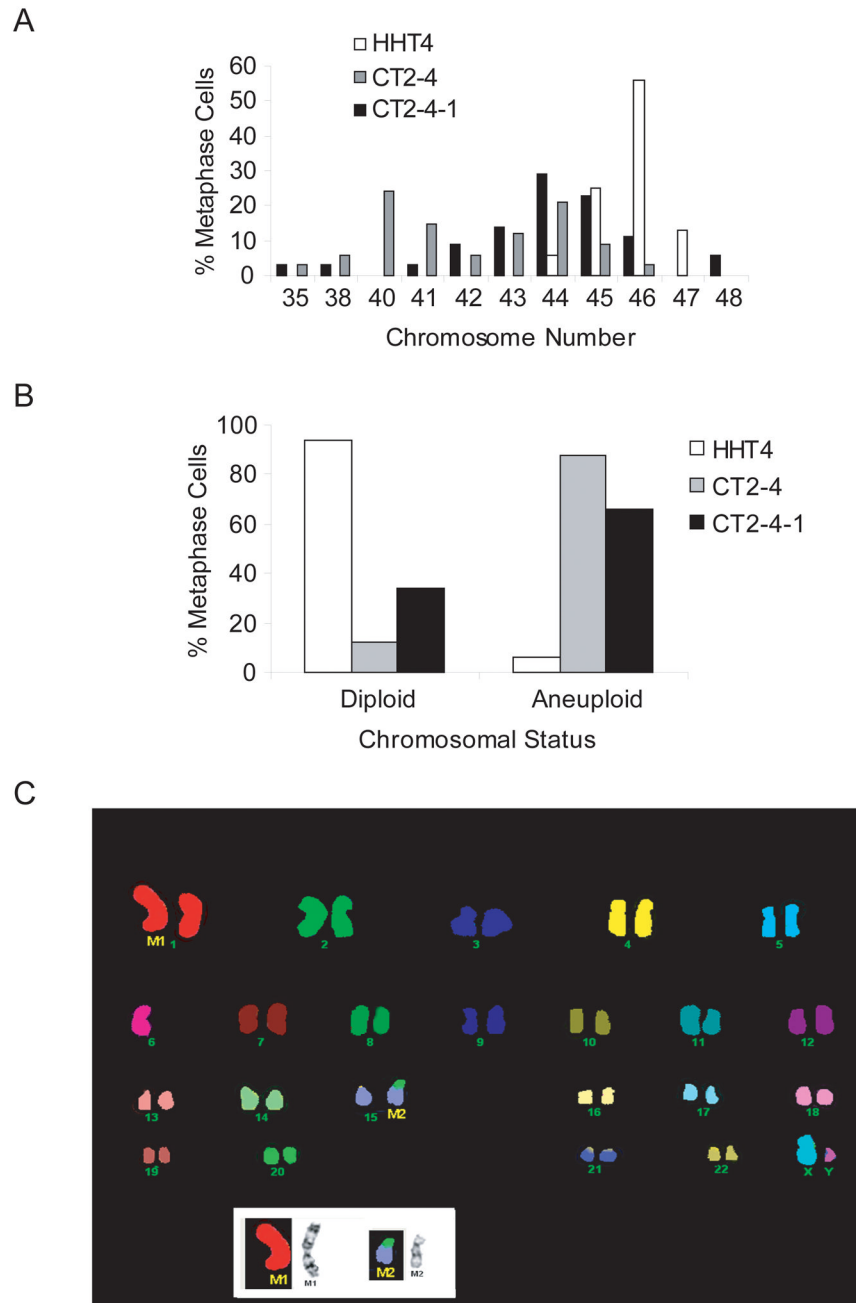


Figure 6. Aneuploidy in hTERT-immortalized normal human hepatocytes expressing a tumor-derived HBx mutant and p53-249ser hot-spot mutant. (A, B) Percent of metaphase cells derived from HHT4, CT2-4 (CT) and CT2-4-1 (CT+p53-249ser) cells with abnormal chromosome numbers. (C) Spectral karyotyping of a representative metaphase from CT-2-4-1 cells.

Table 1

Karyotypes of HHT4 cells expressing CT and p53-249^{ser}

Cells	der1	-4	-6	del9	-13	del15
HHT4	x	-	x	x	-	-
CT2-4	x	-	x	-	-	-
CT2-4-1	x	-	x	-	-	x
CT2-4-2	x	-	x	-	-	x
CT2-4-3	x	-	x	x	-	-
HHT4 + p53-249 ^{ser}	x	x	x	x	x	-