Table 3 Levels of HBV-related markers before, during and after treatment

Case	HBV DNA (log copies/mL)			HBeAg				HBcrAg (log U/mL)				Outcome
	Before	End of Lam	End of IFN	24 w after Tx.	Before	End of Lam	End of IFN	24 w after Tx.	Before	End of Lam	End of IFN	24 w after Tx.	
1	4.8	<3.7	<3.7	<2.6	(+)	(−)	(−)	(−)	5.8	5.1	4.5	4.2	SVR
2	7.3	<2.6	<2.6	2.7	(+)	(−)	(−)	(−)	>7.0	4.3	4.0	ND	SVR
3	8.5	<3.7	4.1	3.8	(+)	(+)	(+)	(−)	>7.0	6.2	5.9	5.7	SVR
4	8.6	<2.6	<2.6	4.2	(+)	ND	(−)	(−)	ND	5.9	5.0	4.2	SVR
5	7.6	<3.7	<3.7	2.6	(−)	(−)	(−)	(−)	6.9	5.4	4.1	3.6	SVR
6	8.5	<2.6	<2.6	2.9	(−)	(−)	(−)	(−)	ND	4.1	3.9	3.6	SVR
7	6.3	<2.6	3.4	3.4	(−)	(−)	(−)	(−)	6.6	4.6	4.6	4.5	SVR
8	8.7	<3.7	<3.7	7.6>	(+)	(−)	(−)	(+)	>7.0	4.4	4.2	>7.0	NR
9	5.7	<3.7	<3.7	5.3	(+)	(−)	(+)	(+)	6.2	5.1	5.0	6.7	NR
10	7.6	<3.7	7.6	7.7	(+)	(+)	(+)	(+)	>7.0	6.3	>7.0	>7.0	NR
11	7.5	<3.7	<3.7	6.1	(+)	(±)	(+)	(+)	6.6	5.2	5.2	5.1	NR
12	6.8	<2.6	3.5	7.6>	(+)	(+)	(+)	(+)	>7.0	4.7	ND	ND	NR

HBcrAg, hepatitis B virus core-related antigen; HBeAg, hepatitis B virus e antigen; HBV DNA, hepatitis B virus DNA; IFN, interferon; Lam, lamivudine; ND, not determined; NR, no response; SVR, sustained virological response; Tx., treatment; w, weeks.

DISCUSSION

ACUTE EXACERBATION OF CHB may cause liver failure, which results in a high mortality rate.[41] IFN monotherapy is contraindicated in this situation because interferon may upregulate the immune response and cause severe liver injury.[8–10] Hence, the immediate treatment with NA for such patients has become standard.[42] Clinical trials of lamivudine have shown that the acute exacerbation of CHB may result in an increased sustained remission rate.[43,44] Therefore, if the safe withdrawal of NA is possible, acute exacerbation may be a good opportunity for sustained remission. However, post-treatment flare, which may result in liver failure, renders lamivudine withdrawal difficult.[45] Consequently, once treatment is initiated, lifelong HBV suppression is necessary in most patients.

Interferon has both antiviral and immunomodulatory functions.[7,8] Additionally, IFN sometimes elevates liver enzymes by depressing hepatic microsomal cytochrome P-450 levels.[46] However, such effects rarely lead to liver failure in humans.[10] Therefore, lamivudine administration and subsequent IFN treatment may be a safe method of lamivudine withdrawal. Additionally, such treatment may result in a high rate of sustained remission of hepatitis. Indeed, Serfaty *et al.* conducted a lamivudine administration and subsequent IFN treatment and achieved a high rate of sustained response.[22]

Yotsuyanagi *et al.* reported the evaluation of short-term lamivudine administration and its withdrawal for genotype C CHB. Decreased serum ALT and HBV DNA level was observed in four of the 12 patients (33.3%) and three of the 12 patients achieved HBe seroconversion 24 weeks after treatment in HBe-positive patients. As for HBe-negative patients, decreased serum ALT and HBV DNA level was observed in five of six patients (83.3%) 24 weeks after treatment.[45] The sustained response in this study was 58.3% (44.4% in HBe-positive and 100% in HBe-negative patients), which is comparable to that shown in Serfaty's study. Furthermore, all our patients were also infected with genotype C HBV, which is resistant to antiviral treatment.[47,48] Although the number of enrolled patients is small, we may conclude that lamivudine administration and subsequent IFN treatment is an effective treatment for CHB.

Lamivudine-interferon combination therapeutic trials have been conducted to date.[19–32] Some large trials, however, did not show the superiority of combination therapy.[19,26] One of the reasons is that, in many such trials, lamivudine and IFN are administrated simultaneously. Lamivudine monotherapy can upregulate

cytotoxic T-cell response.[49] IFN treatment following viral suppression by lamivudine might induce a stronger immune response than a simultaneous treatment.

Another advantage of sequential therapy over monotherapy is that NA can be safely withdrawn. The long-term safety of NA has not been demonstrated yet. Furthermore, NA can generate drug-resistant mutants.[16–18] Hence, the withdrawal of NA is preferred if possible. Our study demonstrates that the safe withdrawal of lamivudine without generating drug-resistant mutants is possible. However, in one patient, a drug-resistant mutant was found at the end of the lamivudine treatment. NA administration during exacerbation might induce the early emergence of drug-resistant mutants. The change from lamivudine to entecavir, which rarely generates drug-resistant mutants in a short time, may prevent the emergence of drug-resistant mutants, but this should be prospectively studied.

Interestingly, all three anti-HBe-positive patients in this study achieved SVR. Previous studies have shown that anti-HBe-positive patients respond poorly to IFN treatment.[50,51] In contrast, our results suggest that a potent antiviral treatment using lamivudine-IFN combination over a long period may result in high SVR rate even in anti-HBe-positive patients. The antiviral effect caused by the sequential therapy may be sufficiently strong to maintain long-term remission. A larger cohort study should be performed to determine whether sequential therapy is really effective for anti-HBe-positive patients.

Comparison between SVR and NR patients showed that the serum albumin levels were lower in SVR patients than in NR patients. Although the difference was not significant, the prothrombin time was longer and the ALT level was higher in SVR patients. These results showed that SVR patients suffered more severe exacerbation than NR patients. Several studies have shown that a high ALT level is predictive of a favorable response to IFN treatment,[21,52,53] which is compatible with our results.

Age is another important determinant of the response to IFN for Japanese patients, as demonstrated in the Japanese national guideline. Therefore, we studied the effect of age on the response to sequential therapy. Younger patients tended to be more responsive to IFN than older patients. If a larger number of patients is studied, the effect of age on the efficacy of sequential treatment may be clarified.

Table 3 shows the time-dependent change in the level of viral markers. In HBeAg-positive patients, HBeAg at the end of the IFN treatment was predictive of the outcome in seven of nine patients; three of four HBeAg-negative patients achieved SVR and four of five HBeAg-positive patients showed NR. HBV DNA and HBcrAg levels were less predictive of the treatment outcome than HBeAg. A marked reduction or clearance of HBeAg during treatment is also important for SVR in the treatment using pegylated IFN.[28,54] HBeAg might affect the efficacy of long-term, potent IFN-based treatment.

We evaluated the temporal change in the HBcrAg level during IFN treatment for the first time. Although the number of enrolled patients was small, a continuous decrease in the HBcrAg level to less than 5 log U/mL may be predictive of the long-term response to IFN treatment, but this should be further studied.

In conclusion, lamivudine-IFN sequential therapy is effective for the treatment of CHB. This treatment may induce long-term remission of both HBeAg-positive and -negative patients and enable safe withdrawal of NA.

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REFERENCES

1 Lai CL, Ratziu V, Yuen MF, Poynard T. Viral hepatitis B. *Lancet* 2003; **362**: 2089–94.
2 Lok AS. Chronic hepatitis B. *N Engl J Med* 2002; **346**: 1682–3.
3 Lau DT-Y, Everhart J, Kleiner DE *et al.* Long-term follow-up of patients with chronic hepatitis B treated with interferon alfa. *Gastroenterology* 1997; **113**: 1660–7.
4 Lin SM, Sheen IS, Chien RN, Chu CM, Liaw YF. Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection. *Hepatology* 1999; **29**: 971–5.
5 Perrillo R. Benefits and risks of interferon therapy for hepatitis B. *Hepatology* 2009; **49** (Suppl 5): S1031–1.
6 Dienstag JL. Benefits and risks of nucleoside analog therapy for hepatitis B. *Hepatology* 2009; **49** (Suppl 5): S112–21.
7 Rang A, Bruns M, Heise T, Will H. Antiviral activity if interferon-α against hepatitis B virus can be studied in non – hepatic cells and independent of MxA. *J Mol Chem* 2002; **277**: 7645–7.
8 Peters M, Walling DM, Waggoner J, Avigan MI, Sjogren M, Hoofnagle JH. Immune effects of alpha – interferon in chronic liver disease. *J Hepatpl* 1986; **3** (Suppl 2): S283–9.

9 Wong DK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B: a meta-analysis. *Ann Intern Med* 1993; **119**: 312–23.
10 Quesada JR, Talpaz M, Rios A, Kurzrock R, Gutterman JU. Clinical toxicity of interferons in cancer patients: a review. *J Clin Oncol* 1986; **4**: 234–43.
11 Dienstag JL, Schiff ER, Wright TL *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; **341**: 1256–63.
12 Marcellin P, Chang TT, Lim SG *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; **348**: 808–16.
13 Chang TT, Gish RG, de Man R *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; **354**: 1001–10.
14 Fontana RJ. Side effects of long-term oral antiviral therapy for hepatitis B. *Hepatology* 2009; **49** (Suppl 5): S185–95.
15 van Nunen AB, Hansen BE, Suh DJ *et al.* Durability of HBeAg seroconversion following antiviral therapy for chronic hepatitis B: relation to type of therapy and pretreatment serum hepatitis B virus DNA and alanine aminotransferase. *Gut* 2003; **52**: 420–4.
16 Feld J, Lee JY, Locarnini S. New targets and possible new therapeutic approaches in the chemotherapy of chronic hepatitis B. *Hepatology* 2003; **38**: 545–53.
17 Leung NW, Lai CL, Chang TT *et al.* Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. *Hepatology* 2001; **33**: 1527–32.
18 Shin JW, Park NH, Jung SW *et al.* Clinical usefulness of sequential hepatitis B virus DNA measurement (the roadmap concept) during adefovir treatment in lamivudine-resistant patients. *Antivir Ther* 2009; **14**: 181–6.
19 Mutimer D, Naoumov N, Honkoop P *et al.* Combination alpha-interferon and lamivudine therapy for alpha-interferon-resistant chronic hepatitis B infection: results of a pilot study. *J Hepatol* 1998; **28**: 923–9.
20 Schalm SW, Heathcote J, Cianciara J *et al.* Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. *Gut* 2000; **46**: 562–8.
21 Barbaro G, Zechini F, Pellicelli AM *et al.* Long-term efficacy of interferon alpha-2b and lamivudine in combination compared to lamivudine monotherapy in patients with chronic hepatitis B. An Italian multicenter, randomized trial. *J Hepatol* 2001; **35**: 406–11.
22 Serfaty L, Thabut D, Zoulim F *et al.* Sequential treatment with lamivudine and interferon monotherapies in patients with chronic hepatitis B not responding to interferon alone: results of a pilot study. *Hepatology* 2001; **34**: 573–7.
23 Shi M, Wang RS, Zhang H *et al.* Sequential treatment with lamivudine and interferon-alpha monotherapies in hepatitis B e antigen-negative Chinese patients and its suppression of lamivudine-resistant mutations. lamivudine-resistant mutations. *J Antimicrob Chemother* 2006; **58**: 1031–5.
24 Tatulli I, Francavilla R, Rizzo GL *et al.* Lamivudine and alpha-interferon in combination long term for precore mutant chronic hepatitis B. *J Hepatol* 2001; **35**: 805–10.
25 Santantonio T, Niro GA, Sinisi E *et al.* Lamivudine/interferon combination therapy in anti-HBe positive chronic hepatitis B patients: a controlled pilot study. *J Hepatol* 2002; **36**: 799–804.
26 Schiff ER, Dienstag JL, Karayalcin S *et al.* Lamivudine and 24 weeks of lamivudine/interferon combination therapy for hepatitis B e antigen-positive chronic hepatitis B in interferon nonresponders. *J Hepatol* 2003; **38**: 818–26.
27 Niro GA, Fontana R, Gioffreda D *et al.* Sequential treatment with lamivudine and alpha-interferon in anti-HBe-positive chronic hepatitis B patients: a pilot study. *Dig Liver Dis* 2007; **39**: 857–63.
28 Janssen HL, van Zonneveld M, Senturk H *et al.* Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. *Lancet* 2005; **365**: 123–9.
29 Chan HL, Leung NW, Hui AY *et al.* A randomized, controlled trial of combination therapy for chronic hepatitis B: comparing pegylated interferon-alpha2b and lamivudine with lamivudine alone. *Ann Intern Med* 2005; **142**: 240–50.
30 Marcellin P, Lau GK, Bonino F *et al.* Peginterferon alfa-2a alone, lamivudine alone, and the two in combination in patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2004; **351**: 1206–17.
31 Lau GK, Piratvisuth T, Luo KX *et al.* Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2005; **352**: 2682–95.
32 Bonino F, Marcellin P, Lau GK *et al.* Predicting response to peginterferon alpha-2a, lamivudine and the two combined for HBeAg-negative chronic hepatitis B. *Gut* 2007; **56**: 699–705.
33 Liaw YF, Chu CM, Su IJ, Huang MJ, Lin DY, Chang-Chien CS. Clinical and histological events preceding hepatitis B e antigen seroconversion in chronic type B hepatitis. *Gastroenterology* 1983; **84**: 216–9.
34 Lok AS, Lai CL, Wu PC, Leung EK, Lam TS. Spontaneous hepatitis B e antigen to antibody seroconversion and reversion in Chinese patients with chronic hepatitis B virus infection. *Gastroenterology* 1987; **92**: 1839–43.
35 Kim HS, Kim HJ, Shin WG *et al.* Predictive factors for early HBeAg seroconversion in acute exacerbation of patients with HBeAg-positive chronic hepatitis B. *Gastroenterology* 2009; **136**: 505–12.
36 Yuki N, Nagaoka T, Nukui K, Omura M, Hikiji K, Kato M. Adding interferon to lamivudine enhances the early virologic response and reversion of the precore mutation in difficult-to-treat HBV infection. *J Gastroenterol* 2008; **43**: 457–63.

37 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40**: 439–45.
38 Kimura T, Rokuhara A, Matsumoto A *et al.* New enzyme immunoassay for detection of hepatitis B virus core antigen (HBcAg) and relation between levels of HBcAg and HBV DNA. *J Clin Microbiol* 2003; **41**: 1901–6.
39 Kobayashi S, Shimada K, Suzuki H, Tanikawa K, Sata M. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). *Hepatol Res* 2000; **17**: 31–42.
40 The French METAVIR Cooperative Study Group. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. *Hepatology* 1994; **20**: 15–20.
41 Sheen IS, Liaw YF, Tai DI, Chu CM. Hepatic decompensation associated with hepatitis B e antigen clearance in chronic type B hepatitis. *Gastroenterology* 1985; **89**: 732–5.
42 Degertekin B, Lok AS. Indications for therapy in hepatitis B. *Hepatology* 2009; **49** (Suppl 5): S129–37.
43 Liaw YF. Results of lamivudine trials in Asia. *J Hepatol* 2003; **39**: S111–5.
44 Chang TT, Lai CL, Chien RN *et al.* Four years of lamivudine treatment in Chinese patients with chronic hepatitis B. *J Gastroenterol Hepatol* 2004; **19**: 1276–84.
45 Yotsuyanagi H, Yasuda K, Iino S. Short-term lamivudine for the treatment of chronic hepatitis B. *Intervirology* 2003; **46**: 367–72.
46 Ghezzi P, Bianchi M, Gianera L, Landolfo S, Salmona M. Role of reactive oxygen intermediates in the interferon-mediated depression of hepatic drug metabolism and protective effect of N-acetylcysteine in mice. *Cancer Res* 1985; **45**: 3444–7.
47 Erhardt A, Reineke U, Blondin D *et al.* Mutations of the core promoter and response to interferon treatment in chronic replicative hepatitis B. *Hepatology* 2000; **31**: 716–25.
48 Sánchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodés J. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. *Gastroenterology* 2002; **123**: 1848–56.
49 Boni C, Bertoletti A, Penna A *et al.* Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. *J Clin Invest* 1998; **102**: 968–75.
50 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2001; **34**: 1225–41.
51 Fattovich G, McIntyre G, Thursz M *et al.* Hepatitis B virus precore/core variation and interferon therapy. *Hepatology* 1995; **22**: 1355–62.
52 Perrillo RP, Schiff ER, Davis GL. A randomized, controlled trial of interferon alfa-2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. *N Engl J Med* 1990; **323**: 295.
53 Lok AS, Wu PC, Lai CL *et al.* A controlled trial of interferon with or without prednisone priming for chronic hepatitis B. *Gastroenterology* 1992; **102**: 2091–7.
54 Fried MW, Piratvisuth T, Lau GK *et al.* HBeAg and hepatitis B virus DNA as outcome predictors during therapy with peginterferon alfa-2a for HBeAg-positive chronic hepatitis B. *Hepatology* 2008; **47**: 428–34.

Expression of Pituitary Tumor–Transforming Gene 1 (PTTG1)/Securin in Hepatitis B Virus (HBV)-Associated Liver Diseases: Evidence for an HBV X Protein–Mediated Inhibition of PTTG1 Ubiquitination and Degradation

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Chronic infection with hepatitis B virus (HBV) is strongly associated with hepatocellular carcinoma (HCC), and the viral HBx protein plays a crucial role in the pathogenesis of liver tumors. Because the protooncogene pituitary tumor–transforming gene 1 (PTTG1) is overexpressed in HCC, we investigated the regulation of this protein by HBx. We analyzed PTTG1 expression levels in liver biopsies from patients chronically infected with HBV, presenting different disease stages, and from HBx transgenic mice. PTTG1 was undetectable in biopsies from chronic hepatitis B patients or from normal mouse livers. In contrast, hyperplastic livers from transgenic mice and biopsies from patients with cirrhosis, presented PTTG1 expression which was found mainly in HBx-expressing hepatocytes. PTTG1 staining was further increased in HCC specimens. Experiments *in vitro* revealed that HBx induced a marked accumulation of PTTG1 protein without affecting its messenger RNA levels. HBx expression promoted the inhibition of PTTG1 ubiquitination, which in turn impaired its degradation by the proteasome. Glutathione S-transferase pull-down and co-immunoprecipitation experiments demonstrated that the interaction between PTTG1 and the Skp1–Cul1–F-box ubiquitin ligase complex (SCF) was partially disrupted, possibly through a mechanism involving protein–protein interactions of HBx with PTTG1 and/or SCF. Furthermore, confocal analysis revealed that HBx colocalized with PTTG1 and Cul1. We propose that HBx promotes an abnormal accumulation of PTTG1, which may provide new insights into the molecular mechanisms of HBV-related pathogenesis of progressive liver disease leading to HCC development. (HEPATOLOGY 2010;51:777-787.)

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide.[1] Chronic infection with hepatitis B virus (HBV) is the main causal factor for HCC.[1] A growing body of evidence suggests that HBV may have a direct oncogenic capacity and that expression of virally encoded proteins, in particular the HBV X protein (HBx), promotes cell growth and tumor development.[2] Importantly, HBx expression is retained after viral integration into hepatocyte DNA[3] and is one of the most prevalent virus antigens in the liver and tumors of HBV carriers, and may induce humoral and cellular immune responses.[2] HBx alters sev-

Abbreviations: Ab, antibody; CFP, cyan fluorescent protein; Dox, doxycycline; GFP, green fluorescent protein; HA, hemagglutinin; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; mRNA, messegner RNA; OA, okadaic acid; PP2A, protein phosphatase 2A; PTTG1, pituitary tumor–transforming gene 1; RT-PCR, reverse-transcription polymerase chain reaction; SCF, Skp1–Cul1–F-box ubiquitin ligase complex; siRNA, small interfering RNA.

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eral host functions that may lead to the carcinogenic process, including cell proliferation, viability, DNA repair, and genome stability.[2] Although HBx does not bind directly to DNA, it may activate the transcription of a wide range of cellular genes by different mechanisms involving activation of signal transduction pathways or direct interaction with components of the transcriptional machinery.[2] Recently, it has been proposed that HBx may also alter gene expression by promoting epigenetic changes in the DNA methylation profile[4] or by enhancing the stability of transcription factors such as HIF-1α[5] and c-myc.[6] Thus, HBx expression results in transcriptional activation of a variety of cellular genes involved in inflammation, angiogenesis, fibrosis, oxidative stress, and tumor development and progression.[2]

Pituitary tumor–transforming gene 1 (PTTG1)-encoded protein, originally isolated from pituitary tumor cells,[7] was later identified as a human securin, a protein implicated in inhibition of sister chromatid separation during mitosis, which has been associated with malignant transformation and tumor development.[8] Furthermore, PTTG1 plays key roles in cellular growth, DNA repair, development, and metabolism.[9] Mechanisms of PTTG1 action include protein–protein interactions, transcriptional activity, and paracrine/autocrine regulation.[9] During mitosis and following chromosome alignment, PTTG1 is degraded by the proteasome at metaphase to anaphase transition through the anaphase-promoting complex/cyclosome, releasing inhibition of separase, which in turn mediates the proteolysis of the cohesins ring that holds sister chromatids together.[8] In nonmitotic cells, the Skp1–Cul1–F-box protein ubiquitin ligase complex (SCF) is involved in the degradation of phosphorylated forms of PTTG1.[10] Furthermore, the SCF complex is involved in PTTG1 turnover in cycle-arrested cells after ultraviolet radiation.[11] PTTG1 overexpression has been reported in a great variety of tumors in which it correlates with invasiveness,[9] and it has been identified as a key signature gene associated with tumor metastasis.[12] In HCC, PTTG1 is overexpressed, and its expression levels have prognostic significance for the survival of postoperative HCC patients.[13] Interestingly, it has been proposed that PTTG1 might be critically involved in the development of HCC through the promotion of angiogenesis.[13] PTTG1 specifically interacts with p53, both *in vitro* and *in vivo*, and inhibits the ability of p53 to induce cell death, demonstrating its oncogenic potential.[14] Additionally, PTTG1 overexpression in hepatoma cell lines negatively regulates the ability of p53 to induce apoptosis.[15] Considering that HBV infection and HBx protein are associated with HCC and that a relationship between PTTG1 expression levels and HCC exists, we analyzed whether HBx may alter PTTG1 expression in chronic HBV-infected patients, HBx transgenic mice, and HBV-containing or HBx-expressing cell lines to provide new insights into our understanding of the molecular pathogenic mechanisms of advanced liver disease associated with HBV chronic infection.

Patients and Methods

Patients, Transgenic Mice, and Immunohistological Assays. Fifteen patients with HBV-related chronic liver disease (five with chronic hepatitis, five with cirrhosis, and five with HCC) were included. HBx transgenic mice were derived by microinjection the HBx gene into fertilized eggs of CD-1 mice.[16] Immunohistological assays were performed by standard procedures.

Cell Culture. Chang liver, Chang liver pX-34 (p34x), AML12 4p and AML12 4pX cells (4pX) were grown as described.[17,18]

Plasmid and Transfections. The indicated expression vectors were transfected employing Lipofectamine Transfection Reagent according to the manufacturer's instructions.

Western Blot Analysis. Proteins were extracted and immunobloted using the indicated antibodies.

Cell Cycle Analysis. Growth profiles of propidium iodide–labeled cells were analyzed by means of flow cytometry.

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis. RNA extraction

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and quantitative reverse-transcription polymerase chain reaction (RT-PCR) were performed as described.[19]

Detection of PTTG1 Ubiquitination, Coimmunoprecipitation and Pull-Down Assays. Cleared lysates were subjected to immunoprecipitation with the indicated antibodies. The immunocomplexes were captured with protein A-sepharose. GST proteins were expressed in *Escherichia coli*, purified with glutathione-sepharose 4B, and incubated with cellular extracts. In both assays, bound proteins were analyzed by means of western blotting.

Immunofluorescence Analysis and Confocal Microscopy. Cells were grown on coverslips and processed as described.[19]

Small Interfering RNAs and Transfections. Cells were transfected with 100 nM ON-TARGET plus SMARTpool small interfering RNAs (siRNAs) directed against human Cul1 or a nonspecific control siRNA.

A detailed description of the protocols and reagents employed is provided in the Supporting Materials and Methods.

Results

PTTG1 Expression Level Increases in HBx-Immunoreactive Cells as Chronic Hepatitis B Progresses to Cirrhosis and HCC. We first investigated the expression of PTTG1 and HBx in human liver biopsies during HBV-related hepatocarcinogenesis by staining serial liver sections with anti-PTTG1 and anti-HBx antibodies (Abs). In specimens from patients with chronic hepatitis B and weak HBx expression, PTTG1 was not detected in hepatocytes (Fig. 1A). As chronic liver disease progressed from chronic hepatitis B to cirrhosis, PTTG1 protein appeared in HBx-immunoreactive hepatocytes (Fig. 1A). PTTG1 staining increased in HCC specimens showing high HBx expression (Fig. 1A). Double immunofluorescence studies in HCC specimens revealed that the distribution of PTTG1 fit well with the pattern shown by HBx immunolabeling (Fig. 1B).

PTTG1 Expression Level Increases as HBx Transgenic Mouse Livers Progress to Hyperplasia and HCC. HBx is considered one of the most important determinants of HBV-induced hepatocarcinogenesis. We further investigated the expression of PTTG1 and HBx during HBx-induced hepatocarcinogenesis in HBx transgenic mouse livers. Beginning at the age of 2 months, HBx transgenic mouse liver showed centrilobular foci of cellular alteration with cytoplasmic vacuolation surrounding the central veins where hepatocytes with increased DNA synthesis were detected.[16] PTTG1 and HBx were not detected in nontransgenic normal mouse livers. In hyperplastic HBx-transgenic mouse livers, expression of PTTG1 was found mainly in the cytoplasm of hepatocytes in the centrilobular region, and distribution of PTTG1 was similar to that of HBx (Fig. 2). Strong expression of both PTTG1 and HBx was observed diffusely in HCC specimens (Fig. 2). Double immunofluorescence studies in transgenic mouse–derived HCC specimens confirmed that PTTG1 and HBx are coexpressed in cancer cells (Supporting Fig. 1).

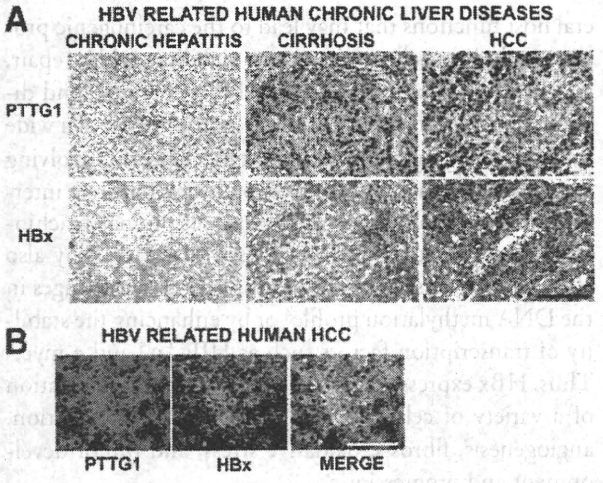

Fig. 1. PTTG1 expression levels increase as human chronic hepatitis B progresses to cirrhosis and HCC. (A) PTTG1 and HBx proteins were detected in human cirrhotic liver and HCC liver biopsies. PTTG1 protein appeared in HBx-immunoreactive hepatocytes in cirrhotic liver, and both proteins were strongly expressed in HCC specimens. Formalin-fixed, paraffin-embedded liver sections were stained with anti-PTTG1 Ab and anti-HBx. The HBx section was paired with an adjacent section stained using anti-PTTG1 Ab. Abs were then bound by goat anti-rabbit immunoglobulin G or by goat anti-mouse immunoglobulin G conjugated with peroxidase-labeled polymer. Peroxidase activity was detected using 3,3'-diaminobenzidine tetrahydrochloride. All sections were counterstained with hemotoxylin (blue). Brown coloring indicates specific Ab reactivity. We investigated five specimens of each chronic hepatitis B, cirrhosis, and HCC. Because HBx staining was not homogenous throughout the liver specimens of HCC and cirrhosis, selected fields with HBx expression are shown. Bar = 50 μm. (B) Hepatocytic PTTG1 in human HCC specimens colocalized with HBx. Human HCC sections were stained for immunofluorescence to simultaneously detect PTTG1 (red) and HBx (green). Yellow color indicates overlap of proteins. Bar = 50 μm.

HBx Expression Induces PTTG1 Accumulation. Because PTTG1 expression was increased during both HBV- and HBx-related chronic liver disease progression, we speculated that HBV and more precisely HBx might induce PTTG1 expression. We first examined whether a HBV replicon could induce PTTG1 expression. We transfected the hepatic-derived Chang liver cells with the plasmid payw1.2, which harbors 1.2 mer of the HBV genome that functions as an HBV replicon, and then evaluated PTTG1 expression by means of western blotting. The complete replicon induced the expression of

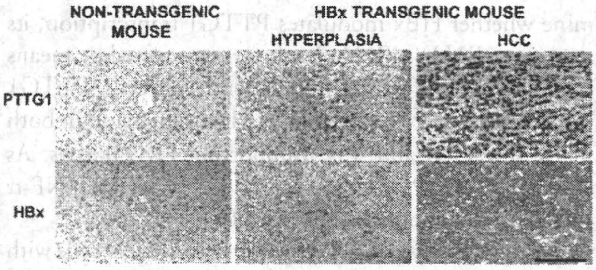

Fig. 2. PTTG1 oncoprotein increases as HBx transgenic mouse livers progress through hyperplasia to HCC. The distribution of PTTG1 and HBx in normal nontransgenic mouse liver, hyperplasia, and HCC specimens from HBx-transgenic mice is shown. PTTG1 was present mainly in the cytoplasm of hyperplastic hepatocytes immunoreactive for HBx oncoprotein surrounding central veins in HBx-transgenic mouse liver. Strong staining of both proteins was observed diffusely in HCC specimens. Immunohistochemical analyses were performed as described above. We investigated five biopsies each of normal, hyperplastic, and HCC liver. Because all of the results were similar among the experiments, representative results are displayed.

PTTG1 protein (Fig. 3A). Interestingly, PTTG1 expression in cells transfected with the HBx-defective whole-genome construct (payw*7) remained unchanged, indicating a role of HBx in PTTG1 induction (Fig. 3A). To further explore the effects of HBx on PTTG1 expression, we employed two hepatocyte-derived cell lines, Chang liver p34X (p34X) and AML12 4pX (4pX), in which HBx expression was controlled by doxicycline treatment (Dox-on) or withdrawal (Dox-off), respectively. Western blot analysis revealed increased PTTG1 expression upon induction of HBx over 48 hours in both Dox-regulated systems (Fig. 3B). Similar results were obtained after 24 hours of Dox treatment (Supporting Fig. 2A). As controls, we included Chang liver and AML12 4p cells—the parental cell lines of p34X and 4pX cells, respectively—and no PTTG1 variation after Dox challenge was observed. PTTG1 levels positively correlate with cell proliferation, and its expression is controlled in a cell cycle–dependent manner.[20] Several studies have also shown that HBx promotes cellular proliferation by triggering DNA synthesis and speeding up cell cycle progression.[21,22] However, evidence regarding the effects of HBx on liver cell proliferation and cell death is controversial, depending on the experimental systems and cell lines employed.[23] To assess the effect of HBx expression on cell cycle progression, we analyzed the growth profiles of Chang liver p34X and AML12 4pX cells with or without Dox treatment by means of flow cytometry. In agreement with previous reports,[24] our data showed that the percentages of p34X cells in G0/G1, S, and G2/M phases of the cell cycle displayed similar profiles 24 hours (Supporting Fig. 2B) and 48 hours (Fig. 3D) after induction of HBx expression. Furthermore, 4pX cells displayed a significant increase in HBx-dependent S phase entry 24 hours (Supporting Fig. 2B)[17] but not 48 hours (Fig. 3D) after induction of HBx expression. Additionally, transient transfection of Chang liver cells with the HBV wild-type and HBx-defective replicons did not induce changes in the cell cycle profile (Fig. 3C). Given that HBx promoted PTTG1 accumulation without significantly affecting cell

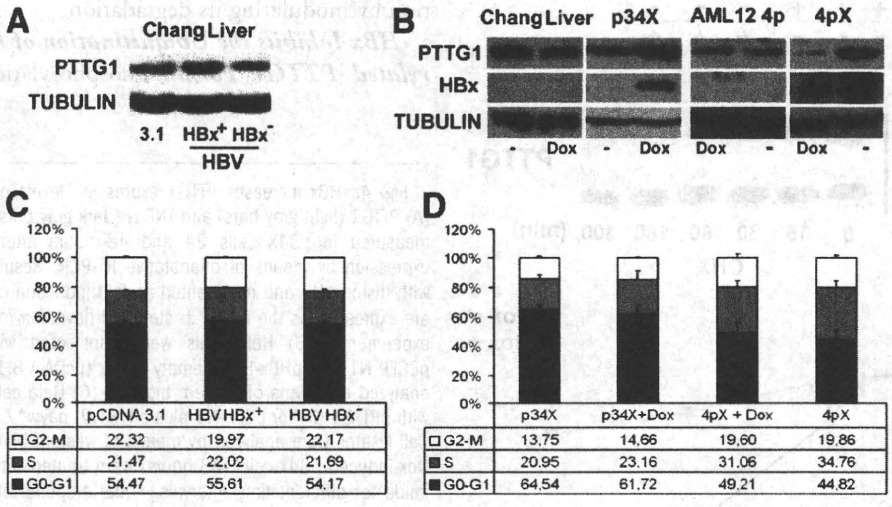

Fig. 3. Effects of HBx on PTTG1 expression. (A) Chang liver cells were transfected with the whole HBV genome payw1.2 (HBx$^+$), an HBx-defective mutant payw7* (HBx$^-$), or control plasmid (pcDNA 3.1), and PTTG1 expression was monitored by means of western blotting. Tubulin expression was assessed to ensure equal protein loading of all samples. (B) PTTG1 protein levels were analyzed in Chang liver, Chang liver p34X, AML12 4p, and AML12 4pX cells grown with or without Dox for 48 hours by means of western blotting. (C) Flow cytometry analysis of cell cycle progression in Chang liver cells 24 hours after transient transfection with payw1.2(HBx$^+$), payw*7(HBx$^-$), or control plasmid (pCDNA3.1). (D) Cell cycle progression in p34X and 4pX cells 48 hours after induction of HBx expression. Values represent the mean ± standard deviation of six independent experiments.

cycle (p34X and HBV complete replicon-transfected Chang liver cells), these results indicated that the HBx-promoted PTTG1 accumulation was not dependent on cell cycle modifications.

HBx-Mediated PTTG1 Accumulation Is Regulated Posttranscriptionally. It is known that HBx transcriptionally induces the expression of viral and cellular genes by activating promoter regulatory sequences.[2] To determine whether HBx modulates PTTG1 transcription, its messenger RNA (mRNA) levels were measured by means of quantitative RT-PCR in p34x and 4pX cells. PTTG1 mRNA levels were unaffected by HBx expression in both p34X (Fig. 4A) and 4px (Supporting Fig. 3) cells. As expected,[25] RT-PCR analysis revealed increased TNF-α mRNA levels upon induction of HBx (Fig. 4A).

Additionally, we transiently transfected Hela cells with both pPTTG1–cyan fluorescent protein (CFP), an expression vector in which PTTG1-CFP transcription is controlled by the CMV promoter, and pHBx-hemagglutinin (HA) plasmids. Western blot analysis using an anti–green fluorescent protein (GFP) Ab revealed that PTTG1-CFP was clearly accumulated in HBx-transfected cells (Fig. 4B). Interestingly, the effect of HBx was not observed when cells were cotransfected with the control plasmid pECFP-N1, coding only for the CFP protein. These results were further confirmed by cotransfecting Hela cells with wild-type or HBx-defective HBV replicons along with the pPTTG1-CFP vector (Fig. 4C). These results strongly suggested that PTTG1 accumulation induced by HBx was not mediated by transcriptional activation.

We next examined whether HBx-induced PTTG1 upregulation could be explained through changes on protein stability by analyzing PTTG1 levels after blocking protein synthesis with cycloheximide. Western blot analysis revealed that PTTG1 protein half-life increased in p34X cells after induction of HBx expression when compared with noninduced cells (Fig. 4D,E). Taken together, these results indicated that HBx promoted PTTG1 accumulation by modulating its degradation.

HBx Inhibits the Ubiquitination of Hyperphosphorylated PTTG1 Forms. Phosphorylation of PTTG1

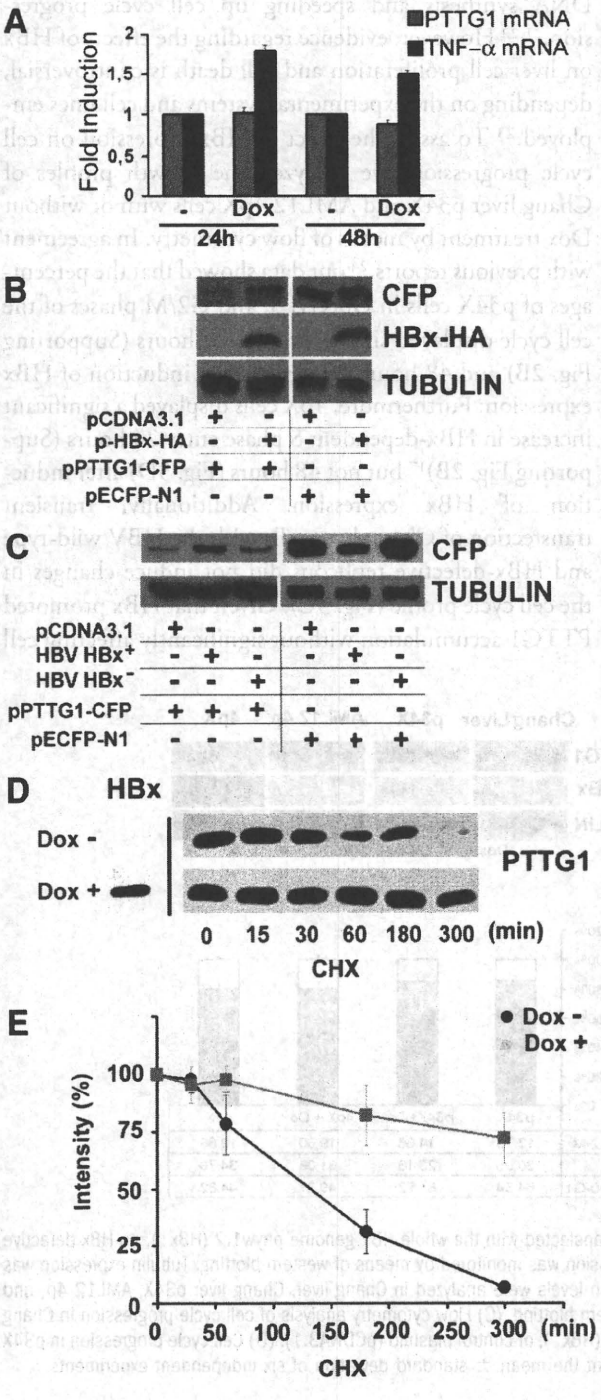

Fig. 4. HBx increases PTTG1 expression levels posttranscriptionally. (A) PTTG1 (light gray bars) and TNF-α (dark gray bars) mRNA levels were measured in p34X cells 24 and 48 hours after induction of HBx expression by means of quantitative RT-PCR. Results were normalized with histone H3 and represented as fold induction over control. Results are expressed as the mean ± standard deviation of three independent experiments. (B) Hela cells were transfected with pPTTG1-CFP or pECFP-N1 plus pHBx-HA or empty vector (pcDNA 3.1). Cell lysates were analyzed by means of western blotting. (C) Hela cells were transfected with pPTTG1-CFP or pCFP-N1 plus payw1.2, payw*7, or control plasmid. Cell lysates were analyzed by means of western blotting. (D) Control or Dox-induced p34x cells (48 hours) were treated with 20 μM cycloheximide for different time intervals. Equal amounts of protein were subjected to Western blot analysis. Representative results of three independent experiments are shown.(E) Analysis of PTTG1 relative levels, assessed by scanning densitometry, was plotted (black circles, control cells; gray squares, HBx-expressing cells). Results are expressed as the percentage of values obtained without cycloheximide treatment for each experimental condition analyzed. Values represent the mean ± standard error of three independent experiments.

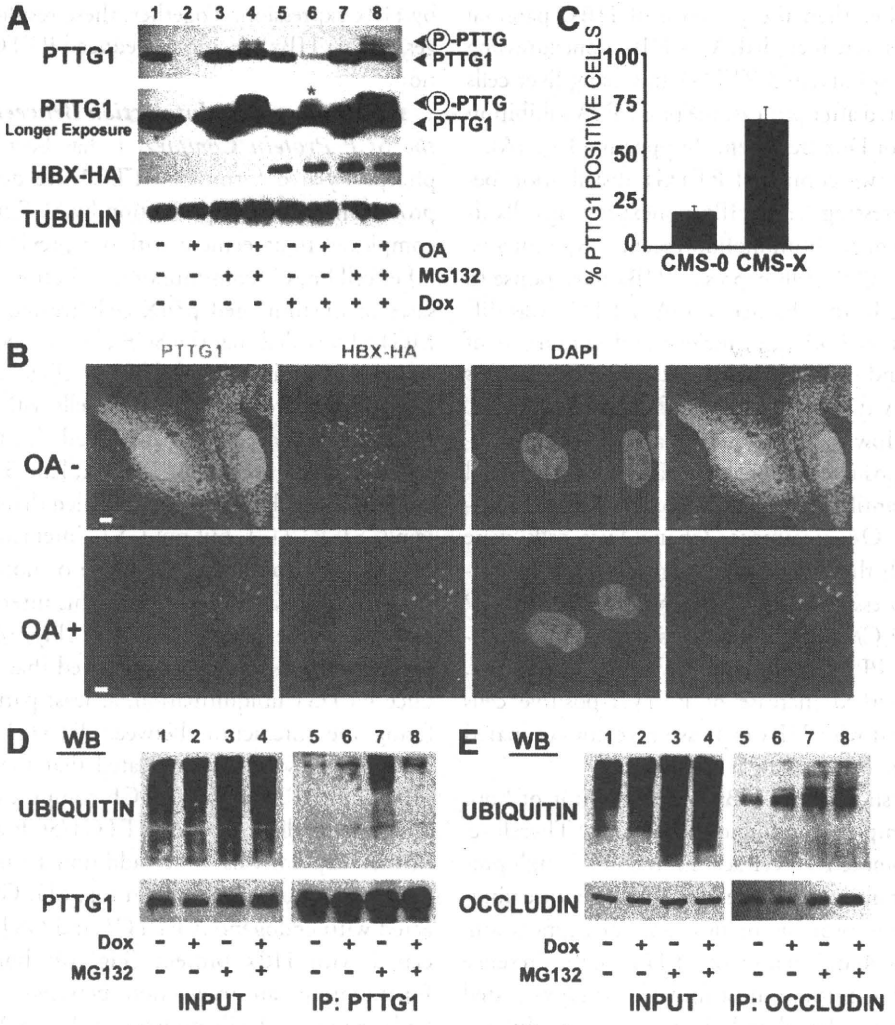

Fig. 5. HBx reduces the ubiquitination of hyperphosphorylated PTTG1 forms. (A) Chang liver p34x cells were treated with OA (1 μM), MG132 (10 μM), or both for 2 hours after 48 hours of Dox induction. Lysates were subsequently analyzed by means of western blotting for the detection of PTTG1. (B) Confocal immunofluorescence analysis of the distribution of PTTG1 (green) and HBx (red) in OA-treated (OA+) or nontreated (OA−) p34x cells. 4',6-Diamidino-2-phenylindole staining is shown in blue. Bar = 5 μm. (C) Twenty-four hours after transfection of Chang liver cells with pCMS-EGFP-HBx (CMS-X) or pCMS-EGFP (CMS-0) plasmids, cells were treated with OA, and PTTG1 accumulation was compared by means of immunofluorescence. Percentages of transfected cells (GFP positive) displaying PTTG1 accumulation are shown. Values are expressed as the mean ± standard deviation of four independent experiments in which at least 50 GFP-positive cells were analyzed. (D) Control or Dox-induced p34X cells were treated or not for 4 hours with MG132 (10 μM). Cell lysates were immunoprecipitated (IP) with anti-PTTG1 pAb and immunoblotted with anti-PTTG1 (bottom) and anti-ubiquitin (top) Abs. (E) Cells were treated as in (D), and lysates were immunoprecipitated with anti-occludin pAb and immunoblotted with anti-occludin (bottom) and anti-ubiquitin (top). Representative results of at least two independent experiments are shown.

leads to its ubiquitination and proteasomal degradation.[10] Thus, we analyzed the levels of phosphorylated forms of PTTG1 in p34X cells treated with okadaic acid (OA), a protein phosphatase 2A (PP2A) inhibitor, and/or MG132, a proteasome inhibitor. As expected, proteasome inhibition by MG132 treatment promoted PTTG1 accumulation independently of HBx expression (Fig. 5A, lane 3 versus lane 1 and lane 7 versus lane 5). As described,[10] MG132 plus OA cotreatment revealed the presence of slower migrating bands corresponding to phosphorylated forms of PTTG1 in both control or Dox-treated cells (Fig. 5A, lanes 4 and 8). OA treatment reduced PTTG1 levels in both HBx-expressing and -nonexpressing cells (Fig. 5A, lane 2 versus lane 1 and lane 6 versus lane 5). However, phosphorylated PTTG1 could be detected in the absence of MG132 after PP2A inhibition (OA treatment) only when HBx was expressed, suggesting that HBx inhibited the degradation of phosphorylated PTTG1 (Fig. 5A, lane 6 versus lane 2). In order to rule out that the differences observed between HBx-expressing and -nonexpressing cells could be due to undefined clonal properties of p34X cells or Dox-associ-

ated effects rather than the presence of HBx, parental Chang liver cells were included. As in HBx-nonexpressing p34X cells, phosphorylated PTTG1 in Chang liver cells were only detected after proteasome plus PP2A inhibition independently of Dox treatment (Supporting Fig. 4A).

Additionally, we compared PTTG1 distribution between HBx-expressing versus HBx-nonexpressing cells after OA treatment by immunofluorescence experiments. Of note, not all p34X cells expressed HBx in response to Dox treatment. In the absence of OA, PTTG1 was diffusely localized in both the nucleus and cytoplasm of HBx-positive and -negative p34X cells (Fig. 5B, top). As mentioned, OA treatment reduced PTTG1 levels (Fig. 5B, bottom). However, we observed a PTTG1 accumulation in HBx-positive cells that colocalized with the viral protein. To quantify the effect of HBx on PTTG1 accumulation after OA treatment, Chang liver cells were transfected with the bicistronic plasmids pCMS-EGFP-HBx (HBx-expressing vector; CMS-X) or pCMS-EGFP (control vector; CMS-O) and processed for immunofluorescence after PP2A inhibition. As shown in Fig. 5C, there was a marked increase of PTTG1-positive cells when transfected with HBx-expressing vector compared with control vector.

It has been shown that HBx is an inhibitor of both proteasome complex[26] and ubiquitin ligases.[6] Therefore, HBx could promote PTTG1 accumulation through proteasome and/or ubiquitin ligase inhibition. Because ubiquitination targets proteins to proteasomal degradation, we analyzed the ubiquitination of PTTG1 in the presence of HBx. For this purpose, unstimulated or Dox-treated p34X cells were incubated with the proteasome inhibitor MG132 and used for immunoprecipitacion using an anti-PTTG1 Ab. Membranes were blotted with anti-ubiquitin monoclonal Ab to detect ubiquitinated forms of PTTG1. As expected, MG132-mediated proteasome inhibition promoted the accumulation of polyubiquinated PTTG1 forms in cells that did not express HBx (Fig. 5D, lane 7 versus lane 5). In contrast, incubation of HBx-expressing cells with MG132 did not significantly increase the levels of ubiquitinated forms of PTTG1 (Fig. 5D, lane 8 versus lane 6). Similar results were obtained in Hela cells cotransfected with expression vectors coding for HA-tagged ubiquitin (HA-ubiquitin), PTTG1 (pcDNA-PTTG1), and either an HBx-coding vector (pSVX) or the control plasmid (pSVHygro) (Supporting Fig. 4B). The tight junction–associated protein occludin is ubiquitinated, and its degradation is sensitive to proteasome inhibition.[27] To analyze whether HBx affected general ubiquitination events, we determined the influence of HBx on occludin ubiquitination. As shown in Fig. 5E, the accumulation of polyubiquitinated occludin was not affected by HBx expression. Together, these results strongly suggested that HBx specifically reduced PTTG1 ubiquitination.

HBx Disrupts the Interaction Between PTTG1 and the SCF Protein Complex. It has been reported that phosphorylated forms of PTTG1 are degraded by the proteasome after ubiquitination by SCF ubiquitin ligase complex.[28] In agreement with our previous results using other cell lines,[11] coimmunoprecipitation assays using lysates of unstimulated p34X cells treated with OA plus MG132 revealed that the SCF core component Cul1 coimmunoprecipitated with PTTG1 (Fig. 6A, top, lane 4). Interestingly, treatment of p34X cells with Dox to induce HBx expression partially disrupted the interaction between PTTG1 and Cul1 (Fig. 6A, lane 5 versus lane 4). GST-based pull-down assays revealed that the fusion protein GST-PTTG1, but not GST, interacted with endogenous Cul1 from a cellular lysate of noninduced p34X (Fig. 6B, top, lane 5). As above, this interaction was also reduced in the presence of HBx (Fig. 6B, top, lane 6 versus lane 5). These data suggested that HBx could reduce PTTG1 ubiquitination, at least partially, by interfering the interaction between PTTG1 and SCF. In addition, these results indicated that the interaction of HBx with PTTG1 and/or SCF complex might be operating in the disruption of PTTG1/SCF association. To further explore this issue, additional pull-down assays were performed. As shown in Fig. 6D, GST-HBx interacted with endogenous PTTG1 and GST-PTTG1 associated with HBx protein (Fig. 6B bottom, lane 6). Furthermore, an interaction between GST-HBx and Cul1 could also be demonstrated (Fig. 6D). The specificity of these GST-HBx interactions was confirmed by observing no interaction of HBx with occludin and other cell cycle–regulating proteins as cyclin B1 or STAG2/SA2 (Fig. 6D). The association between HBx and Cul1 was further confirmed by confocal double-label immunofluorescence in Chang liver p34X cells in which HBx significantly colocalized with Cul1 in dot-like structures (Fig. 6E).

HBx Does Not Affect PTTG1 Stabilization in Cul1 Knockdown Cells. The SCF ubiquitin ligase complex is involved in the degradation of phosphorylated forms of PTTG1.[10] To analyze the specific role of Cul1 on HBx-mediated PTTG1 accumulation, an siRNA-based knockdown approach was employed. First, we determined the levels of PTTG1 in Chang liver cells transiently transfected with control or Cul1-specific siRNAs, and then treated or not with OA and/or MG132. Western blot analysis revealed that Cul1 knockdown promoted PTTG1 accumulation in both control and OA-treated cells. Additionally, OA treatment of Cul1 knockdown

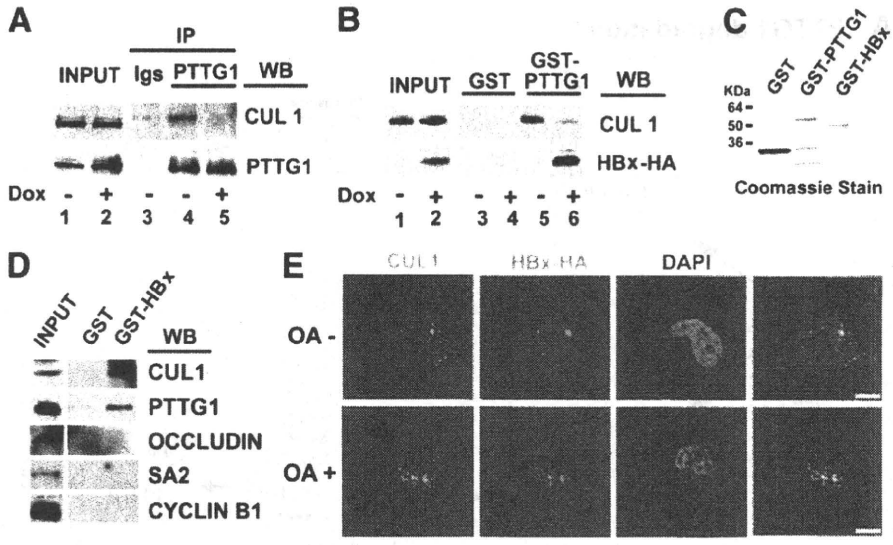

Fig. 6. HBx inhibits the interaction between PTTG1 and SCF complex. (A) Lysates from p34X cells, grown with or without Dox for 48 hours, were immunoprecipitated (IP) using anti-PTTG1 or rabbit immunoglobulin G as a control. Western blot analysis of cell lysates and immunoprecipitates was performed with anti-Cul1 (top) or anti-PTTG1 (bottom) Abs. (B) Pull-down assay with GST or GST-PTTG1 and Dox-treated (+) or untreated (−) p34X extracts. Cell lysates and bound proteins were subjected to western blotting using anti-Cul1 (top) or anti-HA (bottom) Abs. (C) Coomassie brilliant blue staining of 1/10 of the GST proteins used is shown. Molecular weight markers (kDa) are indicated on the left. (D) Pull-down assay with GST or GST-HBx and noninduced p34X cell extracts. Cell lysates and bound proteins were subjected to western blot analysis using anti-Cul1, anti-PTTG1, anti-occludin, anti-SA2, and anti-cyclin B1 Abs. (E) Confocal immunofluorescence analysis of the distribution of Cul1 (green; monoclonal Ab anti-Cul-1) and HBx (red; byotinilated Ab anti-HA epitope) in OA-treated (OA+) or untreated (OA−) p34x cells. 4',6-Diamidino-2-phenylindole staining is shown in blue. Bar = 7.5 μm. All results are representative of at least two independent experiments.

cells resulted in the formation of phosphorylated PTTG1 forms (Supporting Fig. 5). We then analyzed the effect of HBx expression on PTTG1 accumulation in Cul1-silenced cells. In both Chang liver and p34x cells, PTTG1 expression levels were increased after Cul1 silencing (Fig. 7). As above, Dox-induced HBx increased PTTG1 levels in p34X control siRNA-treated cells (Fig. 7, lane 7 versus lane 5). Interestingly, PTTG1 accumulation after Cul1 silencing was not further enhanced by HBx (Fig. 7, lane 8 versus lane 6), suggesting that the stabilization of PTTG1 by HBx was Cul1-dependent, not being likely that other ubiquitin ligase was involved. Given that HBx expression mimicked the effects of Cul-1 knockdown on PTTG1, it can be hypothesized that HBx interferes Cul1-asscociated functions. Overall, these data strongly suggest that HBx promotes the disruption of the PTTG1/SCF association and prevents its ubiquitination and subsequent degradation by the proteasome (Fig. 8).

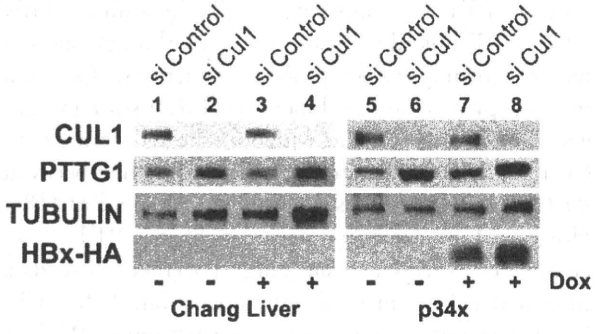

Fig. 7. HBx does not enhance PTTG1 stabilization after Cul1 knockdown. Chang liver and p34x cells were transfected with control or Cul1 siRNA and treated with Dox for 48 hours. PTTG1, Cul1, and tubulin protein levels were analyzed by means of western blotting. Results are representative of two independent experiments.

Discussion

HBV-associated carcinogenesis is a multifactorial process. Liver inflammation results in hepatocellular death and regeneration processes that lead to the accumulation of critical mutations in the host genome. In addition, the regulatory protein HBx has been involved in hepatocarcinogenesis by altering cellular processes. In the present study, we have demonstrated that PTTG1 expression levels increase in HBx-immunoreactive cells as chronic hepatitis B progresses to cirrhosis and HCC. Furthermore, PTTG1 expression increases as HBx transgenic mouse livers progress through hyperplasia to HCC. In addition, PTTG1 accumulates in human and mouse HBx-expressing cell lines and in HBV replicon-containing cells, but not in cells harboring an HBx-defective genome construct. Together, these data strongly suggest that PTTG1

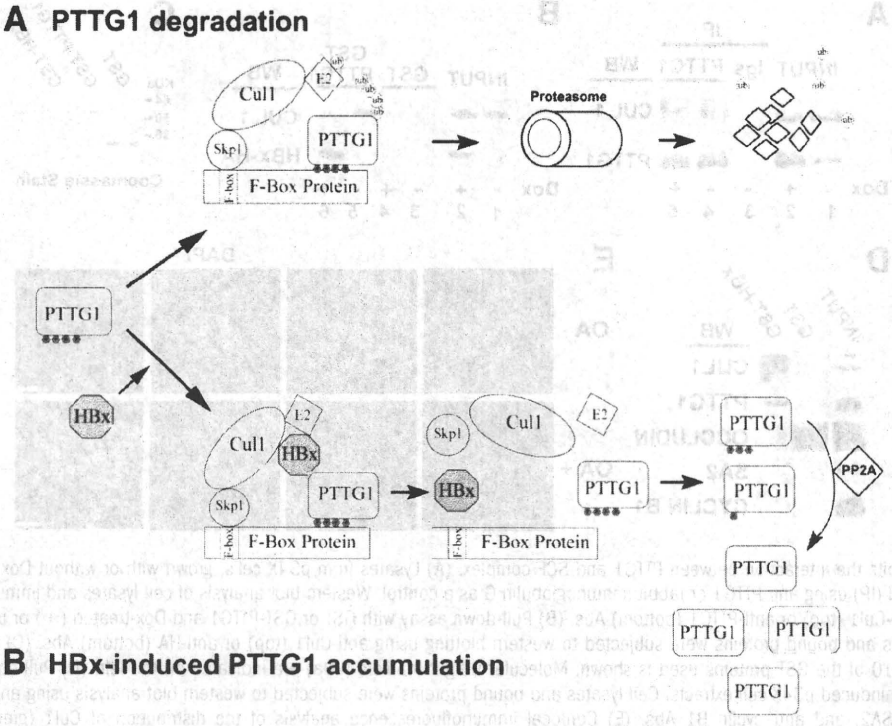

Fig. 8. Possible mechanism for HBx-mediated PTTG1 stabilization. (A) PTTG1 undergoes proteasomal degradation via ubiquitination by the SCF ubiquitin ligase complex (A. PTTG1 degradation). Cul1 interacts with PTTG1, and in the presence of HBx this interaction is disrupted. As a result, there is an impairment of PTTG1 ubiquitination that leads to an increase of its half-life (B. HBx-induced PTTG1 accumulation). The proliferative actions of PTTG1 and HBx could act synergistically in cell transformation.

accumulation is, at least partially, an HBx-mediated effect.

Several viruses, including HBV, have the ability to stimulate the cell cycle progression in order to facilitate their own replication. In doing so, viruses generally disrupt the normal cell cycle checkpoints and in turn extend proliferative signals to host cells to establish a carcinogenic environment.[29] HBx has been demonstrated to suppress serum dependence for cell cycle activation.[30] Furthermore, HBx has been shown to promote transit through G1 in G0-arrested cells and to alter G1-to-S and G2-to-M progression.[17,22] However, in Chang liver p34X cells, the cell cycle profile was unaffected after HBx induction.[24] In addition, it is known that HBx transcriptionally induces the expression of viral and cellular genes.[2] However, our data strongly suggest that HBx-promoted PTTG1 protein accumulation is not strictly dependent on cell cycle modifications or transcriptional up-regulation.

Through interactions with host factors, HBx alters different cellular processes implicated in the development of HCC. Protein degradation by the proteasome complex is a strictly regulated key event of cellular homeostasis. Oncogenic viruses alter the proteasomal activity of target cells, affecting viral entry, replication, and release and enhancing cell survival.[31] Targeting of proteins to the proteasome through interactions with ubiquitin ligases is essential for normal protein turnover. In this context, HBx is able to down-regulate both proteasome[26] and ubiquitin ligase functions.[6] Our data show that HBx induces a marked accumulation of PTTG1 protein by reducing its ubiquitination and subsequent degradation.

It has been demonstrated that the SCF ubiquitin ligase complex is involved in the degradation of phosphorylated forms of PTTG1 in nonmitotic cells. In addition, HBx affects SCF ubiquitin ligase functions through mechanisms involving protein–protein interactions.[6] Confocal microscopy analysis and biochemical data strongly suggest that HBx may interact with both the SCF component Cul1 and PTTG1. Interestingly, the association between PTTG1 and Cul1 is disrupted in the presence of HBx. However, HBx expression does not enhance PTTG1 accumulation after Cul1 silencing. Together, these data suggest that HBx may alter the formation of the SCF/PTTG1 complex, leading to an impairment of PTTG1 ubiquitination. Thus, in the presence of HBx, PTTG1 is not targeted to proteasome-mediated degradation resulting in an abnormal protein accumulation (Fig. 8). It is tempting to speculate that by affecting the normal turn-

over of PTTG1, HBx could alter some of the PTTG1-related functions and promote cellular transformation.

The SCF ubiquitin ligases are mammalian cullin RING ubiquitin ligases in which F-box proteins provide the substrate targeting specificity of the complex . Skp2 is the F-box protein that targets key regulatory proteins, such as c-myc, for degradation.[32] Interestingly, it has been shown that HBx is able to block ubiquitination of c-myc through a direct interaction with Skp2 and destabilization of the SCF/Skp2 complex. An association between HBx-mediated PTTG1 stabilization and HBx/Skp2 interaction may also exist, but this issue requires further study.

PP2A is an important serine/threonine phosphatase family involved in essential cellular processes such as cell division, gene regulation, protein synthesis, and cytoskeleton organization. PP2A enzymes typically exist as heterotrimers comprising a common catalytic subunit (PP2Ac) and different structural and regulatory subunits.[33] It has been shown that hepatotropic viruses, including hepatitis C virus and HBV, alter PP2Ac activity.[34] HBx protein is the most likely candidate responsible for HBV-mediated PP2Ac modulation.[34] Our results show that HBx promotes PTTG1 accumulation, inhibiting the degradation of phosphorylated forms of PTTG1 after chemical inhibition of PP2A. Further experiments are necessary to analyze whether HBx could affect PTTG1 expression levels by up-regulating PP2A activity.

Several lines of evidence suggest that an important transforming mechanism underlying PTTG1 overexpression is the induction of chromosomal instability.[9] Thus, it has been demonstrated that PTTG1 accumulation inhibits mitosis progression and chromosome segregation, but does not directly affect cytokinesis, resulting in aneuploidy.[35] It has been shown that HBx can transform cultured cells[21] and induce liver cancer in transgenic mice.[36] Genetic instability is frequently accompanied with the acquisition of transformation ability and malignant progression of tumors. Moreover, recent reports have shown that HBx expression induces chromosomal aberrations such as chromosome rearrangements and micronuclei formation.[37] Furthermore, HBx promotes multipolar spindle formation and chromosomal missegregation during mitosis, and increases multinucleated cells.[18] Interestingly, it has been determined that HBx binds to BubR1, a component of the mitotic checkpoint complex, and attenuates the association between BubR1 and CDC20, an activator of the anaphase-promoting complex/cyclosome, resulting in chromosomal instability.[38] Our results demonstrate that HBx induces the accumulation of PTTG1 in interphase cells. Further experiments are necessary to study the effects of HBx on PTTG1 functions during mitotic events.

In conclusion, we propose that HBx promotes alterations of PTTG1 expression levels, which may improve our understanding of the molecular mechanisms of HBV-related pathogenesis of progressive liver disease leading to cirrhosis and HCC development.

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References

1. Kremsdorf D, Soussan P, Paterlini-Brechot P, Brechot C. Hepatitis B virus-related hepatocellular carcinoma: paradigms for viral-related human carcinogenesis. Oncogene 2006;25:3823-3833.
2. Tang H, Oishi N, Kaneko S, Murakami S. Molecular functions and biological roles of hepatitis B virus x protein. Cancer Sci 2006;97:977-983.
3. Su Q, Schroder CH, Hofmann WJ, Otto G, Pichlmayr R, Bannasch P. Expression of hepatitis B virus X protein in HBV-infected human livers and hepatocellular carcinomas. HEPATOLOGY 1998;27:1109-1120.
4. Park IY, Sohn BH, Yu E, Suh DJ, Chung YH, Lee JH, et al. Aberrant epigenetic modifications in hepatocarcinogenesis induced by hepatitis B virus X protein. Gastroenterology 2007;132:1476-1494.
5. Moon EJ, Jeong CH, Jeong JW, Kim KR, Yu DY, Murakami S, et al. Hepatitis B virus X protein induces angiogenesis by stabilizing hypoxia-inducible factor-1alpha. FASEB J 2004;18:382-384.
6. Kalra N, Kumar V. The X protein of hepatitis B virus binds to the F box protein Skp2 and inhibits the ubiquitination and proteasomal degradation of c-Myc. FEBS Lett 2006;580:431-436.
7. Pei L, Melmed S. Isolation and characterization of a pituitary tumor-transforming gene (PTTG). Mol Endocrinol 1997;11:433-441.
8. Zou H, McGarry TJ, Bernal T, Kirschner MW. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science 1999;285:418-422.
9. Vlotides G, Eigler T, Melmed S. Pituitary tumor-transforming gene: physiology and implications for tumorigenesis. Endocr Rev 2007;28:165-186.
10. Gil-Bernabe AM, Romero F, Limon-Mortes MC, Tortolero M. Protein phosphatase 2A stabilizes human securin, whose phosphorylated forms are degraded via the SCF ubiquitin ligase. Mol Cell Biol 2006;26:4017-4027.
11. Limon-Mortes MC, Mora-Santos M, Espina A, Pintor-Toro JA, Lopez-Roman A, et al. UV-induced degradation of securin is mediated by SKP1-CUL1-beta TrCP E3 ubiquitin ligase. J Cell Sci 2008;121:1825-1831.
12. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of merastasis in primary solid tumors. Nat Genet 2003;33:49-54.
13. Fujii T, Nomoto S, Koshikawa K, Yatabe Y, Teshigawara O, Mori T, et al. Overexpression of pituitary tumor transforming gene 1 in HCC is associated with angiogenesis and poor prognosis. HEPATOLOGY 2006;43:1267-1275.
14. Bernal JA, Luna R, Espina A, Lazaro I, Ramos-Morales F, Romero F, et al. Human securin interacts with p53 and modulates p53-mediated transcriptional activity and apoptosis. Nat Genet 2002;32:306-311.
15. Cho-Rok J, Yoo J, Jang YJ, Kim S, Chu IS, Yeom YI, et al. Adenovirus-mediated transfer of siRNA against PTTG1 inhibits liver cancer cell growth in vitro and in vivo. HEPATOLOGY 2006;43:1042-1052.
16. Koike K, Moriya K, Iino S, Yotsuyanagi H, Endo Y, Miyamura T, et al. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. HEPATOLOGY 1994;19:810-819.
17. Lee S, Tarn C, Wang WH, Chen S, Hullinger RL, Andrisani OM. Hepatitis B virus X protein differentially regulates cell cycle progression in X-transforming versus nontransforming hepatocyte (AML12) cell lines. J Biol Chem 2002;277:8730-8740.

18. Yun C, Cho H, Kim SJ, Lee JH, Park SY, Chan GK. Mitotic aberration coupled with centrosome amplification is induced by hepatitis B virus X oncoprotein via the Ras-mitogen-activated protein/extracellular signal-regulated kinase-mitogen-activated protein pathway. Mol Cancer Res 2004;2:159-169.
19. Benedicto I, Molina-Jimenez F, Bartosch B, Cosset FL, Lavillette D, Prieto J, et al. The tight junction-associated protein occludin is required for a postbinding step in hepatitis C virus entry and infection. J Virol 2009;83: 8012-8020.
20. Ramos-Morales F, Dominguez A, Romero F, Luna R, Multon MC, Pintor-Toro JA, et al. Cell cycle regulated expression and phosphorylation of hpttg proto-oncogene product. Oncogene 2000;19:403-409.
21. Koike K, Moriya K, Yotsuyanagi H, Iino S, Kurokawa K. Induction of cell cycle progression by hepatitis B virus HBx gene expression in quiescent mouse fibroblasts. J Clin Invest 1994;94:44-49.
22. Benn J, Schneider RJ. Hepatitis B virus HBx protein deregulates cell cycle checkpoint controls. Proc Natl Acad Sci U S A 1995;92:11215-11219.
23. Bouchard MJ, Schneider RJ. The enigmatic X gene of hepatitis B virus. J Virol 2004;78:12725-12734.
24. Yun C, Lee JH, Park H, Jin YM, Park S, Park K, et al. Chemotherapeutic drug, adriamycin, restores the function of p53 protein in hepatitis B virus X (HBx) protein-expressing liver cells. Oncogene 2000;19:5163-5172.
25. Lara-Pezzi E, Majano PL, Gomez-Gonzalo M, Garcia-Monzon C, Moreno-Otero R, Levrero M, et al. The hepatitis B virus X protein up-regulates tumor necrosis factor alpha gene expression in hepatocytes. HEPATOLOGY 1998;28:1013-1021.
26. Huang J, Kwong J, Sun EC, Liang TJ. Proteasome complex as a potential cellular target of hepatitis B virus X protein. J Virol 1996;70:5582-5591.
27. Traweger A, Fang D, Liu YC, Stelzhammer W, Krizbai IA, Fresser F, et al. The tight junction-specific protein occludin is a functional target of the E3 ubiquitin-protein ligase itch. J Biol Chem 2002;277:10201-10208.
28. Romero F, Gil-Bernabe AM, Saez C, Japon MA, Pintor-Toro JA, Tortolero M. Securin is a target of the UV response pathway in mammalian cells. Mol Cell Biol 2004;24:2720-2733.
29. Feitelson MA, Reis HM, Liu J, Lian Z, Pan J. Hepatitis B virus X antigen (HBxAg) and cell cycle control in chronic infection and hepatocarcinogenesis. Front Biosci 2005;10:1558-1572.
30. Mukherji A, Janbandhu VC, Kumar V. HBx-dependent cell cycle deregulation involves interaction with cyclin E/A-cdk2 complex and destabilization of p27Kip1. Biochem J 2007;401:247-256.
31. Banks L, Pim D, Thomas M. Viruses and the 26S proteasome: hacking into destruction. Trends Biochem Sci 2003;28:452-459.
32. Frescas D, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. Nat Rev Cancer 2008;8:438-449.
33. Mumby M. PP2A: unveiling a reluctant tumor suppressor. Cell 2007;130: 21-24.
34. Christen V, Treves S, Duong FH, Heim MH. Activation of endoplasmic reticulum stress response by hepatitis viruses up-regulates protein phosphatase 2A. HEPATOLOGY 2007;46:558-565.
35. Yu R, Lu W, Chen J, McCabe CJ, Melmed S. Overexpressed pituitary tumor-transforming gene causes aneuploidy in live human cells. Endocrinology 2003;144:4991-4998.
36. Kim CM, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 1991;351:317-320.
37. Livezey KW, Negorev D, Simon D. Increased chromosomal alterations and micronuclei formation in human hepatoma HepG2 cells transfected with the hepatitis B virus HBX gene. Mutat Res 2002;505:63-74.
38. Kim S, Park SY, Yong H, Famulski JK, Chae S, Lee JH, et al. HBV X protein targets hBubR1, which induces dysregulation of the mitotic checkpoint. Oncogene 2008;27:3457-3464.

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PEG10 is a probable target for the amplification at 7q21 detected in hepatocellular carcinoma

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Abstract DNA copy number aberrations in human hepatocellular carcinoma (HCC) cell lines were investigated using a high-density oligonucleotide microarray, and a novel amplification at the chromosomal region 7q21 was detected. Molecular definition of the amplicon indicated that *PEG10* (paternally expressed gene 10), a paternally expressed imprinted gene, was amplified together with *CDK14* (cyclin-dependent kinase 14; previously PFTAIRE protein kinase 1, *PFTK1*) and *CDK6* (cyclin-dependent kinase 6). An increase in *PEG10* copy number was detected in 14 of 34 primary HCC tumors (41%). *PEG10*, but not *CDK14* or *CDK6*, was significantly overexpressed in 30 of 41 tumors (73%) from HCC patients, compared with their nontumorous counterparts. These results suggest that *PEG10* is a probable target, acting as a driving force for amplification of the 7q21 region, and may therefore be involved in the development or progression of HCCs.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in men and the eighth most common in women worldwide; it is estimated to cause approximately half a million deaths annually [1]. Although the risk factors for HCC, which include hepatitis B virus, hepatitis C virus, and alpha-toxin, are well characterized, the molecular pathogenesis of this widespread type of cancer remains poorly understood [2].

Amplification of DNA in certain regions of chromosomes plays a crucial role in the development and progression of human malignancies, specifically when protooncogenic target genes within those amplicons are overexpressed. Oncogenes that are often amplified in cancers include *MYC*, *ERBB2*, and *CCND1*. The recent introduction of high-density oligonucleotide microarrays designed for typing of single nucleotide polymorphisms (SNPs) facilitates high-resolution mapping of chromosomal amplifications, deletions, and losses of heterozygosity [3,4].

To identify genes potentially involved in HCC, we investigated DNA copy number aberrations in human HCC cell lines using high-resolution SNP arrays and found a novel amplification at the chromosomal region 7q21. Recurrent amplifications at 7q21 have been observed in human neoplasms [5]. Gains of 7q21 have been associated with the aggressiveness of several tumors, including HCC [6], colorectal cancer [7], prostate cancer [8], Burkitt lymphoma [9], and esophageal squamous cell carcinoma [10]. These data suggest that this chromosomal region may harbor one or more protooncogenes (henceforth referred to as *target genes*) whose overexpression following amplification might contribute to the initiation or progression of HCC. The actual target gene that drives the 7q21 amplification in HCC remains unclear, however, and we therefore conducted a molecular definition study of the amplicon to identify such genes. Three putative oncogenes, *CDK14* (cyclin-dependent kinase 14; previously *PFTK1*, PFTAIRE protein kinase 1), *CDK6* (cyclin-dependent

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kinase 6), and *PEG10* (paternally expressed gene 10), were identified in the 7q21 amplicon.

The serine/threonine-protein kinase PFTAIRE-1 protein (also known as PFTK1) is a member of the cell division cycle-2 (CDC2)-related protein kinase family [11] and acts as a cyclin-dependent kinase that regulates cell cycle progression and cell proliferation [12]. CDK6 is activated in response to increased expression of D-type cyclins in the early G1 phase of the cell cycle and inactivates the retinoblastoma protein by phosphorylation, thereby activating the transcriptional complex E2F-DP1 that regulates the genes for S-phase onset [13]. *PEG10* has been characterized as a paternally expressed, maternally silenced gene [14]. Several research groups have recently reported overexpression of *PEG10* in HCC [15–19].

2. Materials and methods

2.1. Cell lines and tumor samples

A total of 20 HCC cell lines were examined: JHH-1, JHH-2, JHH-4, JHH-5, JHH-6, JHH-7, SNU354, SNU368, SNU387, SNU398, SNU423, SNU449, SNU475, Huh-1, Huh7, Hep3B, PLC/PRF/5, Li7, HLE, and HLF [20]. All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Paired tumor and nontumor tissues were obtained from 36 HCC patients who underwent surgery at the Hospital of Tokyo Medical and Dental University. All specimens were frozen immediately in liquid nitrogen and were stored at −80°C until required. Genomic DNA was isolated using a Puregene DNA isolation kit (Gentra, Minneapolis, MN), and total RNA was obtained using Trizol reagent (Invitrogen, Carlsbad, CA). Thirty-four tumor samples were available for DNA analyses, and 41 paired tumor and nontumor samples were available for mRNA analyses.

Prior to the study, informed consent was obtained and the study was approved by ethics committees.

2.2. SNP array analysis

DNA copy number changes were analyzed by the GeneChip Mapping 100K array set (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions as described previously [21]. In brief, 250 ng of genomic DNA was digested with a restriction enzyme (*Xba*I or *Hin*dIII), ligated to an adaptor and amplified by polymerase chain reaction (PCR). Amplified products were fragmented, labeled by biotinylation, and hybridized to the microarrays. Hybridization was detected by incubation with a streptavidin–phycoerythrin conjugate, followed by scanning of the array; analysis was performed as previously described [22]. After appropriate normalization of mean array intensities, signal ratios were calculated between HCC cell lines and anonymous normal references, and copy numbers were inferred from the observed signal ratios based on the hidden Markov model using CNAG software (Copy Number Analyzer for Affymetrix GeneChip mapping arrays) [23]. The CNAG software is available at http://www.genome.umin.jp.

2.3. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed using five bacterial artificial chromosomes (BACs) as probes, as described previously [24]: RP11-66P5, RP11-412F4, RP11-316P4, RP11-28O23, and RP11-958G24 (Invitrogen, Carlsbad, CA). The BACs were selected based on their homology to locations in the human genome according to the database provided at the University of California, Santa Cruz, Genome Bioinformatics Web site (http://genome.ucsc.edu/).

2.4. Real-time quantitative PCR

Genomic DNA and mRNA were quantified using a real-time fluorescence detection method, as described previously [21]. The primers used for PCR (Table 1) were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) on the basis of sequence data obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) database. *GAPDH* was used as endogenous control for mRNA levels, and the long interspersed nuclear element 1 (LINE-1) was used as an endogenous control for genomic DNA levels.

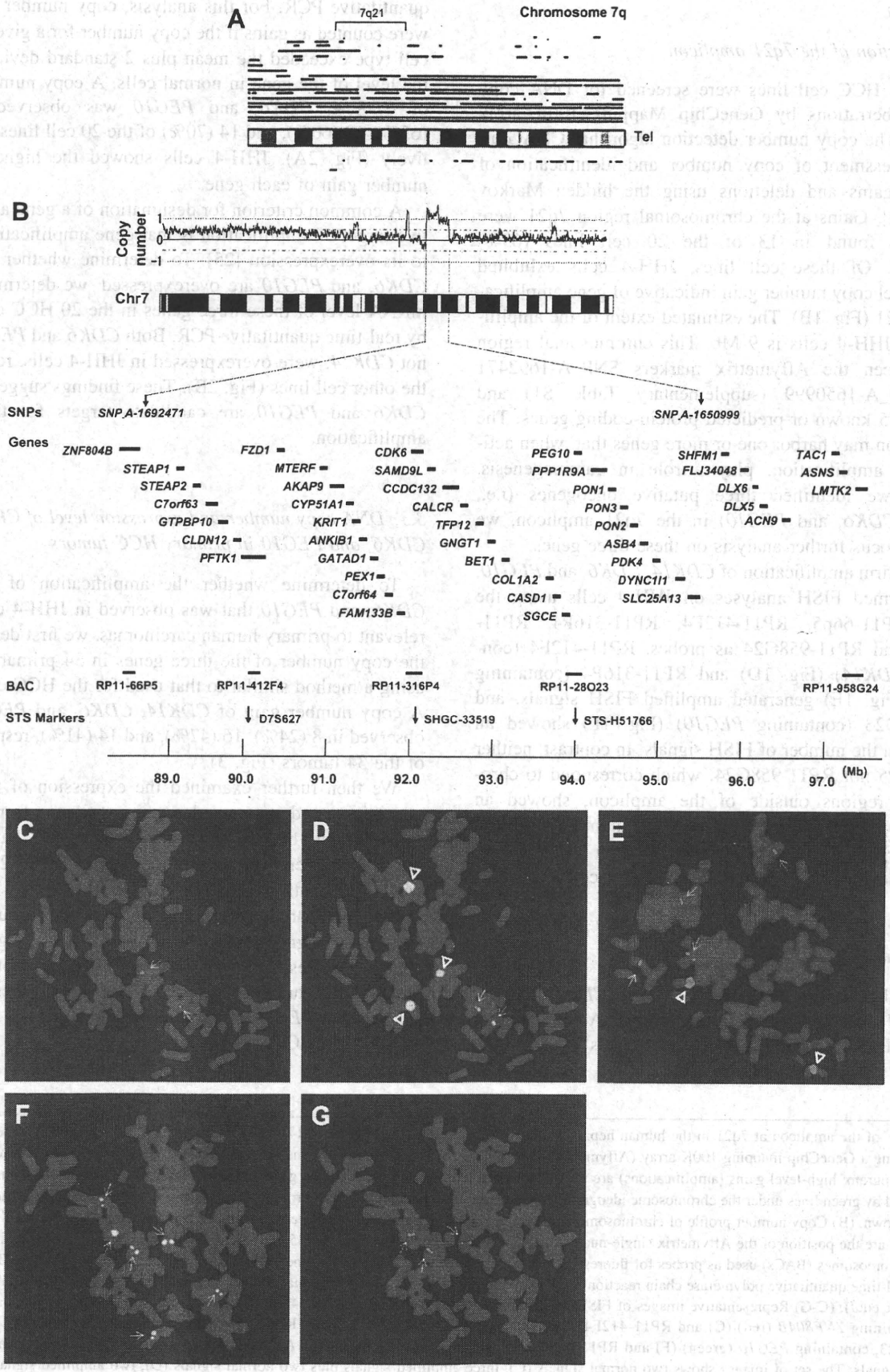
2.5. Statistical analysis

The Wilcoxon signed-rank test was performed using SPSS 15.0 software (SPSS, Chicago, IL). P values of <0.05 were considered significant.

Table 1
Primer sequences used for polymerase chain reaction with sequence-tagged site (STS) markers for the three genes investigated

Gene	STS marker	Forward primer	Reverse primer
CDK14 genomic DNA	D7S627	5′-AAACCAAGAACATTCCAG-3′	5′-ACACATCACATTCTCACC-3′
CDK14 mRNA		5′-CCAAGGAGTTGCTGCTTTTC-3′	5′-GAATGAACTCCAGGCCATGT-3′
CDK6 genomic DNA	SHGC-33519	5′-AAGTCAGAAGGAAAAAAGCTTACTG-3′	5′-TGAGATGTGTTAAAGTAGGTTTTCA-3′
CDK6 mRNA		5′AGCCCAAGATGACCAACATC-3′	5′-AGGTCAAGTTGGGAGTGGTG-3′
PEG10 genomic DNA	STS-H51766	5′-AAAGTTTACATACATTTATGAAGGG-3′	5′- TTCCAGACTGCACCATATAG-3′
PEG10 mRNA		5′-CAGGCCTGAAAAGAAAGTGC-3′	5′-AATGCTTTGTGGAAGCCATC-3′

The gene *CDK14* was previously assigned the symbol *PFTK1* (http://www.genenames.org).


A
7q21
Chromosome 7q
Tel
B
Copy number
1
0
-1
Chr7
SNPs
SNP_A-1692471
SNP_A-1650999
Genes
ZNF804B
STEAP1
STEAP2
C7orf63
GTPBP10
CLDN12
PFTK1
FZD1
MTERF
AKAP9
CYP51A1
KRIT1
ANKIB1
GATAD1
PEX1
C7orf64
FAM133B
CDK6
SAMD9L
CCDC132
CALCR
TFP12
GNGT1
BET1
COL1A2
CASD1
SGCE
PEG10
PPP1R9A
PON1
PON3
PON2
ASB4
PDK4
DYNC1I1
SLC25A13
SHFM1
FLJ34048
DLX6
DLX5
ACN9
TAC1
ASNS
LMTK2
BAC
RP11-66P5
RP11-412F4
RP11-316P4
RP11-28O23
RP11-958G24
STS Markers
D7S627
SHGC-33519
STS-H51766
89.0
90.0
91.0
92.0
93.0
94.0
95.0
96.0
97.0
(Mb)
C
D
E
F
G

3. Results

3.1. Detection of the 7q21 amplicon

Twenty HCC cell lines were screened for DNA copy number aberrations by GeneChip Mapping 100K array analysis. The copy number detection algorithm CNAG allowed assessment of copy number and identification of genomic gains and deletions using the hidden Markov model [23]. Gains at the chromosomal region 7q21 were frequently found in 13 of the 20 cell lines (65%) (Fig. 1A). Of these cell lines, JHH-4 cells exhibited a high-level copy number gain indicative of gene amplification at 7q21 (Fig. 1B). The estimated extent of the amplification in JHH-4 cells is 9 Mb. This chromosomal region lies between the Affymetrix markers SNP_A-1692471 and SNP_A-1650999 (supplementary Table S1) and includes 35 known or predicted protein-coding genes. The 7q21 region may harbor one or more genes that, when activated by amplification, play a role in carcinogenesis. Because we identified three putative oncogenes (i.e., *CDK14*, *CDK6*, and *PEG10*) in the 7q21 amplicon, we chose to focus further analysis on these three genes.

To confirm amplification of *CDK14*, *CDK6*, and *PEG10*, we performed FISH analyses on JHH-4 cells using the BACs RP11-66p5, RP11-412F4, RP11-316P4, RP11-28O23, and RP11-958G24 as probes. RP11-412F4 (containing *CDK14*) (Fig. 1D) and RP11-316P4 (containing *CDK6*) (Fig. 1E) generated amplified FISH signals, and RP11-28O23 (containing *PEG10*) (Fig. 1F) showed an increase in the number of FISH signals. In contrast, neither RP11-66P5 nor RP11-958G24, which correspond to chromosomal regions outside of the amplicon, showed an amplified signal or an increase in the number of FISH signals (Fig. 1C, 1G). These data confirm that *CDK14*, *CDK6*, and *PEG10* are amplified in JHH-4 cells.

3.2. DNA copy number and expression level of CDK14, CDK6, and PEG10 in HCC cell lines

To further analyze the potential role of *CDK14*, *CDK6*, and *PEG10* in HCC, we determined the DNA copy number of these three genes in 20 HCC cell lines by real-time quantitative PCR. For this analysis, copy number changes were counted as gains if the copy number for a given tumor cell type exceeded the mean plus 2 standard deviations of the level of the gene in normal cells. A copy number gain of *CDK14*, *CDK6*, and *PEG10* was observed in 13 (65%), 12 (60%), and 14 (70%) of the 20 cell lines, respectively (Fig. 2A). JHH-4 cells showed the highest copy number gain of each gene.

A common criterion for designation of a gene as a putative target of amplification is that gene amplification leads to its overexpression [25]. To determine whether *CDK14*, *CDK6*, and *PEG10* are overexpressed, we determined the mRNA level of these three genes in the 20 HCC cell lines by real-time quantitative PCR. Both *CDK6* and *PEG10*, but not *CDK14*, were overexpressed in JHH-4 cells, relative to the other cell lines (Fig. 2B). These findings suggested that *CDK6* and *PEG10* are candidate targets for the 7q21 amplification.

3.3. DNA copy number and expression level of CDK14, CDK6, and PEG10 in primary HCC tumors

To determine whether the amplification of *CDK14*, *CDK6*, and *PEG10* that was observed in JHH-4 cells was relevant to primary human carcinomas, we first determined the copy number of the three genes in 34 primary HCCs, using a method similar to that used for the HCC cell lines. A copy number gain of *CDK14*, *CDK6*, and *PEG10* was observed in 8 (24%), 16 (47%), and 14 (41%), respectively, of the 34 tumors (Fig. 3).

We then further examined the expression of the three genes in paired tumor and nontumor tissues from the 41 HCC patients by real-time quantitative PCR. Patient and tumor characteristics are summarized in Table 2. *PEG10* was significantly overexpressed in 30 of the 41 tumors (73%), compared with their nontumorous counterparts (Wilcoxon signed-rank test, $P < 0.001$) (Fig. 4). In contrast, expression of *CDK14* or *CDK6* was not upregulated in HCC tumors (Fig. 4). Taken together, these results suggest that *PEG10* is the most likely target for the 7q21 amplicon in HCC.

Fig. 1. Map of the amplicon at 7q21 in the human hepatocellular carcinoma (HCC) cell line JHH-4. (A) Recurrent copy number gains on the 7q arm as assessed using a GeneChip mapping 100K array (Affymetrix, Santa Clara, CA). Copy number gains are indicated by red horizontal lines above the chromosome ideogram: high-level gains (amplifications) are shown by bright red lines, whereas simple gains are shown by dark red lines. Copy number losses are indicated by green lines under the chromosome ideogram. Each horizontal line represents an aberration detected in a single HCC cell line. The cytobands in 7q are shown. (B) Copy number profile of chromosome 7 in JHH-4 cells. Copy number values were determined by GeneChip mapping 100K array analysis. Shown are the position of the Affymetrix single-nucleotide polymorphism (SNP) probes, the 35 genes included within the amplicon, the five bacterial artificial chromosomes (BACs) used as probes for fluorescence in situ hybridization (FISH) experiments, and the three sequence-tagged site (STS) markers used for real-time quantitative polymerase chain reaction (PCR) based on the University of California, Santa Cruz, Genome Bioinformatics database (http://genome.ucsc.edu/). (C-G) Representative images of FISH on metaphase chromosomes from JHH-4 cells, using the following BAC probes: paired RP11-66P5, containing *ZNF804B* (red) (C) and RP11-412F4, containing *CDK14* (green) (D); single RP11-316P4, containing *CDK6* (red) (E); and paired RP11-28O23, containing *PEG10* (green) (F) and RP11-958G24, containing *LMTK2* (red) (G). Arrows indicate normal signals; arrowheads indicate amplified signals. The set of images shows two normal signals (C), three amplified signals plus two normal signals (D), two amplified signals plus four normal signals (E), six normal signals (F), and three normal signals (G). *Note:* In these figures the gene *CDK14* is identified by the previously approved symbol, *PFTK1*.

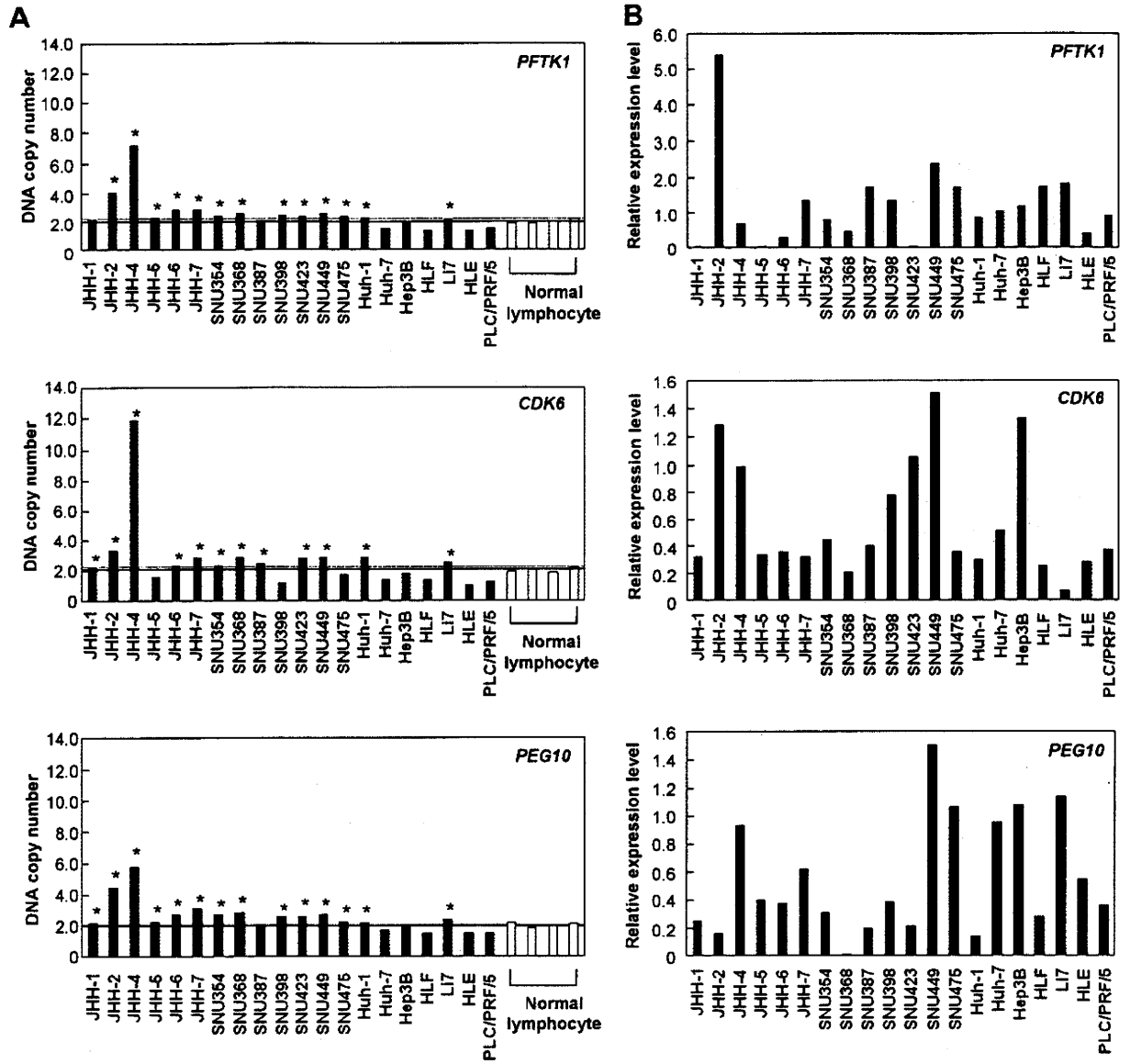


Fig. 2. DNA copy number and expression level of *CDK14* (previously *PFTK1*), *CDK6*, and *PEG10* in HCC cell lines. (A) DNA copy number of *CDK14*, *CDK6*, and *PEG10* in 20 HCC cell lines and four normal peripheral blood lymphocytes as measured by real-time quantitative PCR with reference to LINE-1 controls. Values are normalized such that the average copy number in genomic DNA derived from four normal lymphocytes has a value of 2 (solid horizontal line). A value corresponding to the mean +2 S.D. of the copy number of normal lymphocytes was used as the cutoff value for copy number gain (dotted line). Asterisks indicate cell lines showing copy number gain. (B) Relative expression levels of *CDK14*, *CDK6*, and *PEG10* in 20 HCC cell lines as determined by real-time quantitative PCR. The results are presented as the expression level of each gene relative to a reference gene (*GAPDH*), to correct for variations in the amount of RNA.

4. Discussion

The high-resolution SNP array analysis reported in this study identified amplification at the chromosomal region 7q21 in JHH-4 HCC cells. A copy number gain at this region was frequently observed, not only in HCC cell lines, but also in primary HCCs. Of the three genes identified in the amplicon (i.e., *PEG10*, *CDK6*, and *CDK14*), subsequent experiments suggested that *PEG10* is the most likely target for the amplicon, in that the *PEG10* transcript was both overexpressed in JHH-4 cells and significantly upregulated in primary HCC tumors, compared with their nontumorous counterparts. In contrast, although the highest level of copy number gain was found at the *CDK6* locus in JHH-4 cells and primary HCC tumors, *CDK6* expression was not upregulated in primary HCC tumors.

Contrary to these data, a recent report indicated that expression of *CDK14* was higher in HCC tumors than in