reported in specific geographic areas and in some inherited cases. For example, homozygous deletion of the INK4a/ARF locus has been detected in 25 of 41 HCCs (61 %) in Korean subjects with HCC [40], and it has been reported that four of 26 patients with familial HCC (15 %) carried hemizygous germ-line point mutations of the p16 gene [41]. In contrast in Taiwan, homozygous deletion of the p16 gene was only identified in one of 30 HCCs (3 %) and no aberrant DNA methylation and mutation were detected [42], suggesting that the genetic alteration pattern of p16 is diverse among different geographic areas. Thorough examinations of their etiological backgrounds are important for further understanding.

With respect to the observations in Japanese cases, we previously examined 60 HCC patients and found no allelic loss of the p16 gene [20]. Point mutation of the p16 gene was only detected in four of 60 HCCs (6.6 %), suggesting that genetic changes of the p16 gene are rare in Japanese subjects. Immunohistochemical analysis showed that p16 expression was negative in 29 of 60 tumors (48 %), and the degree of p16 expression was inversely correlated with the histopathological grading of the tumors: negative immunostaining was observed in one (20 %) of five well-differentiated HCCs, 13 (50 %) of 26 moderately differentiated HCCs, 11 (48 %) of 23 poorly differentiated HCCs, and four (67 %) of six undifferentiated HCCs. The results of methylation-specific PCR (MSP) revealed that 24 (83 %) of the 29 HCCs with negative p16 immunostaining exhibited DNA methylation in the promoter region, indicating that DNA methylation is the main reason for p16 inactivation. We addressed the degree of DNA methylation in the p16 gene using a methylation-sensitive single nucleotide primer extension (Ms-SNuPE) method, which is a previously reported protocol based on the measurement of the band intensities of the methylated p16 gene incorporating [^{32}P]dCTP and the un-methylated p16 gene incorporating [^{32}P]TTP [43]. Our results showed that the degrees of CpG methylation in HCC with p16-negative expression ranged from 60 to 85 %, indicating that the p16 gene was extensively methylated at multiple sites in HCC.

Intriguingly, epigenetic changes of the p16 gene can be also observed in non-tumorous liver tissues. Kaneto et al. [44] reported that DNA methylation of the p16 gene was detected in five of 17 liver cirrhosis (29.4 %) and four of 17 chronic hepatitis (23.5 %). Li et al. [45] observed that the p16 gene was methylated in six of 38 (16 %) cases with chronic hepatitis or cirrhosis. We examined the genetic and epigenetic changes of the p16 gene in chronic liver injury and found that it was not mutated in any of the cases (unreported data), while methylated p16 promoter was detected in four of 112 (4 %) cases of liver cirrhosis [29]. Although the reported frequencies of p16 gene methylation in non-tumorous liver tissues differ among the studies, it should be kept in mind that techniques with higher sensitivity and specificity are required for the precise detection of methylated DNA. Methylation-specific PCR is a commonly used standard method for detecting methylated DNA, and is based on the examination of bisulfite-converted genomic DNA using methylated DNA-specific primers. This technique has been used in many studies to examine the methylation status of the p16 gene; however, false-negative results due to contamination of the tissue samples with PCR inhibitors cannot be ruled out. Recently, bisulfite sequencing has been applied for the screening of the methylation status of different DNA regions, and the MethyLight method (a sensitive and quantitative fluorescent real-time PCR technique) has been reported as the best method for the practical screening of methylated PCR. Using this new technique, Csepreg et al. [46] identified that methylation of CpG islands at the first exon of p16 gene could be detected by MethyLight but not by MSP. Therefore, to understand the clinical significance of p16 in non-tumorous liver tissues, surveillance using highly sensitive methods such as MethyLight technique would be useful.

p27 as a tumor suppressor

p27 is a member of the Cip/Kip family of CDKIs involved in G1 arrest and is encoded by the cyclin-dependent kinase inhibitor 1B (*CDKN1B*) gene [47, 48]. p27 binds and inhibits cyclinE-CDK2 complexes, leading to the hypophosphorylation of pRb in arrested cells. During the cell cycle, the level of p27 expression is stable and post-translational mechanisms mainly regulate its activity including protein expression levels, sequestration between the nucleus and cytoplasm, protein phosphorylation, and proteasome-mediated protein degradation. Of note, it has been unveiled that the role of p27 is not restricted to a CDK inhibitor, and different phosphorylation sites independently determine its activity. For example, mitogenic stimuli result in phosphorylation of p27 at serine 10 (S10) and sequestration of p27 from the nucleus to the cytoplasm, which leads to repressed inhibition of the cyclin E/CDK2 complex. When p27 is phosphorylated at threonine 157 and 198 (Thr157 and Thr198) through AKT signaling, p27 is located in the cytoplasm and sequestered from cyclin E/CDK2 to cyclin D1/CDK4 complexes, which leads p27 to act as an activator of CDK4. In turn, p27 is phosphorylated at threonine 187 (T187) by cyclin E/CDK2 complexes in late G1–S, which causes protein degradation by the recruited S phase kinase-associated protein 2 (SKP2) ubiquitin ligase-bound SCF complexes (consisting of Cullin-1, Skp1 and RBX1) [49, 50]. Recent studies have reported that decreased expression of p27 is correlated with poor prognosis in cancer patients, suggesting that it might

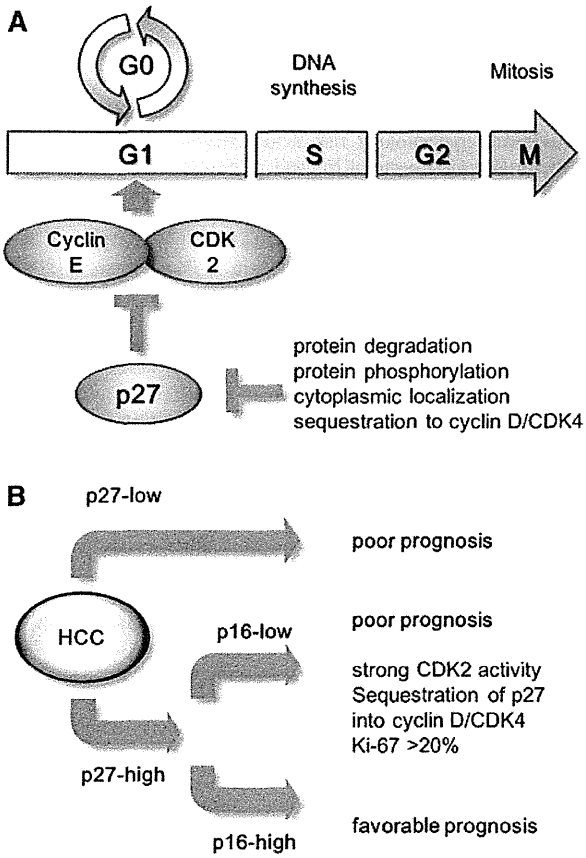

Fig. 2 **a** Key regulatory mechanisms controlling p27 activity. p27 inhibits cyclin E/CDK2 complexes at the G1–S phase. p27 is inactivated by translational modifications, including protein degradation, protein phosphorylation, cytoplasmic localization and sequestration into cyclin D/CDK4 complexes. **b** p27 is an adverse prognostic biomarker of HCC. Cases with high p27 expression can be categorized into two groups according to the labeling index of p16. In cases with high p27 and low p16 expression, CDK2 activities are strong and p27 is sequestered into cyclin D/CDK4 complexes, leading to poor prognoses

be a useful prognostic biomarker. In many types of cancer cells, gene mutations of p27 are rare but p27 expression is decreased through proteasome-mediated protein degradation. In fact, an inverse relationship between the levels of SKP2 and p27 has been observed in many types of human malignancies with poorer prognosis [51, 52] (Fig. 2).

p27 and hepatocellular carcinoma

To date, accumulating evidence has suggested that decreased p27 expression can be considered as an adverse prognostic biomarker in HCC [53–57]. Consistent with previous studies, we found that the 5-year survival rates were 62 and 93 % in low p27-expressing patients [labeling index (LI) of <50 by immunohistochemical analysis] and high p27-expressing patients (LI of >50 %), respectively. Low-p27 expressers represented a relative risk of dying from HCC as compared to high-p27 expressers ($P = 0.002$) [10]. Intriguingly, we also found that the cases of high-p27 expressers represented diverse levels of Ki-67 LI (a cell proliferation marker) and could be categorized into two groups with low and high Ki-67 expression. Of 40 cases with high-p27 expressors, 26 (65 %) showed a Ki-67 LI above 20 % (22–42 %), while 14 (35 %) showed a Ki-67 LI less than 20 % (2–13 %). To address the diverse cell proliferation status among the cases of high-p27 expressers, we set out to examine the activities of CDK2 in these cases. As expected, a relatively close relationship between the level of CDK2 activities and Ki-67 LI was observed, and the CDK2 kinase activities were significantly strong in all HCC cases in a subgroup with a Ki-67 LI of >20 %. In cases of p27 expressers representing strong CDK2 activities, heating the lysates of the protein samples effectively recovered the inhibitory function of p27, indicating that p27 alone was functional. In these tumors, p27 was found to be exclusively bound to cyclin D1/CDK4 complexes, supporting the idea that p27 can be inactivated by inappropriate protein–protein interaction in HCC. More importantly, we found that p16 was undetectable in all HCCs ($n = 8$), representing exclusive binding of p27 with cyclin D1/CDK4 complexes. Taken together, our data may indicate that the level of p27 expression does not accurately indicate the degree of functional activity, and in tumors with positive p27 expression p16 might act as potent determinant of the conformational change of p27. Moreover, when the cases of high p27 expression ($n = 40$) were categorized according to p16-negative and p16-positive status, the 5-year overall survival rates were 70 and 100 %, respectively ($P = 0.0004$). Multivariate analysis showed that p16 was an independent adverse prognostic factor in HCC ($P = 0.003$). Taken together, we surmise that combined examination of p16 and p27 might be useful for the precise prognostic diagnosis of HCC.

Cytoplasmic p27: another aspect of cell cycle inhibition

Although p27 has been widely considered a favorable prognostic factor in patients with different types of cancer, it has been shown that p27 exerts cell cycle-independent oncogenic properties [58, 59]. Post-translational modifications through phosphatidylinositol-3 kinase (PI3K)/AKT and RAS/mitogen-activated protein kinase (MAPK) signaling have been reported to be the main determinants of the function of p27 [60–65]. In the case of HCC, several studies have reported that the level of p27 in the cytoplasmic fraction of tumor cells was associated with cancer progression. For example, Nan et al. [66] reported an association with

clinical stage and the degree of invasion of HCC. Zhou et al. [67] examined 528 cases of HCC and reported that phosphorylated AKT and cytoplasmic p27 expression showed the strongest associations with pathological parameters of HCC. We previously reported that seven of 40 (17.5 %) HCCs exhibited cytoplasmic p27 expression and all the cases with cytoplasmic p27 had a Ki-67 LI of >20 % (22–42 %) [68]. Therefore, it is highly likely that nuclear p27 is a favorable prognostic marker, while cytoplasmic p27 is an adverse prognostic marker. For precise evaluation of the clinical significance of p27 in HCC, a comprehensive analysis of p27 including its expression level, localization, and the site of phosphorylation is required.

Future perspective

Recent progress has enabled improved diagnosis and management of HCC, but its prognosis still remains dismal. Identification of biomarkers is an important step for developing an efficient treatment. Dysfunction of either p16 or p27 causes abnormal cell proliferation, eventually leading to cancer development. The modality and mechanism for inactivating p16 and p27 are unique: epigenetic changes are the main cause of p16 inactivation, and either protein degradation or inappropriate protein–protein interaction results in the inactivation of p27. We have extensively examined the statuses of both CDK inhibitors in HCC, and our results show that their role in tumor aggressiveness is complex. Our hypothesis that p16 rather than p27 might be a useful prognostic factor in HCC should be investigated in the near future.

Dr. Yasunobu Matsuda, of the Department of Medical Technology, Niigata University Graduate School of Health Sciences, Niigata, is the winner of the Japanese Society for Clinical Molecular Morphology Award for Promoting Young Researchers in 2011. Dr. Matsuda is recognized for his great contribution in molecular morphological analysis of human hepatocellular carcinoma and advancement, and also for his contribution in the prediction of the prognosis in hepatocellular carcinoma patients, using biomarkers such as p16 and p17.

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ORIGINAL ARTICLE

Value of Highly Sensitive Fucosylated Fraction of Alpha-Fetoprotein for Prediction of Hepatocellular Carcinoma Recurrence After Curative Treatment

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Abstract
Background The fucosylated fraction of alpha-fetoprotein (AFP-L3) has been used as a diagnostic marker for hepatocellular carcinoma (HCC). Recently, a highly sensitive immunoassay using an on-chip electrokinetic reaction and separation by affinity electrophoresis (micro-total analysis system; μTAS) has been developed.
Aim The aim of this study was to investigate the relationship between changes in the serum AFP-L3 level measured by μTAS assay and recurrence of HCC after curative treatment.
Methods A total of 414 HCC patients who met the Milan criteria and underwent hepatectomy or radiofrequency ablation were investigated prospectively for the relationship between HCC recurrence and values of tumor markers.
Results There were significant differences in recurrence-free survival between groups with and without AFP-L3 elevation measured before and after treatment ($p = 0.024$ and $p = 0.001$ for before and after treatment, respectively). Multivariate analysis revealed that AFP-L3 status ($p = 0.002$) measured 1 month after treatment was a significant independent predictor of HCC recurrence after curative treatment.
Conclusions Elevation of the serum AFP-L3 level before treatment is a predictor of HCC recurrence, and sustained elevation of the AFP-L3 level after treatment is an indicator of HCC recurrence. Repeated measurement of μTAS AFP-L3 should be performed for surveillance of HCC recurrence after curative treatment.

Keywords Hepatocellular carcinoma · Alpha-fetoprotein · Fucosylated fraction of alpha-fetoprotein · Des-gamma-carboxy prothrombin

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third most common cause of cancer-related death [1]. Three serum markers, alpha-fetoprotein (AFP), fucosylated fraction of AFP (AFP-L3), and des-gamma-carboxy prothrombin (DCP), have been widely used for HCC surveillance and diagnosis. Among these markers, AFP is the most frequently used as a diagnostic marker for HCC in Japan [2, 3]. However, AFP levels are sometimes elevated in patients with chronic hepatitis and cirrhosis who have no evidence of HCC [4–6]. In contrast, AFP-L3 has been accepted as a specific marker for HCC by several investigators [7–9]. Moreover, its level predicts the malignant potential of HCC, and thus the expected outcome after treatment [10–15]. However, measurement of AFP-L3 has not always been reliable using serum samples with a low total AFP concentration determined by conventional lectin affinity electrophoresis, or using a liquid-phase binding assay system (LiBASys assay) [16]. Recently, a highly sensitive immunoassay for AFP-L3 using the on-chip electrokinetic reaction and separation by affinity electrophoresis (micro-total analysis system; μTAS) has been developed [17, 18]. We have already reported that the μTAS AFP-L3 is more sensitive for discriminating HCC from benign liver disease than the conventional LiBASys AFP-L3, particularly in subgroups with lower AFP concentrations and early stage HCC [19]. However, there has been little information about the μTAS AFP, AFP-L3 and DCP measured after curative treatment of HCC.

In the present study, we investigated the relationship between the tumor markers μTAS AFP, AFP-L3, and DCP, measured before and after curative treatment, and the likelihood of HCC recurrence. Additionally, we examined the changes in these markers in relation to treatment outcome.

Methods

Patients

A total of 420 HCC patients who met the Milan criteria (single tumor ≤5 cm in size or ≤3 tumors each ≤3 cm in size, and no macrovascular invasion) and who underwent hepatectomy or radiofrequency ablation at ten participating hospitals (Niigata University Medical and Dental Hospital, Medical Hospital of Tokyo Medical and Dental University, Kurume University Hospital, Tokyo Medical University Hospital, Juntendo University Hospital, Musashino Red Cross Hospital, Nihon University Itabashi Hospital, The University of Tokyo Hospital, Tokyo Women's Medical University Hospital, Hyogo Medical College Hospital), between May 2008 and November 2009, were investigated prospectively. HCC recurrence was assessed using several imaging modalities every 3 months after treatment, and all recurrences were evaluated up to the end of January 2012. The mean observation time after treatment was 594 ± 319 days. Six patients were excluded from the study because their follow-up periods were less than 1 month, giving a final total of 414 patients who were enrolled. Among these patients, HCC recurrence was observed in 236 patients, and the remaining 178 showed no HCC recurrence within the study period. Informed consent was obtained from each patient, and the study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the a priori approval granted by each institution's human research committee.

Diagnosis of HCC and Laboratory Examination

Hepatocellular carcinoma patients were diagnosed using several imaging modalities, including computed tomography (CT), magnetic resonance imaging (MRI), and CT, during hepatic arteriography, giving due consideration to hyperattenuation in the arterial phase with washout in the late phase. Vascular invasion was evaluated on the basis of imaging modalities. In some cases that showed atypical features on imaging, ultrasound-guided biopsies were performed.

Simultaneous quantitative measurement of AFP-L1 (ng/ml) and AFP-L3 (ng/ml) was performed using the μTAS assay (Wako Pure Chemical Industries Ltd., Osaka, Japan) [17, 18]. Total AEP concentration (ng/ml) in the serum sample was determined by summation of AFP-L3 and AFP-L1, and then the percentage of the AFP-L3 level was calculated. The serum level of des-gamma-carboxy prothrombin (DCP) was also measured using a μTAS assay, except for patients who had taken warfarin, a DCP-inducing agent. The lower limits of quantitation for AFP (L1 and L3) and DCP were 0.3 ng/ml and 5 mAU/ml, respectively [17, 18]. When AFP-L3 was not detectable, the percentage of AFP-L3 was defined as 0.5 %. AFP, AFP-L3, and DCP were measured in the same serum sample before and 1 month after treatment. Cut-off positivity values for AFP, AFP-L3, and DCP were set at 20 ng/ml, 10 %, and 40 mAU/ml, respectively. Hepatic functional reserve was ranked using the criteria of the Child-Pugh scoring system.

Treatment of HCC

Therapeutic modalities for HCC patients were chosen on the basis of hepatic functional reserve, tumor multiplicity, and tumor size. Among the 414 HCC patients, 228 underwent hepatic resection and 186 underwent

radiofrequency ablation (RFA). The efficacy of RFA treatment was evaluated using dynamic CT or dynamic MRI within a few days after treatment. Complete ablation of HCC was defined as non-enhancement of the lesion relative to the surrounding liver parenchyma. Patients received additional sessions of an ablative therapy until the treatment was judged as complete.

Statistical Analysis

Differences in the proportions of patients with elevated levels of AFP, AFP-L3, and DCP before and after treatment were determined by Fisher's exact test. Recurrence-free survival of patients with HCC was determined by the Kaplan–Meier method. Logrank test was used to test for equality of recurrence-free survival between the groups. Multivariate analysis of prognostic factors among the clinical features was performed using the Cox stepwise proportional hazards model. The factors included in multivariate analysis were patient age (years), gender (female/male), HBsAg (negative/positive), anti-HCV (negative/positive), alcohol abuse (negative/positive), Child-Pugh class (A/B,C), platelet count ($\times 10^4/\mu l$), tumor size (cm), number of tumors (single/multiple), treatment method (hepatic resection/RFA), pretreatment AFP (ng/ml) (<20/≥20), pretreatment AFP-L3 (%) (<10/≥10), pretreatment DCP (mAU/ml) (<40/≥40), post-treatment AFP (ng/ml) (<20/≥20), post-treatment AFP-L3 (%) (<10/≥10), and post-treatment DCP (mAU/ml) (<40/≥40). Statistical analyses were performed with the SPSS version 17.0 software package (SPSS Japan Inc., Tokyo, Japan). Differences at $p < 0.05$ were considered to be statistically significant.

Results

Clinical Features of Patients

Table 1 summarizes the demographics, etiology of liver disease, hepatic functional reserve ranked by the Child-Pugh classification, platelet count, serum concentration of AFP (ng/ml), AFP-L3 (%), DCP (mAU/ml) tumor size, number of tumors, and treatment modality for the study patients.

This population comprised 136 females and 278 males with a median age of 71 (range 33–89) years. The majority of patients had a viral etiology for their liver disease, anti-HCV being positive in 287 (69.3 %) and HBsAg being positive in 53 (12.8 %). Most patients (86.2 %) were Child-Pugh class A. The median tumor size was 1.9 (range, 0.6–5.0) cm and multiple tumors were present in 17.4 % of the patients. When the cut-off values were set at 20 ng/ml for AFP, 10 % for AFP-L3 and 40 mAU/ml for DCP, the positivity rates for AFP, AFP-L3 and DCP were 40.1, 25.4, and 33.3 %, respectively.

Table 1 Clinical features of patients ($n = 414$)

Age (years) [median (range)]	71 (33–89)
Gender (female/male)	136/278
Etiology	
HBsAg negative/positive	361/53
Anti-HCV negative/positive	127/287
Alcohol abuse negative/positive	396/18
Child-Pugh classification	
A/B/C	357/37/4
Platelet ($10^4/\mu l$) [median (range)]	12.2 (3.6–59.2)
AFP (ng/ml) [median (range)]	11.1 (0.3–14,597)
<20 ng/ml/≥20 ng/ml	248/166
AFP-L3 (%) <10 %/≥10 %	309/105
DCP (mAU/ml) [median (range)]	23 (5–33,283)
<40 mAU/ml/≥40 mAU/ml	273/136
Tumor size (cm) [median (range)]	1.9 (0.6–5.0)
Tumor number (single/multiple)	342/72
Therapeutic modalities	
Hepatic resection/RFA	228/186

Range or percent are shown in *parentheses*

HCC hepatocellular carcinoma, *HBsAg* hepatitis B surface antigen, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin, *RFA* Radiofrequency ablation

Impact of AFP, AFP-L3, and DCP on Recurrence-Free Survival of Patients with HCC

Among the 414 patients enrolled in this study, HCC recurrence was observed in 236, and the remaining 178 showed no HCC recurrence within the study period. Recurrence-free survival rates of patients with HCC were evaluated in terms of serum AFP, AFP-L3, and DCP levels before and after treatment. Comparison of the three tumor markers measured before treatment showed that patients with an elevated AFP-L3 level had a significantly lower recurrence-free survival rate than patients without such an elevation of the AFP-L3 level ($p = 0.024$) (Fig. 1). In contrast, there was no significant difference in recurrence-free survival between the groups with and without elevation of the AFP and DCP levels before treatment (AFP, $p = 0.171$; DCP, $p = 0.208$) (Fig. 1). The recurrence-free survival rate of patients with an elevation of AFP, AFP-L3, and DCP measured after treatment were significantly lower than that of patients without such elevation (AFP, $p = 0.009$; AFP-L3 and DCP, $p = 0.001$) (Fig. 2).

In addition, we evaluated the recurrence-free survival rates of patients according to AFP-L3 status measured 9 months after treatment. In patients who had no

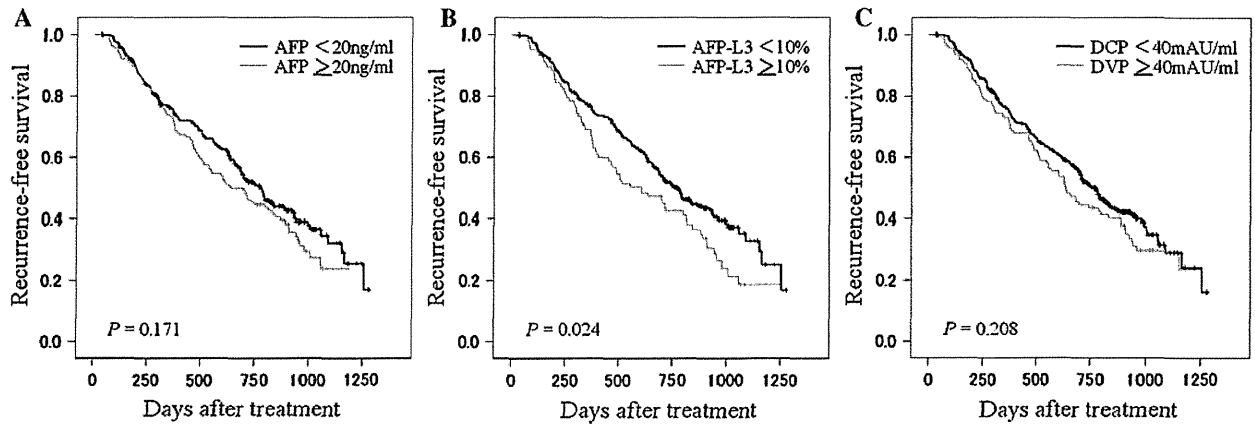

Fig. 1 Comparison of recurrence-free survival rates between patients with and without elevation of the AFP, AFP-L3, and DCP levels before treatment. Recurrence-free survival rates according to AFP (**a**), AFP-L3 (**b**), and DCP (**c**)

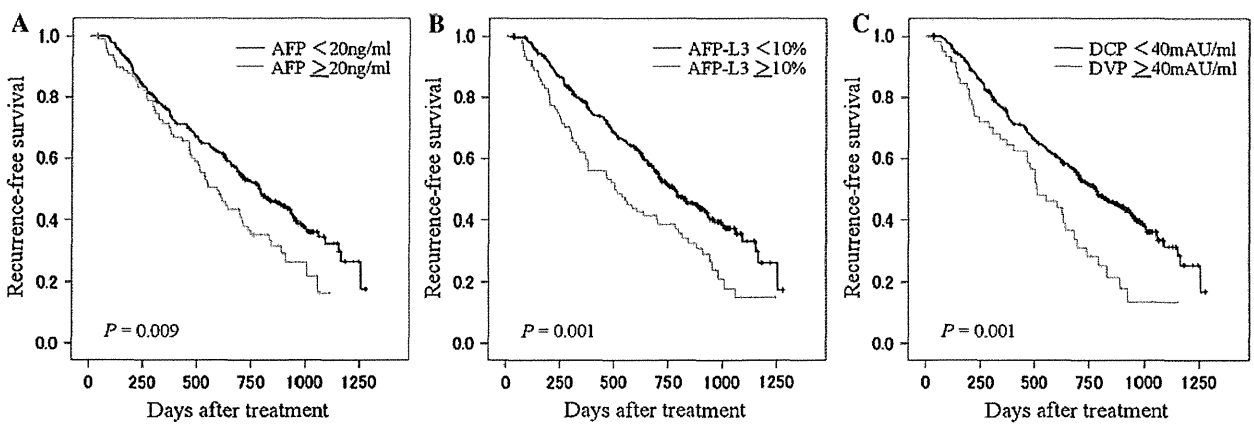

Fig. 2 Comparison of recurrence-free survival rates between patients with and without elevation of the AFP, AFP-L3, and DCP levels at one month after treatment. Recurrence-free survival rates according to AFP (**a**), AFP-L3 (**b**), and DCP (**c**)

recurrence for at least 9 months, a significant difference was also observed between those with and without elevation of the AFP-L3 level ($p = 0.003$) (Fig. 3).

Multivariate analysis using the Cox stepwise proportional hazard model revealed that post-treatment AFP-L3 status ($p = 0.002$) and post-treatment DCP status ($p = 0.004$) were significant independent factors predictive of recurrence-free survival in patients with HCC (Table 2).

Relationship Between HCC Recurrence and Change in Positivity Rates for Serum AFP, AFP-L3, and DCP Before and After Treatment

We evaluated the changes in positivity rates for serum tumor markers before and after treatment in 308 of the 414 patients who were enrolled in this study. These 308 patients comprised 193 who suffered recurrence within 2 years and 115 in whom no recurrence was observed for more than 2 years. Positivity rates for serum AFP, AFP-L3, and DCP were investigated in relation to HCC recurrence, and the results are shown in Table 3. Regardless of HCC recurrence, the proportions of patients showing elevation of the AFP and DCP levels (AFP, ≥20 ng/ml; DCP, ≥40 mAU/ml) for 1 month after curative treatment were significantly lower in comparison to the situation before treatment (AFP, $p = 0.004$ for patients with HCC recurrence and $p = 0.001$ for patients without HCC recurrence; DCP, $p < 0.001$ for both patients with and without HCC recurrence). On the other hand, there was no significant decrease in the proportion of patients showing an elevated AFP-L3 level 1 month after treatment in comparison to the situation before treatment in both of the groups with and without HCC recurrence. Among the 193 patients in the HCC recurrence group, 15 (26.8 %) of 56 patients with an elevated AFP-L3 level (≥10 %) before treatment became negative for AFP-L3 (<10 %) after treatment. In the recurrence-free group, eight (38.1 %) of 21 patients with an elevated AFP-L3 level before treatment became negative for AFP-L3 after treatment. In addition, we investigated the long-term changes

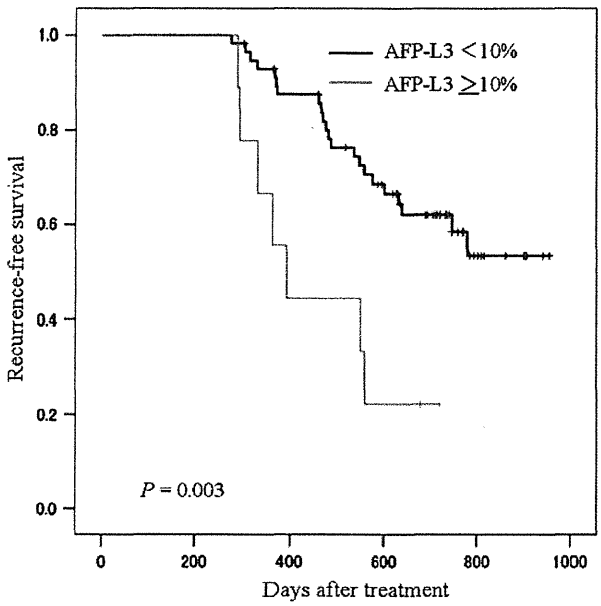

Fig. 3 Comparison of recurrence-free survival rates between patients with and without elevation of the AFP-L3 level at 9 months after treatment

Table 2 Multivariate analysis of factors associated with HCC recurrence after curative treatment

Variables	Hazard ratio (95 % CI)	*p* value
Post-treatment AFP-L3		
<10 %	1	0.002
≥10 %	1.592 (1.183–2.144)	
Post-treatment DCP		
<40 mAU/ml	1	0.004
≥40 mAU/ml	1.687 (1.187–2.398)	

Hazard ratio and *p* value were calculated using Cox's stepwise proportional hazard model

HCC hepatocellular carcinoma, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin, *CI* confidence interval

in the level of AFP-L3 after treatment. Among patients who suffered recurrence of HCC after 9 months or later of treatments, the proportion of those who were still AFP-L3-positive 6 and 9 months after treatment was 28.6 % (4/14) and 24.1 % (7/29), respectively, being similar to the proportion of such patients before treatment (29.2 %; 69/236). On the other hand, in the group who remained recurrence-free for more than 2 years, no patient was AFP-L3-positive at 6 and 9 months after treatment (6 months; 0 %, 0/5: 9 months; 0 %, 0/18).

Discussion

Many studies have shown that AFP-L3 status is a specific marker for HCC, and that the level of AFP-L3 predicts the malignant potential of HCC, and thus the expected outcome after treatment [10–15]. Recently, μTAS AFP-L3 has been newly developed as an automated immunoassay for AFP-L3. A number of investigators, including our group, have shown that the μTAS AFP-L3 is more sensitive for discriminating HCC from benign liver diseases than the conventional LiBASys AFP-L3, particularly in subgroups with lower AFP concentrations and early stage HCC [19–21]. In addition, we have previously reported that μTAS AFP-L3 status is a statistically significant independent prognostic indicator of long-term survival in patients with HCC [19]. On the other hand, there has been little information about the levels of μTAS AFP, AFP-L3, and DCP measured after curative treatment of HCC.

In the present study, we demonstrated that there was a significant difference in recurrence-free survival between groups with and without elevation of the AFP-L3 level before and after treatment. Multivariate analysis revealed that AFP-L3 status measured 1 month after treatment was a significant independent predictor of HCC recurrence after curative treatment. On the other hand, we also found that, irrespective of HCC recurrence, there was no significant decrease in the proportion of patients showing an elevated AFP-L3 level 1 month after treatment in comparison to the situation before treatment. In our present examination of long-term changes in the level of AFP-L3 after treatment, the proportion of patients with an elevated level of AFP-L3 at 6 and 9 months after treatment was decreased relative to that before treatment in the recurrence-free group. By contrast, the proportion of patients who were still AFP-L3-positive after treatment was similar to that before treatment in the HCC recurrence group. Taken together, these results suggest that an elevated AFP-L3 level before treatment is a predictor of HCC recurrence, and that sustained elevation of AFP-L3 after treatment should be considered to indicate HCC recurrence.

The decrease in the total concentration of AFP within 1 month after treatment, in terms of half-life, is reported to be 4–6 days [22, 23]. However, the percentage decrease in the level of AFP-L3 basically does not change, and the lower limit for quantitation of μTAS AFP-L3 is quite low (0.3 ng/ml). We suggest that the minimal change in AFP-L3 positivity in some patients without HCC recurrences within the first month after treatment was attributable to serum AFP that had been produced by HCC just before treatment. Therefore, repeated combination measurement of μTAS AFP, AFP-L3, and DCP should be performed for surveillance of HCC recurrence after curative treatment.

Recently, Yamamoto et al. [24] have investigated the relationship between HCC recurrence and changes in the levels of three tumor markers—AFP, AFP-L3, and DCP—measured using the LiBASys assay. They reported that, among these markers, AFP-L3 positivity after treatment

Table 3 Serum AFP, AFP-L3, and DCP before and 1 month after treatment

Patient group and tumor markers	Before treatment	1 month after treatment	*p* value
Patients with HCC recurrence within 2 years ($n = 193$)			
AFP			
Patient number (≥20 ng/ml/<20 ng/ml)	83/110	55/138	0.004
Percent of patients with AFP ≥20 ng/ml	43.0 %	28.5 %	
AFP-L3			
Patient number (≥10 %/<10 %)	56/137	52/141	0.734
Percent of patients with AFP-L3 ≥ 10 %	29.0 %	26.9 %	
DCP			
Patient number (≥40 mAU/ml/<40 mAU/ml)	69/123	35/155	<0.001
Percent of patients with DCP ≥ 40mAU/ml	34.3 %	18.4 %	
Patients without HCC recurrence for more than 2 years ($n = 115$)			
AFP			
Patient number (≥20 ng/ml/<20 ng/ml)	43/72	20/95	0.001
Percent of patients with AFP ≥ 20 ng/ml	37.4 %	17.4 %	
AFP-L3			
Patient number (≥10 %/<10 %)	21/94	17/98	0.595
Percent of patients with AFP-L3 ≥10 %	18.3 %	14.8 %	
DCP			
Patient number (≥40 mAU/ml/<40 mAU/ml)	31/83	6/109	<0.001
Percent of patients with DCP ≥40 mAU/ml	27.0 %	5.2 %	

p values were calculated by Fisher's exact test

HCC hepatocellular carcinoma, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin

had the highest risk ratio of 5.0 for HCC recurrence after curative treatment. Kobayashi et al. [25] have investigated the relationship between changes in the serum AFP-L3 level measured by μTAS assay 30–120 days after curative treatment and HCC recurrence. They reported that 29 of 37 patients (78.4 %) with preoperative AFP elevation (>20 ng/ml) showed a decrease in the AFP level to <20 ng/ml, although 16 of 42 patients (38.1 %) with preoperative AFP-L3 elevation (>5 %) showed a decrease in the level to <5 %. On this basis, they concluded that it was rare for AFP-L3 to become negative after treatment. Toyoda et al. [26] also investigated the value of AFP, AFP-L3, and DCP, measured before and 1–2 months after treatment, for prediction of survival and recurrence in patients who had undergone hepatectomy for HCC. They concluded that the combination of tumor markers measured by μTAS assay after hepatectomy had excellent ability to predict postoperative survival and recurrence. Our present results support their findings, although the AFP-L3 cut-off value used in our study was 10 %, unlike their value of 5 %. In our previous study, we suggested that a cut-off value of 7 % was most appropriate for discriminating HCC from benign liver disease using μTAS AFP-L3 [19]. In our present study, however, we chose a cut-off value of 10 % for μTAS AFP-L3 in view of a recently published report by Kanke et al. [27] in which intra-individual biological variation of the μTAS AFP-L3 level was relatively high, at 29.0 %.

Some factors other than AFP-L3 and DCP could potentially have been associated with HCC recurrence. Several studies have implicated obesity and diabetes as risk factors for HCC [28–30]. However, we did not investigate these factors in the present study.

The difficulty in the treatment of HCC is related to the underlying impairment of hepatic functional reserve and high rate of recurrence, even after curative treatment. Therefore, early detection of HCC recurrence after treatment is an important issue for improving the survival of patients with HCC. From this viewpoint, the μTAS assay is an extremely powerful tool for detection of HCC at an early stage. Additionally, clinicians should interpret the values of tumor markers measured by the μTAS assay giving due consideration to their property. In conclusion, the present study has demonstrated that patients with an elevated AFP-L3 level determined before treatment have a high risk of HCC recurrence, and that sustained elevation of AFP-L3 after treatment is a strong predictor of HCC recurrence. In addition, short-term AFP-L3 status is not a reliable indicator of incomplete treatment for HCC. Accordingly, repeated and combined measurement of μTAS AFP, AFP-L3, and DCP should be performed for surveillance of HCC recurrence after curative treatment.

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Conflict of interest None.

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VIRAL HEPATITIS

Hepatitis B virus X induces cell proliferation in the hepatocarcinogenesis via up-regulation of cytoplasmic p21 expression

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Keywords
HBV X protein – hepatocarcinogenesis – IFN-β – p21 (Cip1/WAF1) – PKCα

Abbreviations
CDK, cyclin-dependent kinase; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FBS, foetal bovine serum; HBV, hepatitis B virus; HBx, HBV X protein; HCC, hepatocellular carcinoma; IFN, interferon; MEK, mitogen-activated protein kinase/ERK kinase; PI-3K/Akt, phosphatidylinositol 3-kinase/protein kinase B; PKC, protein kinase C; pRb, retinoblastoma protein; qRT-PCR, quantified real-time PCR; WM, wild mice; Xg, HBx transgenic mice.

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Abstract

Background: Hepatitis B virus X protein (HBx) has been shown to induce hepatocarcinogenesis by disrupting the functions of intracellular molecules. Cyclin-dependent kinase inhibitor p21 (Cip1/WAF1), known as a tumour-suppressor gene, has been reported to have paradoxical function, that is, acting as an oncogene, particularly when expressed in the cytoplasm. The effects of HBx on the expression and function of p21 also remain controversial. *Aims:* We attempted to investigate the role of HBx in the hepatocarcinogenic process, focusing on the association with this paradoxical function of p21. The results obtained were further verified with experiments using the antihepatocarcinogenic action of interferon (IFN)-β. *Methods:* HBx transgenic mice (Xg) and HBx-transfected hepatoma cell lines were used. Intracellular localization of p21 was determined by Western blot analysis and immunofluorescence. *Results:* Xg and HBx-transfected cells exhibited increased expression of p21. Up-regulation of p21 was positively correlated with the expression of cyclin D1 and inactive phosphorylation of retinoblastoma protein (pRb). These HBx-induced cell proliferative responses were cancelled by knockdown of p21, which resulted in growth reduction in HBx-expressing cells, suggesting the oncogenic properties of HBx-induced p21. HBx induced accumulation of p21 in the cytoplasm, and activation of PKCα was involved. Finally, IFN-β-treated Xg liver, as well as hepatoma cells, showed a shift of cytoplasmic p21 to the nucleus, accompanied by the abrogation of HBx-induced oncogenic modulation. *Conclusions:* Our results suggest that HBx induces hepatocarcinogenesis via PKCα-mediated overexpression of cytoplasmic p21 and IFN-β suppressed these molecular events by shifting p21 to the nucleus.

Persistent infection with hepatitis B virus (HBV) is a prevalent aetiology for chronic liver disease, and may lead to a premalignant condition preceding the development of hepatocellular carcinoma (HCC) (1, 2). Elimination of this virulent pathogen is a solid clinical strategy for prevention of HCC formation. Despite recent advances in anti-HBV therapy, such as interferon (IFN)-based therapy and use of nucleic acid analogue drugs, there remain a number of problems in the establishment of preventive strategies for HBV-related HCC (3). These problems result from unresolved questions regarding the molecular mechanisms of HBV-induced hepatocarcinogenesis (4, 5). Among the four proteins (S, C, P and X) encoded by the four open reading frames in HBV genome, HBV X protein (HBx), consisting of 154 amino acids, has been found to disrupt cell proliferative and apoptotic processes through the modulation

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of intracellular signal transduction pathways, including the protein kinase C (PKC), phosphatidylinositol 3-kinase/protein kinase B (PI-3K/Akt) and extracellular signal-regulated kinase (ERK)1/2 pathways (6–8). These aberrant molecular effects of HBx during HBV infection are likely to render normal hepatocytes susceptible to neoplastic and malignant transformation.

Mammalian cell proliferation is controlled by various protein kinase complexes, including cyclin/cyclin-dependent kinase (CDK) complexes that regulate distinct phases of the cell cycle by repressing anticell proliferative functions of retinoblastoma protein (pRb) (9). Although induction of p21 (also known as Cip1/WAF1) as a universal inhibitor of cyclin/CDK activity downstream of active p53 generally results in inhibition of cell proliferation (10), more recent studies have revealed the opposite effect. Such studies have suggested that p21 renders normal cells susceptible to transformation by leading to an anti-apoptotic or a cell proliferative status, which is unrelated to p53 (11–13) and controlled by protein kinase activity (14–16). This oncogenic potential of p21 has also been confirmed *in vivo* and in clinical studies (17, 18). It has been suggested that the intracellular distribution pattern of p21 in particular may determine whether it has anti-oncogenic or oncogenic property. Zhou *et al.* reported that cytoplasmic accumulation of p21 resulted in a loss of the cell cycle arrest induced by p21 located in nuclei (19). Furthermore, forced cytoplasmic localization of p21, using a nuclear localization signal-deficient p21 construct, triggered cell cycle progression as well as protection from apoptosis (20).

Previous reports have revealed that HBx triggers hepatocarcinogenesis by modulation of p21 expression and function (21–24). However, they showed different results and interpretations. Kwun and Jang demonstrated that, using several HBx clones from the sera of patients with chronic HBV infection, natural variants of HBx determined the expression level of p21 in a p53-dependent or -independent pathway, suggesting that these variants show different effects on the cell cycle progression (25). However, these studies were not confirmed by comparative assays using experimental models, which showed the suppressive state against HBx-induced hepatocarcinogenesis after anticancerous treatment. Thus, the role of p21 in HBx-triggered HCC development remains uncertain.

PKC plays a central role in signal transduction and has been implicated in a wide range of physiological functions, including cell growth, transformation and differentiation. HBx protein was found to increase the levels of endogenous PKCs and subsequently activate AP-1 and NF-κB transcription factors (6, 7). As oncogenic p21 was reported to be regulated by PKCα activation, resulting in cell cycle progression by incorporation of p21 into cyclin/CDK complexes (14), we hypothesize that HBx-induced p21 up-regulation may be mediated through active PKCα. We here report that HBx promotes cell proliferation via up-regulation of PKCα-mediated overexpression of cytoplasmic p21 using both HBx transgenic mice (Xg) (26, 27) and HBx-transfected cells. Because our previous study found that treatment with IFN-β prevented HBx-induced HCC development in Xg (28), we verified our results using the anticell proliferative action of IFN-β.

Materials and methods

Mouse liver samples

Frozen mouse livers for quantified real-time PCR (qRT-PCR) and Western blot analyses were obtained as described previously (28). mRNA and proteins were extracted from the livers of 6-month-old wild mice (WM) and Xg treated with either saline (control) or IFN-β. RNA (1 μg) purified with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was subjected to reverse transcription and qRT-PCR using a Light Cycler System (Roche, Manheim, Germany). Specific primers and probes for the genes were designed based on the Universal Probe Library (Roche). All the experimental protocols were approved by the Institutional Animal Care and Use Committee of Niigata University and performed in accordance with the National Institute of Health Guidelines.

Cell culture and reagents

HepG2 and Hep3B were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and antibiotic–antimycotic solution (Sigma Aldrich, St. Louis, MO, USA). IFN-β was kindly provided by Toray Medical Co., Ltd. (Chiba, Japan). Specific kinase inhibitors, GF109203X, U0126 and LY294002, were purchased from Calbiochem (San Diego, CA, USA), and dimethyl sulfoxide (DMSO) was from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Rabbit-polyclonal antibodies specific for p21, used for mouse liver samples, and p53 and anti-GAPDH mouse-monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse-monoclonal antibodies specific for FLAG M2 and β-actin were purchased from Sigma, and other specific antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Plasmid construction and transfection

For HBx overexpression in cell lines, we generated pCAG-FLAG-HBx expressing FLAG-tagged HBx under the CAG promoter. The blunted *Not*I-*Bgl*II fragment containing the FLAG-HBx coding sequence from pNK-FLAG-HBx (kind gift from Dr Murakami, Kanazawa University, Japan) was inserted into the blunted *Eco*RI site downstream of the CAG promoter in pCAG-IRES-Puro. After sequence verification, transfection with

pCAG-IRES-Puro, used for mock transfection, or pCAG-FLAG-HBx was performed using FuGENE6 (Roche) and MA lipofection enhancer (IBA, St. Louis, MO, USA) according to the manufacturer's protocols. To establish stable expressing cell lines, pCAG-IRES-Puro and pCAG-FLAG-HBx were linearized with *Pvu*I and transfected into HepG2, followed by selection with 2 μg/ml puromycin (Cayla, Toulouse, France).

Western blot analysis

Frozen mouse livers or culture cells were lysed using Tissue Ruptor or Cell Scraper with RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitor cocktail and phospho-stop tablets (Roche). Lysates were sonicated and collected by centrifugation to remove debris. Nuclear and cytoplasmic fractions of liver tissues and culture cells were prepared by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's protocols. Next, 20 μg of mouse samples and 10 μg of cellular lysates, respectively, were subjected to Western blotting.

Knockdown of p21 with siRNA

For knockdown assay of p21, the FlexiTube GeneSolution for CDKN1A (Entrez gene ID: 1026) containing four different siRNA duplexes of p21 or negative control siRNA was transfected into HepG2 cells using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocols. At 24 h of siRNA delivery, mock or HBx expression plasmids were transiently expressed. To examine the effects of p21 knockdown on cell growth or cell cycle, 5×10^5 HepG2 cells were plated onto six-well plates in triplicate or a 10 cm dish and transfected with siRNA. For cell growth analysis, at 24 or 48 h after plasmid expression, cells were harvested and then the number of viable cells was counted after trypan blue dye treatment as described previously (29). For cell cycle analysis, 48-h HBx-expressed cells were stained with BrdU using a FITC BrdU Flow Kit (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's protocols. The FITC intensity and total DNA content were measured by FACScan (BD Pharmingen) and analysed using FlowJo software (Ver. 7; Tree Star Inc., Ashland, OR, USA).

Immunofluorescence

Cultured cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. After washing, cells were blocked in Image-iT FX Signal Enhancer (Invitrogen). For double immunofluorescence staining, fixed cells were reacted with anti-FLAG and anti-p21 antibody at a dilution of 1/50, followed by incubation with fluorescence-conjugated AlexaFluor secondary antibody (Cell Signaling) at a dilution of 1/1000. Nuclei were counterstained with DAPI (Invitrogen) and slides were examined by fluorescence microscopy.

Immunoprecipitation

Cell extracts were prepared by incubation in 0.5% modified NP40 Cell Lysis Buffer (Invitrogen) for 30 min on ice. After centrifugation, 500 μg of cell extracts was immunoprecipitated with anti-FLAG or negative control mouse IgG (Thermo Scientific) using Catch and Release v2.0 system (Millipore, Billerica, MA, USA) according to the manufacturer's protocols. Eluted samples were separated by SDS-PAGE and subjected to immunoblotting.

Statistical analysis

Statistical analysis was performed using commercial SPSS software (Version 15.0; SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to compare the differences among mRNA levels on qRT-PCR analysis and cell growth data. P values <0.05 were considered to be statistically significant.

Results

Effects of HBx on p21 *in vivo* and *in vitro*

Using qRT-PCR, we first compared the intrahepatic expression of p21 mRNA between 6-month-old WM and Xg. p21 mRNA was highly up-regulated in Xg liver, showing about 10-fold more expression than in WM (Fig. 1A). As shown in Fig. 1B, we also verified this difference by assessing p21 protein levels in 6- and 12-month-old mouse liver lysates. Notably, expression of p53, a general upstream regulator of p21, was not enhanced in Xg liver, suggesting that p21 was p53-independently overexpressed in Xg.

Next, using *in vitro* assays, we determined whether HBx-induced p21 overexpression could be demonstrated in HBx-expressing hepatoma-derived cells as shown in Xg liver. Transient transfection with HBx constructs, but not mock constructs, resulted in dose-dependent enhancement of p21 expression without changes in p53 expression, in HepG2 and in p53-null Hep3B cells (Fig. 1C). We further confirmed that HBx caused p21 up-regulation in stable cell lines, similar to that in transiently expressing cells (Fig. 1D). These results indicate that culture cells transfected with HBx constructs also showed enhancement of p21 expression in a p53-independent manner.

Effects of HBx on p21-associated molecules

p21 has been well known as a tumour suppressor directly inhibiting activation of cyclin D/CDK complexes, which promote the cell cycle following

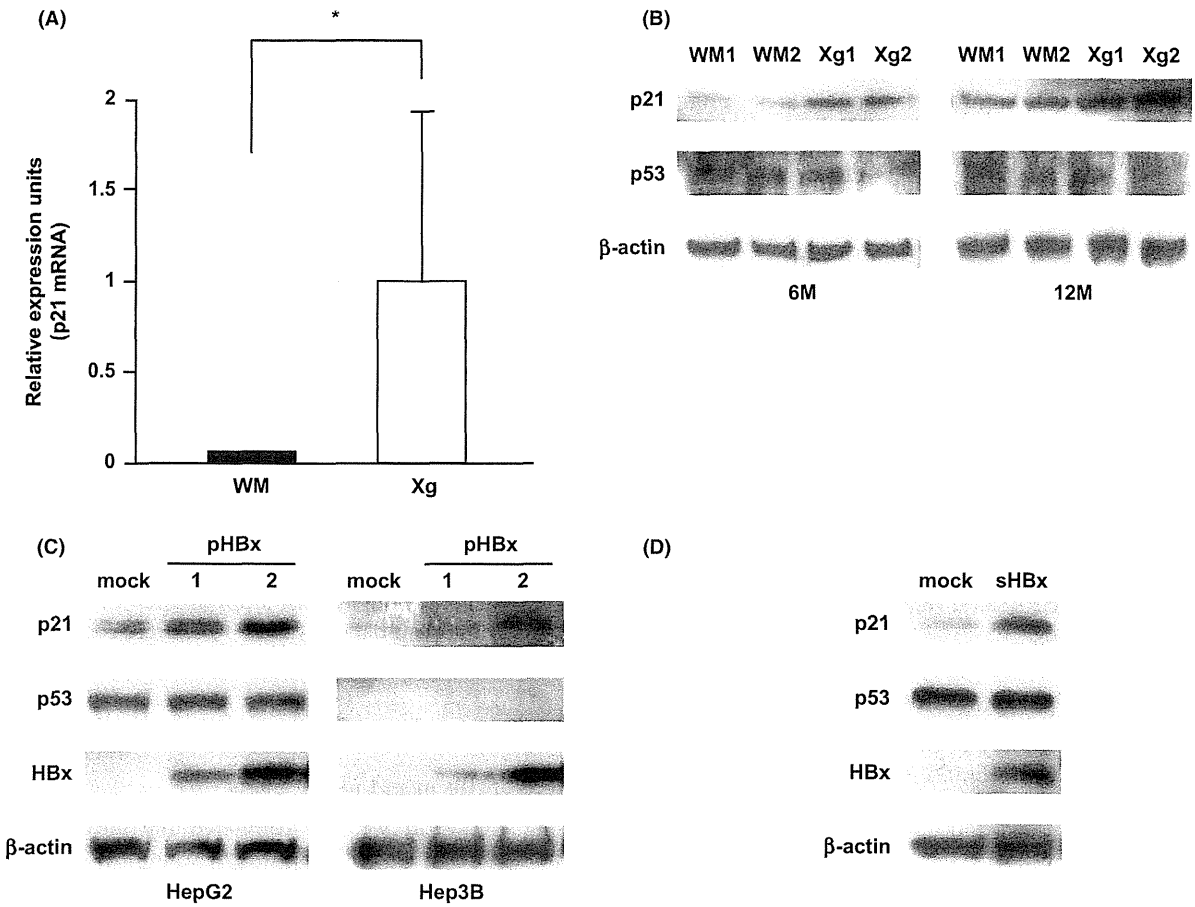

Fig. 1. p53-independent p21 expression is up-regulated by HBx in Xg liver and HBx-expressing hepatoma cells. (A) Comparative analysis of p21 mRNA in livers from WM and Xg at age 6 months by qRT-PCR; GAPDH, internal control (*$P < 0.01$) Liver mRNAs from seven mice in each group were used. (B) Western blot analysis showing p53-independent up-regulation of p21 in Xg liver. Two liver samples from each mouse group at age 6 months (6 M; left panel) or 12 months (12 M; right) were immunoblotted with antibodies specific for p21 and p53. (C, D) Western Blot analysis showing p53-independent up-regulation of p21 in HBx-expressing hepatoma cells. HepG2 (left panel) or Hep3B (right) cells were transiently transfected with mock or increasing amounts of HBx plasmid (pHBx) for 40 h (C), and mock- or HBx-expressing stable HepG2 cells (sHBx) were established as described in Materials and methods (D). After incubation and harvest, levels of p21, p53 and FLAG (HBx) were analysed by Western blot. Membranes were stripped and reprobed with anti-β-actin antibody to control for protein-loading variations.

phosphorylation and inactivation of pRb (10). However, recent studies focusing on intracellular functions of p21 found that it could also facilitate the assembly of cyclin D/CDK complexes via a p53-independent pathway (12, 13). Hence, we examined the expression and phosphorylation of these proteins in Xg liver and HBx-transfected cells. As shown in Fig. 2A, Xg liver exhibited increased the expression of cyclin D1, while cyclin D3 and CDKs expression were not enhanced. Consistent with this, HBx-transfected hepatoma cells showed increases in cyclin D1 and phosphorylation, inactivated form, of pRb, which may be mediated by active cyclin D1, in both transient and stable cell lines (Fig. 2B,C). These observations implied a positive link among p21, cyclin D1 expression and pRb inactivation in HBx-induced hepatocarcinogenesis.

Knockdown and localization analysis of p21 in HBx-transfected cells

Both *in vivo* and *in vitro* experiments indicated the involvement of p53-independent up-regulation of p21 expression in the hepatocarcinogenic action of HBx, but the mechanisms of HBx-induced p21 remain uncertain. To evaluate whether HBx-induced p21 promotes cell growth and cell cycle, we investigated the effects of p21 knockdown with siRNA on cell proliferation and oncogenic events in this setting. Figure 3A shows attenuations of pRb phosphorylation and cyclin D1 expression by p21 knockdown in HBx-transfected cells, and reciprocally increased induction of those in mock-transfected cells. These results suggest that the opposite function of p21, acceleration of the cell cycle, was induced by HBx.

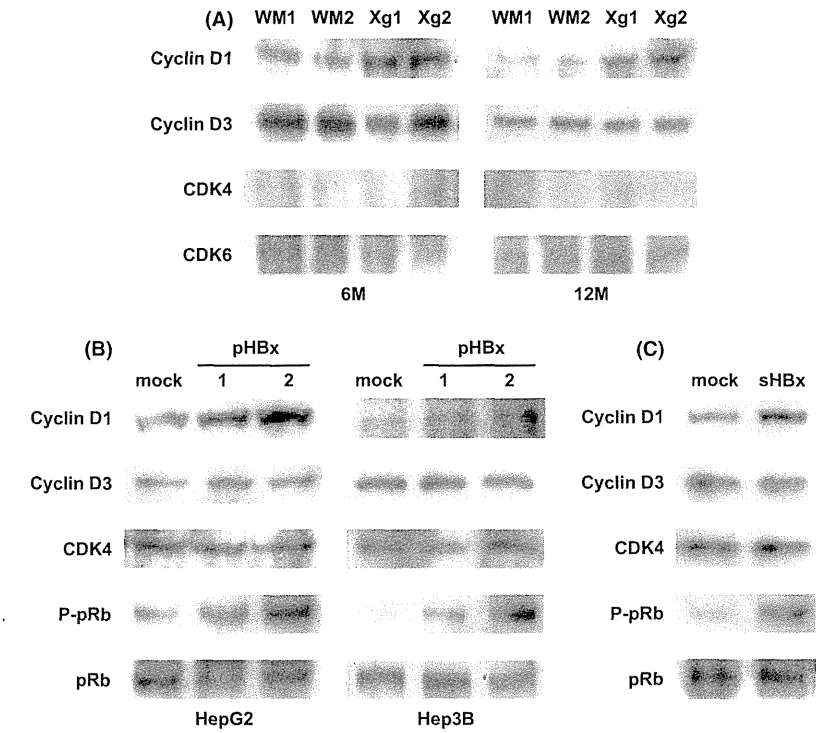

Fig. 2. Up-regulations of cyclin D1 expression and pRb phosphorylation are induced by HBx. (A) Western blot analysis showing cyclin D1 up-regulation in Xg liver. The same liver samples as shown in Fig. 1B were immunoblotted with antibodies specific for cyclin D/CDKs. (B, C) Western blot analysis showing increases in cyclin D1 and phosphorylated-pRb (P-pRb) in HBx-expressing cells. The same HBx-expressing transient (B) or stable (C) cell samples as shown in Fig. 1C or 1D were immunoblotted with antibodies specific for cyclin D/CDKs, pRb (phosphorylated form and total) and FLAG (HBx). Anti-β-actin antibody was used as protein-loading control.

Reflecting these observations, in cell growth and cell cycle assays, significant reductions in cell proliferation and S phase cell population were observed in HBx-transfected cells with the knockdown of p21 in contrast to control siRNA (Fig. 3B,C).

Previous reports indicated that the regulatory role of p21 in the cell cycle may be dependent upon its intracellular localization (19, 20). We therefore performed Western blot analysis using nuclear and cytoplasmic fractions to verify the intracellular localization of p21 in liver lysates from Xg and in HBx-transfected cells. We found that intracellular localization of p21 became predominantly cytoplasmic in both Xg liver and in HBx-expressing cells (Fig. 4A,B). These results suggest that the aberrant overexpression of cytoplasmic p21 by HBx results in pRb inactivation and cell cycle progression, which may lead to an oncogenic state.

Involvement of PKCα in HBx-induced cytoplasmic p21 overexpression

As there are previous reports that this oncogenic localization of p21 in cytoplasm requires the activation of PKC (14), mitogen-activated protein kinase/ERK kinase (MEK)-ERK1/2 (16) or Akt (15), which have been known to be aberrantly stimulated by HBx and lead to hepatocarcinogenesis (6–8), we tested the effects of an inhibitor specific for each kinase on molecular results caused by HBx in HBx-expressing stable cells. Treatment with GF109203X (PKC inhibitor) or U0126 (MEK1/2 inhibitor), but not LY294002 (PI-3K inhibitor), attenuated HBx-enhanced p21 expression, and only GF109203X treatment resulted in the attenuations of both cyclin D1 and phosphorylated-pRb (Fig. 5A). As shown in Fig. 5B, GF109203X also inhibited HBx-induced overexpression of cytoplasmic p21, whereas U0126, which decreased total expression of p21 in HBx cells (Fig. 5A), did not alter the intracellular distribution of p21, indicating that HBx enhanced this oncogenic cytoplasmic p21 via PKC activation, not through MEK-ERK1/2 or PI-3K/Akt. We confirmed the up-regulation of PKCα phosphorylation in Xg liver as well as in HBx-expressing cells (Fig. 5C,D), but other PKC isoforms were not phosphorylated in any experimental models (data not shown).

HBx reportedly exerts hepatocarcinogenic functions via direct association with various molecules, including p53 (7). We hypothesized that up-regulation of cell proliferative p21 by HBx is mediated through their direct interaction in cytoplasm (see Fig. 4C). However, HBx

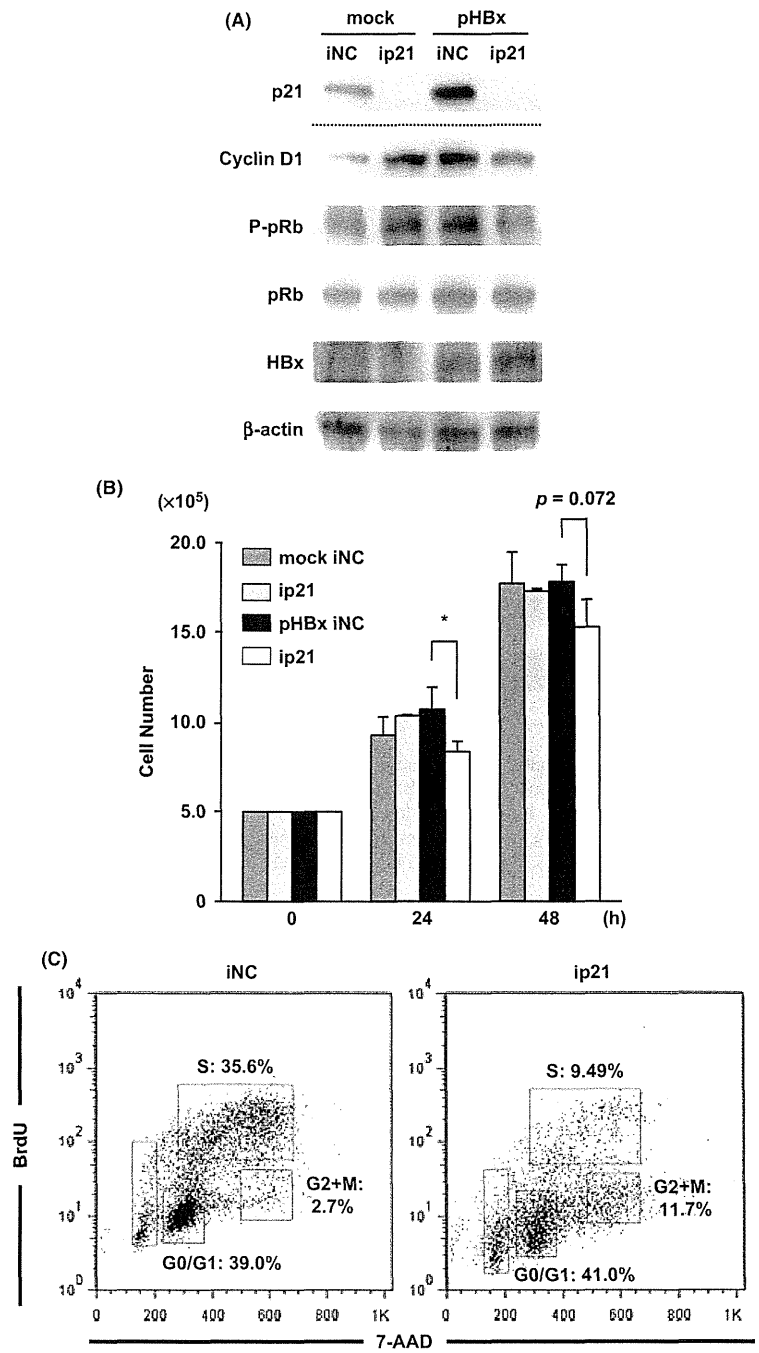

Fig. 3. HBx-induced p21 overexpression positively regulates cell proliferation and cell cycle. (A) Effects of p21 knockdown with siRNA on HBx-caused cell proliferative up-regulations of cyclin D1 and phosphorylated-pRb (P-pRb). HepG2 cells were transfected overnight with negative control (iNC) or p21 siRNA (ip21) and then mock or HBx plasmid (pHBx) was transiently expressed in iNC or ip21-transfected cells respectively. After incubation for 40 h, cells were analysed by Western blot for levels of p21, cyclin D1, pRb (phosphorylated form and total) and FLAG (HBx). Anti-β-actin antibody controlled for protein loading. (B, C) Effects of p21 knockdown on cell growth and cell cycle. Transfection with siRNA and expression plasmid into HepG2 cells was performed as described in (A); at 24 and 48 h post-transfection, cells were harvested and number of viable cells was determined. *P* values were compared for pHBx-transfected cells with iNC (black bars) and those with ip21 (white bars) (B). At 48 h post-pHBx transfection, cells with iNC (left) or ip21 (right) were labelled with 10 μM BrdU for 30 min and harvested at 3 h after changing the medium to remove BrdU. The samples were subjected to incubation with a FITC-conjugated anti-BrdU antibody and DNA staining with 7-AAD. FACS analysis was performed as described in Materials and methods (C).

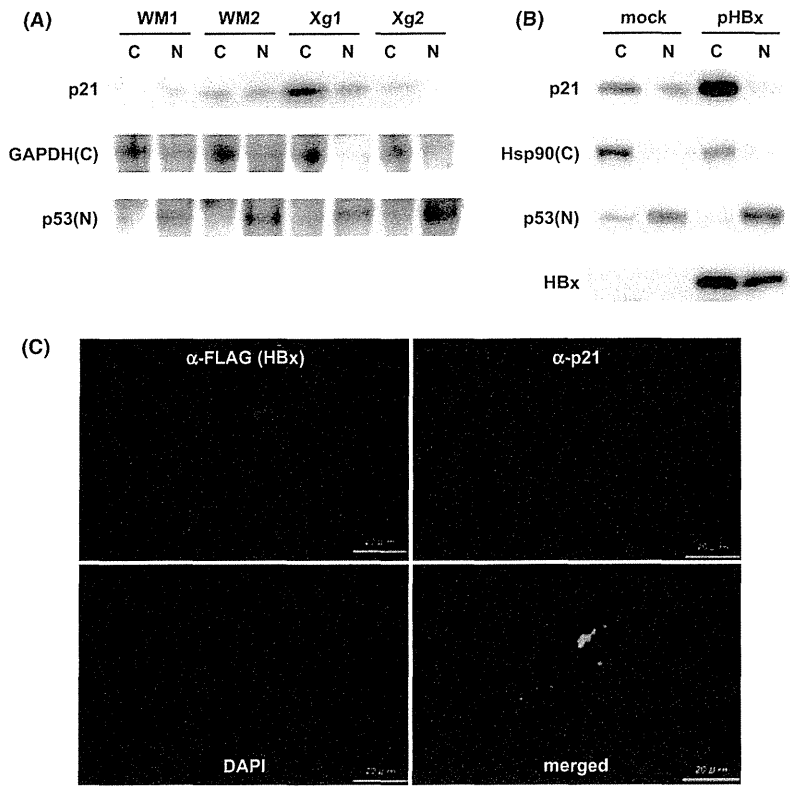

Fig. 4. HBx induces overexpression of cytoplasmic p21 in Xg liver and HBx-expressing cells. (A) Intracellular localization of p21 in livers from WM and Xg. Cytoplasmic (C) and nuclear (N) fractions of two liver samples from each of two mouse groups (WM and Xg at the age of 6 months) were immunoblotted with antibodies specific for p21, GAPDH (cytoplasmic protein marker) and p53 (nuclear marker). (B) Intracellular localization of p21 in transiently mock- or HBx plasmid (pHBx)-transfected HepG2 cells. After incubation for 40 h, C/N fractions were immunoblotted with antibodies specific for p21, Hsp90 (C), p53 (N) and FLAG (HBx). (C) Immunofluorescence of p21 localization in transiently pHBx-transfected HepG2 cells. Intracellular localization after 40 h of expression; HBx (FLAG; red) and p21 (green). Nuclei were stained with DAPI (blue).

did not associate with p21, whereas direct interaction between HBx and PKCα was found by immunoprecipitation assay (Fig. 5E). Immunofluorescence staining further revealed colocalization of HBx with PKCα in the cytoplasm of HBx-transfected cells (Fig. 5F). These results suggest that HBx indirectly promotes cell-proliferative accumulation of p21 in the cytoplasm via direct stimulation of PKCα.

Analysis using antihepatocarcinogenic models provided by IFN-β

We previously reported that treatment with IFN-β prevented the occurrence of HCC in Xg (28). To confirm the involvement of PKCα-mediated cytoplasmic p21 overexpression in hepatocarcinogenesis by HBx, we used this antihepatocarcinogenic state model induced by IFN-β. Xg, as well as HBx-expressing cells, treated with IFN-β exhibited suppressions of HBx-induced oncogenic modulations (i.e. reductions in p21, phosphorylated-PKCα, cyclin D1 and phosphorylated-pRb) (Fig. S1A,B). Finally, we found that Xg liver treated with IFN-β showed higher levels of nuclear p21 than non-treated liver (Fig. 6A). Shifts of cytoplasmic p21 to the nucleus by IFN-β were also observed in HBx-expressing cells (Fig. 6B,C), a finding consistent with our *in vivo* experimental results. Interestingly, this regulation of intracellular localization of p21 by IFN-β was also observed in mock-transfected cells.

Discussion

This study indicates that p21 plays an important role in hepatocarcinogenecity by HBx. p21 mediates the antiproliferative response through arrest of the G1/S transition in the cell cycle under the control of the p53 tumour suppressor (10). Although many previous studies have explored the relevance of HBx-modified p21 expression to HBV-triggered hepatocarcinogenesis, the results and interpretations are contradictory. Some studies revealed that HBx promotes the hepatocarcinogenic process by suppression of anti-oncogenic p21

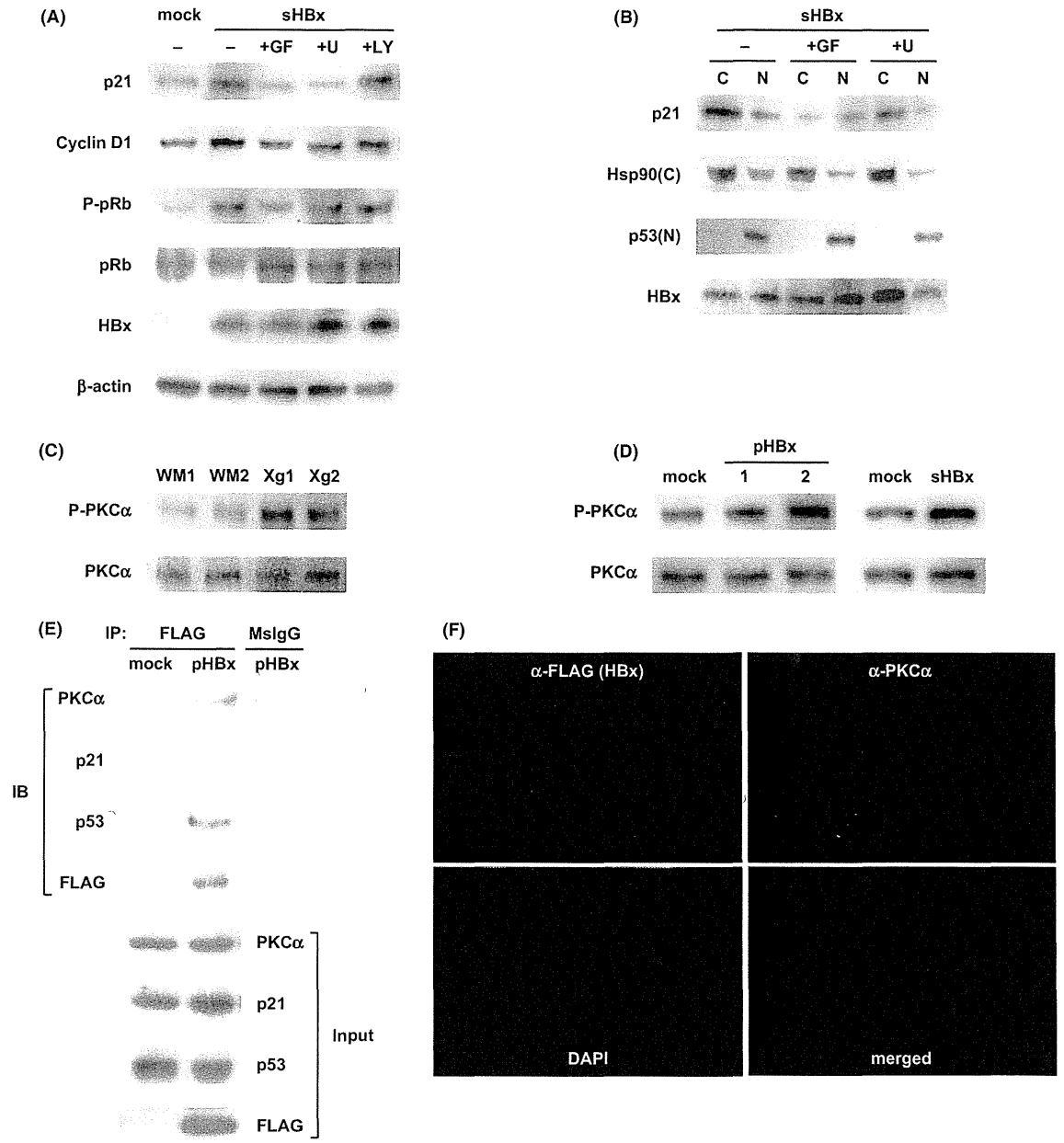

Fig. 5. PKCα activation through direct interaction with HBx mediates HBx-induced cytoplasmic p21 overexpression and cell proliferative states. (A) Effects of kinase inhibitors on HBx-induced up-regulations of p21, cyclin D1 and phosphorylated-pRb (P-pRb). Mock- or HBx-expressing stable cells (sHBx) were precultured in FBS-free medium overnight before each treatment. Mock-expressing cells were treated with DMSO (–), and sHBx with DMSO (–), 1 μM GF109203X (+GF), 2 μM U0126 (+U) or 5 μM LY294002 (+LY) for 24 h, and then analysed by Western blot for levels of p21, cyclin D1, pRb (phosphorylated form and total) and FLAG (HBx). Anti-β-actin antibody controlled for protein loading. (B) Effects of kinase inhibitors on intracellular localization of p21 in sHBx. sHBx were precultured and treated as described in (A). After treatment, cytoplasmic (C) and nuclear (N) fractions were immunoblotted with antibodies specific for p21, Hsp90 (cytoplasmic protein marker), p53 (nuclear marker) and FLAG (HBx). (C, D) Western blot analysis showing increase in phosphorylated-PKCα (P-PKCα) in Xg liver and HBx-expressing cells. Two liver samples from each of two mouse groups (WM and Xg at the age of 6 months), same as shown in Fig. 1B left panel (C) and HBx-plasmid (pHBx; left panel) expressing transient cells or sHBx (right) samples, same as in Fig. 1C, left panel or 1D (D) were immunoblotted with antibodies specific for phosphorylated and total PKCα. (E) Immunoprecipitation (IP) assay for interaction with HBx. HepG2 cells were transiently transfected with mock or pHBx for 40 h; lysates were immunoprecipitated with antibody directed against FLAG or mouse negative control IgG (MsIgG). Immunocomplexes and input lysates were immunoblotted (IB) with antibodies specific for PKCα, p21, p53 and FLAG. (F) Immunofluorescence of PKCα localization in transiently pHBx-transfected HepG2 cells. Intracellular localization after 40 h of expression; HBx (FLAG; red) and PKCα (green). Nuclei were stained with DAPI (blue).