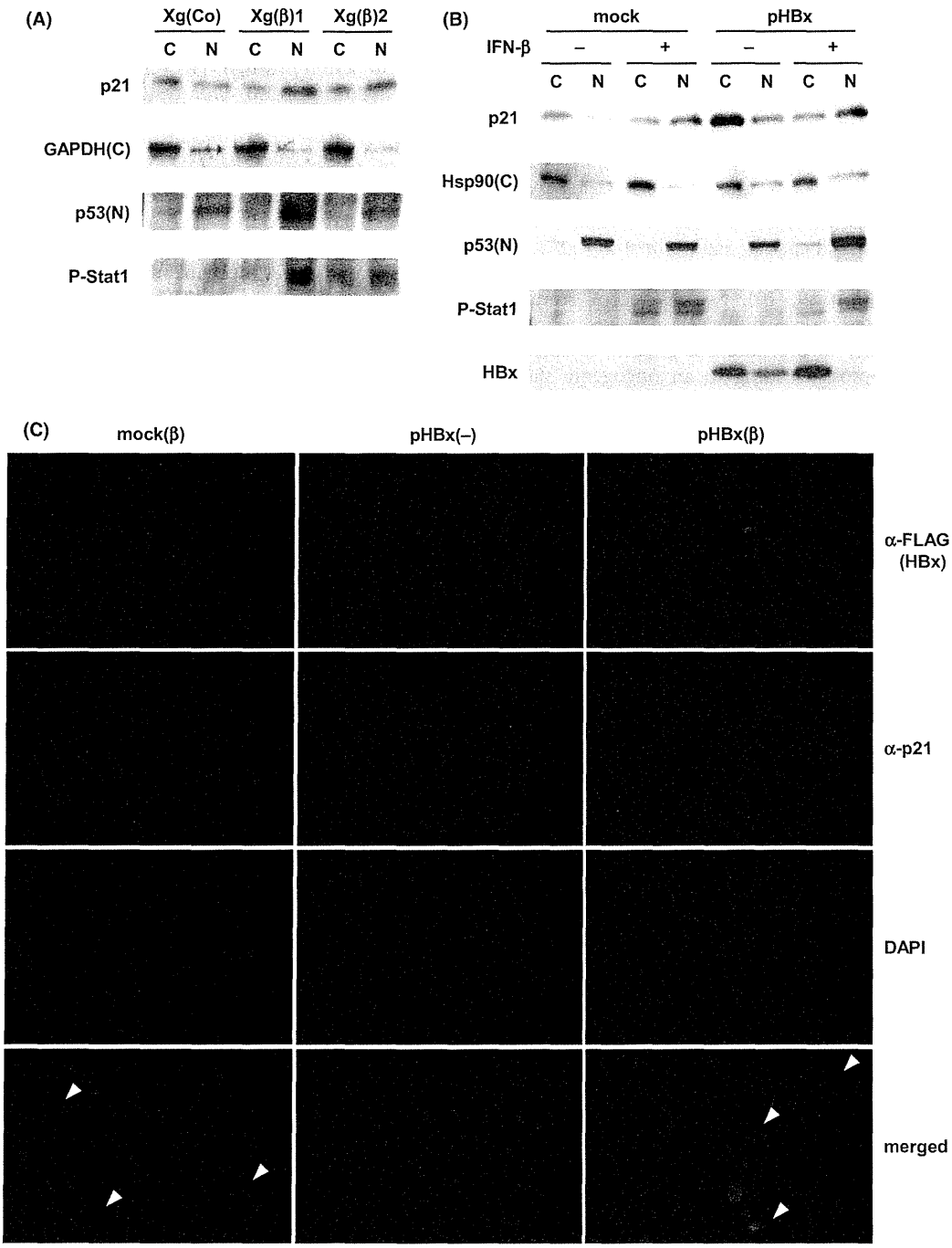

Fig. 6. Cytoplasmic p21 is suppressed and shifted into the nucleus in antihepatocarcinogenic Xg liver and HBx-expressing cells treated with IFN-β. (A) Western blot analysis showing suppression and nuclear shift of cytoplasmic p21 in IFN-β-treated Xg liver. Cytoplasmic (C) and nuclear (N) fractions of liver samples from two mouse groups (control Xg (Co) and IFN-β-treated Xg (β) at the age of 6 months) were immunoblotted with antibodies specific for p21, GAPDH (cytoplasmic protein marker), p53 (nuclear marker) and P-Stat1. (B) Effects of IFN-β treatment on intracellular localization of p21 in transiently mock- or HBx plasmid (pHBx)-transfected HepG2 cells. After 20 h, transfected cells remained untreated (–) or were treated (+) with 100 IU/ml IFN-β for 20 h, and then C/N fractions were immunoblotted with antibodies specific for p21, Hsp90 (C), p53 (N) and FLAG (HBx). Phosphorylated-Stat1 (P-Stat1) was used to confirm activation of IFN signalling. (C) Untreated (–) or IFN-β-treated (β) cells were analysed by immunofluorescence to determine intracellular localization of HBx (FLAG; red) and p21 (green). Nuclei were stained with DAPI (blue). White arrows in the merged images indicate IFN-β-caused shift of cytoplasmic p21 to nucleus (cyan).

expression (21, 24), whereas others showed that HBx exhibited p53-independent up-regulation of p21 expression (22), thereby facilitating the acquisition of gene mutations by prolonging the G1/S phase transition in the cell cycle (23).

Our current study also showed p53-independent enhancement of p21 by HBx in both *in vivo* and *in vitro* experiments. Consistent with this enhancement of p21, we observed increases in cyclin D1 expression and pRB phosphorylation, which enabled E2F to be unrestrained, thus activating E2F-dependent gene expression facilitating cell proliferation (9, 10). Several recent studies revealed that under certain molecular conditions, p21 itself has the ability to promote cell growth or confer resistance to apoptosis, leading to an oncogenic state (11–13), which is in marked contrast to the normal function of p21. To confirm this observation, we investigated whether knockdown of p21 suppressed cell growth and cell cycle. As a result, we found that suppressions of cell growth and cell cycle progression, accompanied by attenuation of pRB phosphorylation, occurred in HBx-transfected cells with p21 siRNA. Hence, these findings confirm that HBx-induced p21 overexpression positively regulates cell cycle progression in our experimental model.

Recent investigations have proposed that the molecular properties of p21 are controlled by its intracellular localization, which is also regulated by several upstream kinases. When localized in the cytoplasm, p21 appears to exert oncoprotein-like properties by inhibiting apoptosis or facilitating cell proliferation. For instance, it has been suggested that cytoplasmic p21 interacts with and inhibits apoptotic procaspase-3 or apoptosis-signal-regulating kinase 1 (13, 20). Moreover, in 3T3 cells overexpressing the HER-2/neu gene (also known as *c-erbB2*), Akt-induced phosphorylation of p21 results in stabilization and accumulation of cell proliferative p21 in cytoplasm (19), and in monocytic leukaemia cells, cytoplasmic localization of anti-apoptotic p21 is regulated in a PKC-dependent manner (30). In addition, ERK1/2-phosphorylated p21 is reported to be translocated from the nucleus to cytoplasm and degraded in 3T3 and HEK293 cells (31). We investigated which signal pathways are involved in the HBx-induced p21 up-regulation and found that PKCα, but not PI-3K/Akt and MEK-ERK1/2, is specifically important for this up-regulation and cytoplasmic-predominant localization of p21. Because of the direct association of HBx with PKCα, but not with p21, HBx may indirectly up-regulate cell proliferative p21 expression via direct association with PKCα. In other words, HBx-activated PKCα through interaction in the cytoplasm stimulates the overexpression of cytoplasmic p21, although the detailed mechanisms are still unknown. This p21 overexpression is followed by pRB inactivation, which may induce the oncogenic state.

The preventive effects of IFNs against HCC progression have been known to result from several events, including (i) hepatitis virus suppression (32, 33); (ii) stimulative immunomodulation (34); and (iii) direct antitumour action through cell cycle inhibition or apoptosis induction (35, 36). Our results demonstrate that IFN-β, a more potent type I IFN (37, 38), exerts a preventive effect against HBx-induced hepatocarcinogenesis by negatively regulating PKCα-mediated cell proliferative cytoplasmic p21 overexpression and restoring pRb activation. To the best of our knowledge, this is the first report of IFN regulating p21 intracellular localization as a suppressive mechanism against hepatitis viral protein-induced hepatocarcinogenesis. Since the regulation of intracellular localization of p21 by IFN-β treatment was observed in both HBx-transfected cells and mock-transfected cells (Fig. 6B,C), we speculate that this is a general molecular mechanism involved in the antihepatocarcinogenic effect of IFN-β, which may be irrelevant to HBx. In our previous study, we noted that IFN-β treatment reduced cell proliferation, but not induced apoptosis, in the Xg liver (28). Thus, the results of our studies suggest that HBx-induced aberrant cytoplasmic p21 may be a cell cycle promoter through pRb inactivation, rather than an anti-apoptotic signaller. Further studies are necessary to elucidate the regulatory mechanisms involved in these different functions of cytoplasmic p21.

In conclusion, we confirmed the involvement of PKCα-mediated cytoplasmic p21 overexpression in the molecular pathway leading to a cell proliferative hepatocarcinogenesis of HBx. Our novel findings may contribute to new therapeutic approaches, focusing on the intracellular expression of p21, for the prevention of HBV-related HCC.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. HBx-induced p21 overexpression and cell proliferative states are down-regulated in antihepatocarcinogenic Xg liver and HBx-expressing cells treated with IFN-β. (A) Western blot analysis showing down-regulations of p21, phosphorylated-PKCα (P-PKCα) and cyclin D1 in IFN-β-treated Xg liver. Two liver samples from two mouse groups {control Xg [Co] and IFN-β-treated Xg (β) at the age of 6 months} were immunoblotted with the indicated antibodies. (B) Dose effects of IFN-β on HBx-induced molecular events. HepG2 cells transiently transfected with 2 μg of mock or HBx plasmid (pHBx) for 20 h (left panel) and mock- or HBx-expressing stable HepG2 cells (sHBx) (right) were treated with increasing amounts of IFN-β for 20 h, then analysed by Western blot using the indicated antibodies. Phosphorylated-Stat1 (P-Stat1) was used to confirm activation of IFN signalling. Anti-β-actin antibody controlled for protein loading.

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Clinical Study

DNA Damage Sensor γ-H2AX Is Increased in Preneoplastic Lesions of Hepatocellular Carcinoma

Yasunobu Matsuda,[1] Toshifumi Wakai,[2] Masayuki Kubota,[3] Mami Osawa,[3] Masaaki Takamura,[4] Satoshi Yamagiwa,[4] Yutaka Aoyagi,[4] Ayumi Sanpei,[1] and Shun Fujimaki[1]

[1] *Department of Medical Technology, Niigata University Graduate School of Health Sciences, 2-746 Asahimachi-dori, Niigata 951-8518, Japan*
[2] *Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, 2-746 Asahimachi-dori, Niigata 951-8518, Japan*
[3] *Division of Pediatric Surgery, Niigata University Graduate School of Medical and Dental Sciences, 2-746 Asahimachi-dori, Niigata 951-8518, Japan*
[4] *Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 2-746 Asahimachi-dori, Niigata 951-8518, Japan*

Correspondence should be addressed to Yasunobu Matsuda; yasunobu@med.niigata-u.ac.jp

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Background. Phosphorylated histone H2AX (γ-H2AX) is a potential regulator of DNA repair and is a useful tool for detecting DNA damage. To evaluate the clinical usefulness of γ-H2AX in hepatocellular carcinoma (HCC), we measured the level of γ-H2AX in HCC, dysplastic nodule, and nontumorous liver diseases. *Methods.* The level of γ-H2AX was measured by immunohistochemistry in fifty-eight HCC, 18 chronic hepatitis, 22 liver cirrhosis, and 19 dysplastic nodules. Appropriate cases were also examined by fluorescence analysis and western blotting. *Results.* All cases with chronic liver disease showed increased levels of γ-H2AX expression. In 40 (69.9%) of 58 cases with HCC, the labeling index (LI) of γ-H2AX was above 50% and was inversely correlated with the histological grade. Mean γ-H2AX LI was the highest in dysplastic nodule (74.1 ± 22.1%), which was significantly higher than HCC ($P < 0.005$). Moreover, γ-H2AX was significantly increased in nontumorous tissues of HCC as compared with liver cirrhosis without HCC (62.5 ± 24.7%, from 5.1 to 96.0%, $P < 0.005$). *Conclusions.* γ-H2AX was increased in the preneoplastic lesions of HCC and might be a useful biomarker for predicting the risk of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in developing and industrial countries and is increasing worldwide [1–4]. HCC is unique as it frequently reoccurs after treatment irrespective of the different etiological factors including hepatitis virus B (HBV) and C (HCV), alcohol abuse, and nonalcoholic steatohepatitis [2, 4, 5]. One possible reason for the frequent recurrence of HCC might be due to many patients being affected with hepatitis virus-associated chronic liver inflammation [1–3].

To date, many reports have described a possible relationship between hepatitis virus and DNA damage. For example, HBV has been reported to directly regulate the DNA damage response in host cells [6]. HBV stimulates ATM- and Rad3-related protein kinase (ATR)and checkpoint kinase 1 (Chk1) pathways [7], leading to the acquisition of strengthened survival against DNA damage. Moreover, HBV X gene product (HBX), widely recognized as a possible viral carcinogen [8, 9], plays a critical role in the phosphorylation and inactivation of Rb via activating p38 mitogen-activated protein kinase [10]. HBX also binds and inhibits the functional efficiency of p53 [11, 12], leading to DNA damage accumulation in HBV-infected cells. HCV has been also reported to be involved in the deregulation of the DNA repair system. HCV nonstructural proteins, NS3 and NS4A, inhibit Ataxia-telangiectasia-mutated (ATM) kinase in response to DNA damage [13]. HCV core protein inhibits the functional formation of

the Mre11/NBS1/Rad50 complex, which causes the ATM-mediated DNA repair system to be markedly impaired in HCV-infected cells [14]. Together, these lines of evidence strongly suggest that DNA damage response machinery is significantly involved in hepatocarcinogenesis and might be used as biomarkers for predicting the risk of HCC development.

Recently, several studies reported that the level of oxidative DNA damage is a good biomarker. For example, hepatic 8-oxo-2′-deoxyguanosine (8-OHdG), an oxidized derivative of deoxyguanosine, which reflects oxidative stress, was closely correlated with the risk of HCC recurrence after surgery [15, 16]. To search for more sensitive and reliable biomarkers of DNA damage, we investigated the levels of γ-H2AX in HCC tissues, which mark the site of DNA double-strand breaks and evoke the DNA repair system [17, 18]. To address whether γ-H2AX might be a good indicator for the risk of HCC development, we also examined and compared the level of γ-H2AX in nontumorous chronic liver diseases.

Table 1: Clinical characteristics of the patient groups of DN and HCC.

Clinical variables	DN ($n = 19$)	HCC ($n = 58$)	P value*
Mean age (year)	63 ± 8	62 ± 9	0.792
Gender			
Male	13	49	0.117
female	6	9	
HBs Ag			
+	2	7	0.610
−	17	51	
Anti-HCV			
+	12	35	0.525
−	7	23	

DN: dysplastic nodule; HCC: hepatocellular carcinoma.
*P value of independent Student's t-test for continuous data and Chi^2 test for categorical data.

2. Materials and Methods

2.1. Patients. The pathological diagnoses and analyses of dysplastic nodule and HCC were made according to the General Rules for the Clinical and Pathological Study of Primary Liver Cancer [19]. HCC tissue samples were obtained from 58 patients (7 cases with hepatitis B virus-positive, 35 with hepatitis C virus-positive, and 16 with unknown etiology; 9 females, 49 males; mean age, 62 ± 9) that underwent hepatic resection at Niigata University Medical and Dental Hospital. Tissue samples of dysplastic nodules were obtained from 19 patients (2 cases with hepatitis B virus-positive, 12 with hepatitis C virus-positive, and 5 with unknown etiology; 13 males and 6 females; mean age, 63 ± 8) by ultrasound-guided biopsy (Table 1). Tissue samples of 18 cases with chronic hepatitis (6 with hepatitis B virus-positive and 10 with hepatitis C virus-positive; 14 males and 4 females) and 22 with liver cirrhosis (4 with hepatitis B virus-positive and 18 with hepatitis C virus-positive; 19 males and 3 females) were obtained by ultrasound-guided or laparoscopic biopsy. All tissue samples were fixed in formalin, and the tissue sections were subjected to hematoxylin and eosin staining for histopathological evaluation by two pathologists. Freshly frozen tissues were obtained from 12 cases with HCC and 8 with liver cirrhosis and were used for western blot analysis. Normal liver tissue samples were surgically obtained from 5 individuals without liver disease. Informed consent was obtained from all the human subjects included in the study under an Institutional Review Board-approved protocol, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the institution's human research committee.

2.2. Immunohistochemical Analysis. Tissue sections were deparaffinized in xylene, rehydrated in alcohol, and quenched in 3% hydrogen peroxide with methanol to block endogenous peroxidase activity. Slides were heated in a microwave in 10 mm sodium citrate (pH 6.5) for antigen retrieval. Immunohistochemical reactions were performed by immersing tissue sections in 5% normal goat serum for 60 minutes and incubating them at 4°C overnight with mouse anti-phosphohistone H2AX monoclonal antibody (Ser139) clone JBW301 (Upstate Biotech, Charlottesville, VA) at a dilution of 1 : 500 in blocking buffer. As a negative control, control mouse immunoglobulin G (Dako Cytomation, Glostrup, Denmark) was used instead of the primary antibody. After the sections were rinsed, a secondary antibody from the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) was applied, and color development was performed using 3,3-diaminobenzidine (Sigma Chemical Co., St Louis, MO). Counterstain was provided by staining with hematoxylin. Labeling indices (LIs) for γ-H2AX were determined as the number of positive nuclei in 100 hepatocytes or tumor cells in 3 randomly selected fields. In HCC cases, the patients were divided into two groups according to the levels of γ-H2AX LI, as low expressors (LI <50%) and high expressors (LI >50%).

2.3. Immunofluorescence Staining. For immunofluorescence analysis, appropriate tissue slides were incubated in 100 mm glycine for 15 minutes, three times to reduce fluorescent background. Slides were reacted with the same primary antibody as used for immunohistochemistry and washed in tris-buffered saline containing 0.05% Tween-20 3 times for 5 minutes to reduce background. They were incubated for 30 minutes with Alexa Fluor 488 goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, OR) in the dark and mounted with 0.2 μg/mL 4′-6-diamidino-2-phenylindole (DAPI). Immunofluorescence images were visualized by fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).

2.4. Western Blotting. Liver tissues were homogenized using a TissueRuptor (Qiagen, Valencia, CA, USA) with a buffer containing 20 mm Tris-HCl (pH 7.4), 150 mm NaCl, 2 mm EGTA, 5 mm β-glycerophosphate, 1 mm $MgCl_2$, 1% Triton X-100,

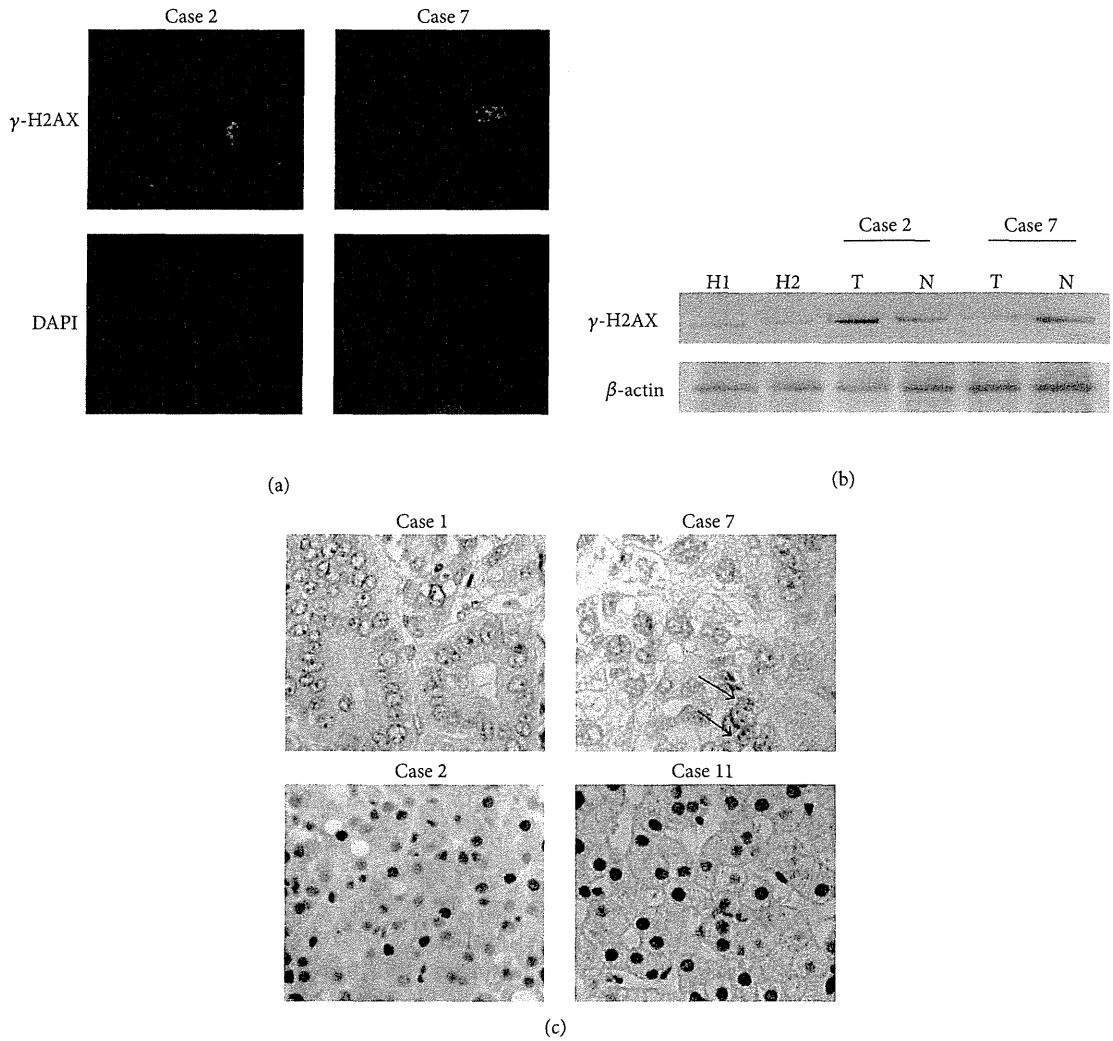

FIGURE 1: γ-H2AX is expressed at different levels in HCC. (a) Immunofluorescence staining shows that phosphorylated histone H2AX (γ-H2AX) is located in the nuclei of HCC cells (green) (original magnification ×100). Case 2: HCC with increased γ-H2AX expression; Case 7: HCC with sparse expression of γ-H2AX. DAPI (blue): nucleus counterstain. (b) Representative data of western blotting for γ-H2AX in liver tissues. H1 and H2: healthy livers; Cases 2 and 7: HCC cases. T: tumor tissues; N: adjacent nontumorous liver tissues. (c) Immunohistochemical staining of γ-H2AX. Cases 1 and 7: HCC cases with negative to low expression of γ-H2AX. Cases 2 and 11: HCCs with high expression of γ-H2AX (original magnification ×40). Arrows indicate positive staining in the nuclei.

1 mm sodium orthovanadate, 10 μg/mL protease inhibitors, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin. Lysates were cleared by centrifugation, and the supernatants containing 20 μg of protein were electrophoresed on 5–20% SDS-polyacrylamide gels. After samples were blotted onto Hybond-P membranes (GE Healthcare, Milwaukee, WI), membranes were incubated with rabbit anti-γ-H2AX polyclonal antibody (Bethyl Lab. Inc., Montgomery, TX). Protein blots were visualized using an enhanced ECL Western blotting detection system (GE Healthcare), and equal amounts of the protein loading were confirmed by reprobing with anti-β-actin antibody (Sigma Chemical Co.).

2.5. Statistical Analysis. Data were analyzed using SPSS software (Statistical Product and Service Solutions 11.5 for Windows; SPSS Inc., Chicago, IL). Chi-square test was used for examining the association between the status of γ-H2AX and clinic-pathologic features in HCC. When appropriate, a Mann-Whitney *U*-test or independent Student's *t*-test was used to test for statistical differences between the groups.

3. Results

3.1. γ-H2AX Expression Is Increased in HCC. Immunofluorescence analysis demonstrated that γ-H2AX appeared as diffuse and discrete foci in the nuclei of HCC cells (Figure 1(a)). The results of western blotting correlated with the immunofluorescence staining (Figure 1(b)), confirming the potential reliability for detecting γ-H2AX in the HCC samples. Immunohistochemical analysis showed that

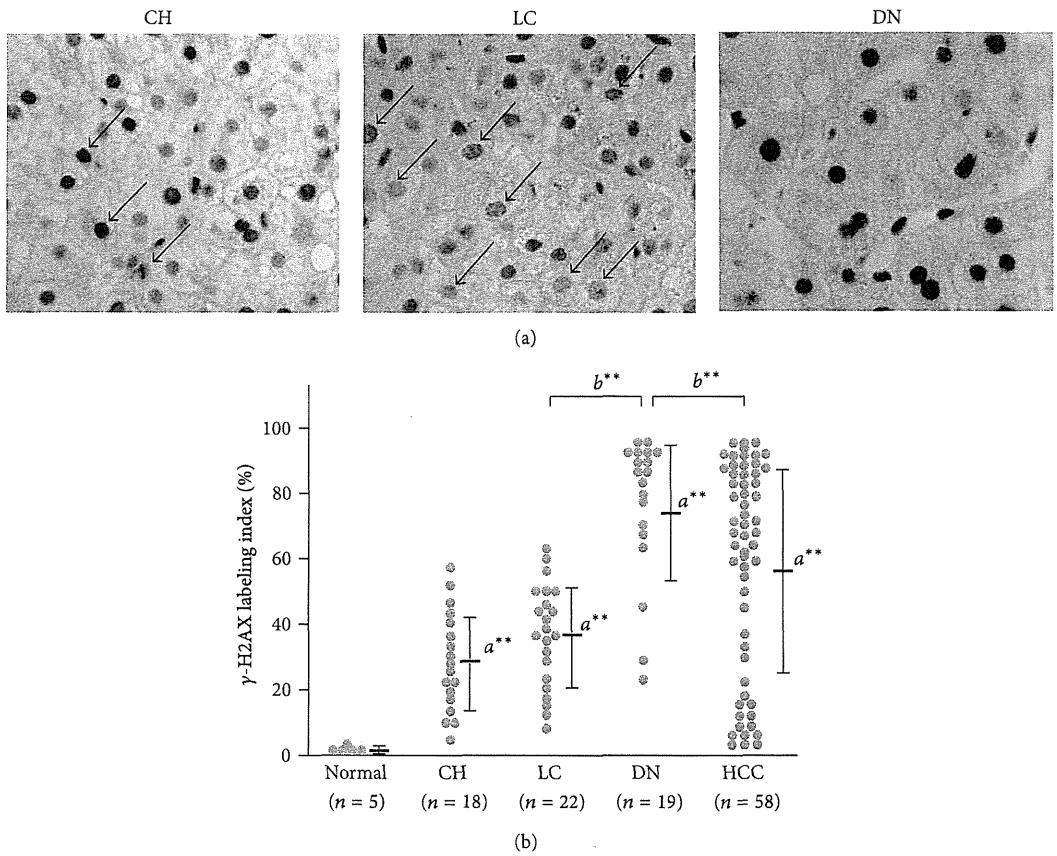

FIGURE 2: γ-H2AX is significantly increased in preneoplastic lesions in the liver. (a) Representative images of immunostaining for γ-H2AX in non-HCC tissues. CH: chronic hepatitis; LC: liver cirrhosis; DN: dysplastic nodule (original magnification ×40). (b) Dot plots showing the γ-H2AX labeling index in normal livers ($n = 5$), chronic hepatitis (CH; $n = 18$), liver cirrhosis (LC; $n = 22$), dysplastic nodule (DN; $n = 19$), and HCC ($n = 58$). Horizontal bars depict the mean value, and vertical bars indicate the standard deviation. a^{**}: P value of <0.01 versus normal livers; b^{**}: P value of <0.01 versus dysplastic nodule.

the mean value of LI for γ-H2AX in HCC was 56.2 ± 31.4% (range from 3.0 to 95.1%), which was significantly increased compared with normal livers, LI 1.0 ± 0.6%. Fifty-eight HCC patients were categorized into two groups, 40 (69.0%) characterized by significantly increased levels of γ-H2AX expression (high expressors; LI >50%), and 18 (31.9%) with very low to negative expression (low expressors; LI <50%) (Figure 1(c)). γ-H2AX expression levels showed no positive correlation with clinical features in HCC patients (Table 2); however, there was an inverse relationship between the histological grade of the tumors ($P = 0.011$) (Table 2).

3.2. γ-H2AX Is Increased in Chronic Liver Diseases and Dysplastic Nodules. Immunohistochemical analysis showed that the LI of γ-H2AX in chronic hepatitis and liver cirrhosis was increased compared with normal livers (chronic hepatitis, 27.5 ± 15.8%, range from 5.0 to 59.3%, $P < 0.005$; liver cirrhosis, 56.2 ± 31.4%, range from 7.2 to 63.0%, $P < 0.005$, resp.). Intriguingly, dysplastic nodules showed a significantly increased γ-H2AX expression (74.1 ± 22.1%, range from 20.1 to 94.0%), which was significantly increased compared with those in liver cirrhosis ($P < 0.005$) and HCC ($P < 0.005$) (Figures 2(a) and 2(b)).

3.3. γ-H2AX Is Increased in Adjacent Nontumorous Liver Tissues from HCC Patients. To investigate the clinical significance of γ-H2AX, we determined whether fundamental differences existed between the nontumorous liver tissues with and without the coexistence of HCC. Western blotting detected γ-H2AX in 3 (37.5%) of 8 liver cirrhosis patients with no evident HCC occurrence, while 9 (75%) of 12 HCC cases showed a detectable protein band for γ-H2AX in the adjacent nontumorous liver tissues (Figure 3(a)). Immunohistochemical analysis showed that the mean LI of γ-H2AX in nontumorous liver tissues obtained from HCC patients with chronic hepatitis was relatively but not statistically increased as compared with that obtained from individuals with chronic hepatitis without HCC occurrence (35.7±17.2%, range from 8.0 to 65.5%, $P = 0.11$). Moreover, the mean LI of γ-H2AX in nontumorous tissues from HCC patients with

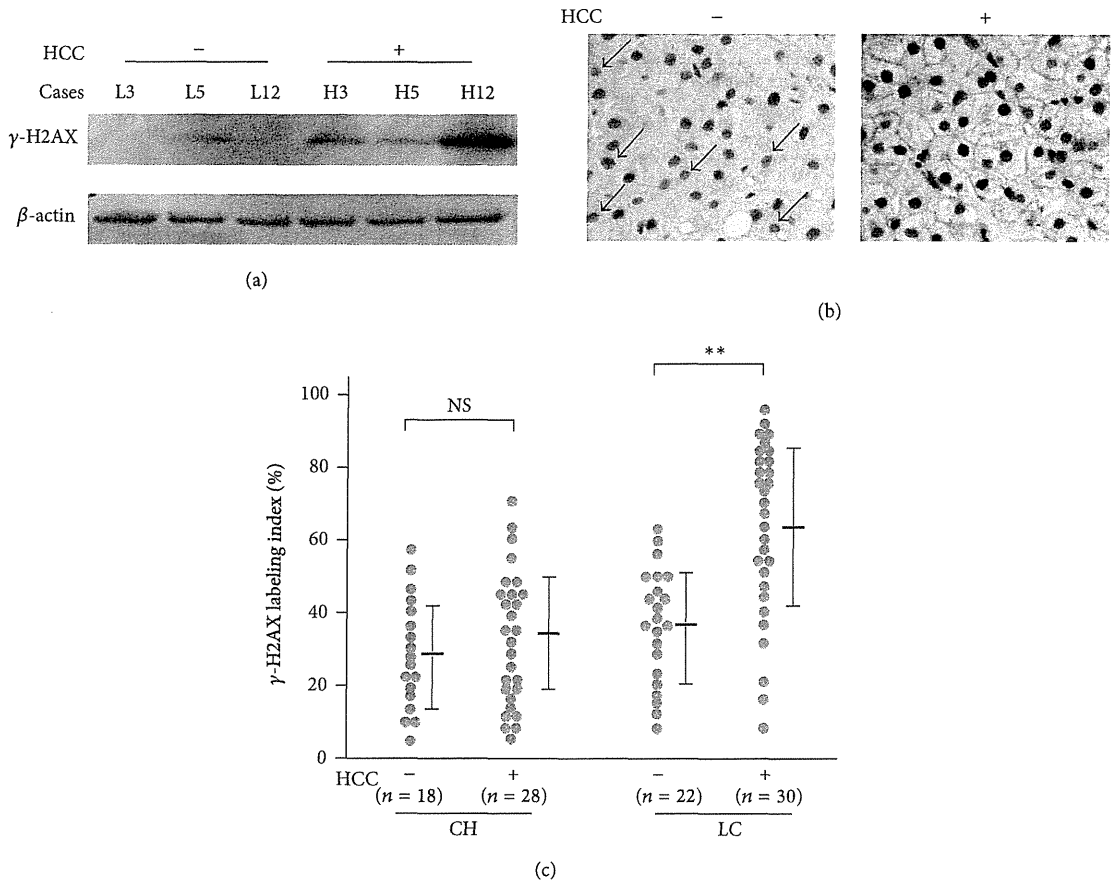

FIGURE 3: γ-H2AX is increased in the adjacent nontumorous liver tissues of HCC patients. (a) Representative data of western blotting for γ-H2AX. L3, 5, and 12: cases with liver cirrhosis without the coexistence of HCC; H3, 5, and 12: adjacent nontumorous liver tissues obtained from HCC patients. (b) Representative images of γ-H2AX immunostaining in liver tissues with and without the coexistence of HCC (original magnification ×40). (c) Dot plots showing the γ-H2AX labeling index. CH: chronic hepatitis without the coexistence of HCC ($n = 18$) and with HCC ($n = 28$); LC: liver cirrhosis without HCC ($n = 22$) and with HCC ($n = 30$). NS: not significant; $^{**}P < 0.01$.

liver cirrhosis was significantly increased when compared with the cases with liver cirrhosis without HCC (62.5±24.7%, range from 5.1 to 96.0%, $P < 0.005$) (Figure 3(c)).

4. Discussion

Over the last few decades, alpha-fetoprotein (AFP), normally produced by the fetal liver and yolk sac in pregnant individuals, has commonly been used as a clinically available biomarker for HCC. Unfortunately, serum levels of AFP do not correlate well with the risk of the development of HCC, and reliable biomarkers have been long awaited for improving the poor prognosis of HCC. As DNA double-strand breaks accumulate during long periods of chronic inflammation, investigating the molecules involved in the DNA repair system for use as biomarkers for the risk of HCC development would be of value.

During the period of chronic inflammation, intracellular reactive oxygen species (ROS) are increased and have a strong capability to induce oxidative DNA damage [20]. In this setting, the highly conserved phosphatidylinositol-3-related kinases ATM and ATR play critical roles in regulating the cell cycle checkpoints and DNA repair [21]. Of note, both of these kinases have a strong capability to increase the level of phosphorylated histone H2AX (γ-H2AX), which immediately traffics to the damaged sites of DNA. γ-H2AX plays a key role in the DNA repair process, because it recruits many other molecules involved in the DNA repair [17, 22]. Intriguingly, γ-H2AX is now regarded as a useful biomarker for assessing radio sensitivity of cancer cells after treatment [23], and more recently, it was reported that this unique molecule might be increased in preneoplastic lesions such as colon adenoma [24] and dysplastic lesions in the bronchial epithelium [25].

There have been few reports of γ-H2AX during hepatocarcinogenesis. Kim et al. [26] reported that γ-H2AX foci were significantly increased in HBV-related liver cirrhosis and HBV-related HCC compared with normal hepatocytes, but there have been no following reports and no such studies to investigate the clinical significance of γ-H2AX in individuals with a risk of HCC. In this study, we found that γ-H2AX

Table 2: Associations of γ-H2AX with clinicopathological features in HCC.

Clinicopathological variables	γ-H2AX immunoreactivity		P value*
	Low ($n = 18$)	High ($n = 40$)	
Age (year)			
<50	2	4	
≥50	16	36	0.613
Gender			
male	15	34	
female	3	6	0.576
Tumor size (cm)			
<3	5	11	
≥3	13	29	0.609
Intrahepatic metastasis			
−	14	30	
+	4	10	0.550
Venous invasion			
−	16	37	
+	2	3	0.497
Histological grade†			
I/II	10	35	
III/IV	8	5	0.011*

†Histological grade was assessed according to the Edmondson-Steiner grade.
*P value of Chi^2 test.

was sequentially increased from normal to chronic hepatitis and liver cirrhosis. Importantly, dysplastic nodule showed a significantly high level of γ-H2AX LI, which was increased compared with HCC (74.1 ± 22.1% versus 56.2 ± 31.4%, $P < 0.005$). Together with our data suggesting that histological grades of HCC were inversely correlated with the level of LI, γ-H2AX might play a critical role in the development of HCC, especially during the early stages of carcinogenesis. Furthermore, the levels of LI increased in CC-coexisting tissues with liver cirrhosis than those without tumors (62.5 ± 24.7% versus 56.2 ± 31.4%, $P < 0.005$). Together, γ-H2AX may be a useful biomarker for predicting individuals with a high risk of HCC.

Until recently, there have been no useful biomarkers for predicting the potential of HCC development. Because γ-H2AX foci can be representatively detected using standard pathological techniques, this may be a promising and standard biomarker for HCC surveillance. To confirm the clinical usefulness of this DNA damage sensor, repeated liver biopsies and a long followup study of individuals with chronic liver diseases would be required.

Conflict of Interests

None of the authors has any financial or other interest with regard to the submitted paper that might be construed as a conflict of interests.

Acknowledgment

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p21-activated kinase-2 is a critical mediator of transforming growth factor-β-induced hepatoma cell migration

Munehiro Sato,* Yasunobu Matsuda,§ Toshifumi Wakai,† Masayuki Kubota,‡ Mami Osawa,§ Shun Fujimaki,§ Ayumi Sanpei,† Masaaki Takamura,* Satoshi Yamagiwa* and Yutaka Aoyagi*

Divisions of *Gastroenterology and Hepatology, †Digestive and General Surgery and ‡Pediatric Surgery, Niigata University Graduate School of Medical and Dental Sciences, and §Department of Medical Technology, Niigata University Graduate School of Health Sciences, Niigata, Japan

Key words
cell migration, hepatocellular carcinoma (HCC), p21-activated kinase-2 (PAK2), transforming growth factor-β (TGF-β).

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Correspondence
Dr Yasunobu Matsuda, Department of Medical Technology, Niigata University Graduate School of Health Sciences, 2-746 Asahimachi-dori, Niigata 951-8518, Japan.
Email: yasunobu@med.niigata-u.ac.jp

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Abstract

Background and Aim: Transforming growth factor-β (TGF-β) has been shown to play a central role in the promotion of cell motility, but its functional mechanism has remained unclear. With the aim of investigating the diagnostic and treatment modalities for patients with hepatocellular carcinoma (HCC), the signaling pathway that may contribute to TGF-β-mediated cell invasion in hepatoma cells was evaluated.

Methods: Three hepatoma cell lines, HepG2, PLC/PRF/5, and HLF, were treated with TGF-β, and the involvement of the non-canonical TGF-β pathway was analyzed by cell migration assays. HepG2 cells were treated with a p21-activated kinase-2 (PAK2)-targeting small interfering RNA and analyzed for their cell motility. The relationships between the PAK2 status and the clinicopathological characteristics of 62 HCC patients were also analyzed.

Results: The cell migration assays showed that Akt is a critical regulator of TGF-β-mediated cell migration. Western blotting analyses showed that TGF-β stimulated Akt and PAK2 in all three hepatoma cell lines, and phosphorylated PAK2 was blocked by Akt inhibitor. Suppression of PAK2 expression by small interfering RNA resulted in increased focal adhesions with significantly repressed cell migration in the presence of TGF-β. Clinicopathological analyses showed that the phosphorylation level of PAK2 was closely associated with tumor progression, metastasis, and early recurrence of HCC.

Conclusions: PAK2 may be a critical mediator of TGF-β-mediated hepatoma cell migration, and may represent a potential target for the treatment of HCC.

Introduction

The majority of hepatocellular carcinoma (HCC) patients present with advanced cancer stages at the time of diagnosis.[1–4] The etiology of HCC is unique on the point that it mainly occurs in liver cirrhosis where transforming growth factor-β (TGF-β), a central player involved in cell migration, is implicated in the disease progression.[1,5] TGF-β signaling has been functionally categorized into two distinct pathways: canonical Smad-mediated signaling and non-canonical (non-Smad) signaling mainly mediated by mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K)/Akt signaling.[6,7] It should be noted that non-canonical TGF-β signaling plays an important role in cell migration. For example, TGF-β-induced cell migration of fibroblasts and transformed keratinocytes has been reported to be exclusively dependent on p38 MAPK.[8,9] More recently, it was reported that TGF-β-mediated Akt signaling results in fibroblast cell migration through activation of p21-activated kinase-2 (PAK2).[10–12] To date, however, the clinical significance of these signaling pathways in HCC remains unclear.

In this study, we addressed the role of non-canonical TGF-β signaling in hepatoma cell migration, and found that Akt/PAK2 signaling is a potent inducer of hepatoma cell invasion. Evaluation of clinicopathological data indicated a close correlation between PAK2 expression and poorer prognosis of HCC patients, suggesting that PAK2 may be a useful target for the treatment of HCC.

Materials and methods

Reagents. TGF-β (R&D Systems, Minneapolis, MN, USA) was used at 5 ng/mL. N-acetyl-L-cysteine (NAC) (LKT Laboratories, St. Paul, MN, USA) was freshly prepared by dissolving in water, adjusted to pH 7.4, and used at 5 mmol/L as an antioxidant. The PI3K/Akt inhibitor LY294002 (Cell Signaling Technology, Beverly, MA, USA), extracellular signal-regulated kinase1/2 inhibitor U0126 (Calbiochem, San Diego, CA, USA), p38 MAPK inhibitor SB203580 (Enzo Life Sciences, Farmingdale, NY, USA), and c-Jun N-terminal kinase inhibitor SP600125 (Enzo Life Sciences) were dissolved in dimethylsulfoxide and used at 25, 10,

20, and 50 μmol/L, respectively. Polyclonal antibodies recognizing phospho-Smad2 (Ser465/467) (p-Smad2), phospho-Akt (Thr308) (p-Akt), phospho-PAK1 (Ser144)/PAK2 (Ser141) (p-PAK1/2), phospho-PAK2 (Ser20) (p-PAK2 (S20)), PAK1, and PAK2 were obtained from Cell Signaling Technology. A polyclonal antibody against β-actin and a mouse monoclonal antibody against vinculin labeled with fluorescein isothiocyanate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G was from Molecular Probes (Carlsbad, CA, USA).

Cell culture. HepG2 and PLC/PRF/5 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). HLF cells (Health Science Research Resources Bank, Tokyo, Japan) were cultured in DMEM containing 5% FBS. The cells were treated with TGF-β for 2 days. If noted, the cells were serum-deprived for 48 h and treated with TGF-β for 2 h with or without pretreatment with chemical reagents for 1 h. PAK2 gene expression was knocked down by treatment with a small interfering RNA (siRNA).[13] Transwell cell migration assay and wound healing assay were performed according to our previous report[14] (details are in Supplemental Methods).

Patients. Tissue samples were obtained from 62 HCC patients (14 females, 48 males; mean age, 66.8 ± 11.4 years) who underwent a hepatic resection. Frozen tissue samples from 36 cases were used for Western blot analyses. Normal liver tissue samples were surgically obtained from five individuals without liver disease. Informed consent was obtained from each patient included in the study under an Institutional Review Board-approved protocol (approval no. 323), and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Western blot analysis. Protein samples (20 μg) were separated in sodium dodecylsulfate–polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene difluoride membranes. The membranes were probed with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The protein band intensities were normalized by the β-actin band intensity and quantified using Image-J analysis software (ver. 1.44; NIH, Bethesda, MD, USA).

Immunohistochemistry. Tissue sections were reacted with an anti-p-PAK2 (S20) antibody (1:50) overnight at 4°C using a Vector Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). The immunohistochemical staining was evaluated by two independent observers by multiplying the staining intensity graded as 0 (negative), 1 (faint), 2 (moderate), and 3 (strong) by the percentage of stained cells as 0 (< 5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (> 75%), to give a composite "histoscore" as previously reported.[15,16] HCCs were classified as p-PAK2 high expressors or low expressors based on the median histoscore of 2.5 calculated in our study.

Statistical analysis. Data for the *in vitro* analyses were expressed as the means ± SD of individual triplicate experiments and analyzed by Student's *t*-test for the significance of differences between two groups. The clinicopathological features of the HCC patients classified as p-PAK2 low expressors and high expressors were compared using Pearson's chi-square test for categorical variables and an independent Student's *t*-test for continuous data. Survival times were calculated from the data for curative surgery to HCC recurrence, death, or last follow-up of the patients. Kaplan–Meier plots and log-rank tests were used for survival analyses. All analyses were performed using SPSS software (Statistical Product and Service Solutions ver. 20; SPSS Inc., Chicago, IL, USA). Differences were considered significant for values of $P < 0.05$.

Results

TGF-β induces hepatoma cell migration through Akt. The cell migration assays showed that TGF-β treatment led to increased cell migration of HepG2 and PLC/PRF/5 cells. Increased cell migration was also observed in HLE cells with low expression of TGF-β receptors.[17] These effects of TGF-β were significantly suppressed by treatment with the PI3K inhibitor LY294002 (TGF-β *vs* LY294002 plus TGF-β: HepG2, 245 ± 23% *vs* 70 ± 11%; PLC/PRF/5, 204 ± 18% *vs* 46 ± 13%; HLF, 151 ± 19% *vs* 70 ± 11%; $P < 0.05$) (Fig. 1a). Western blotting analyses showed that TGF-β phosphorylated Akt in all three hepatoma cell lines (Fig. 1b). The level of p-Akt in TGF-β-treated HepG2 cells was observed to be repressed by the antioxidant NAC (Fig. 1c).

TGF-β activates Akt/PAK2 signaling in hepatoma cells. Western blotting analyses using an anti-p-PAK2 (S20) antibody showed two bands owing to concomitant phosphorylation at Thr402.[18] In all hepatoma cell lines, p-PAK2 (S20 and S141) was significantly increased by TGF-β treatment (Fig. 2a). Western blotting showed that p-PAK1 (Ser144) was increased by 1.4- and 2.0-fold in HepG2 and PLC/PRF/5 cells, respectively, and undetectable in HLF cells (Fig. 2a). In contrast, the level of p-PAK2 (Ser141) was significantly increased by TGF-β, with 8.0- ($p \leq 0.01$), 4.5- ($p \leq 0.01$), and 4.5- fold ($P \leq 0.01$) increase in HepG2, PLC/PRF/5, and HLF cells, respectively (Fig. 2a). When HepG2 cells were exposed to TGF- β for 6 days in serum-containing media, p-PAK2 (Ser141) was continuously increased to six times the level of untreated cells ($p \leq 0.01$), and the level of p-PAK1 (Ser144) was 1.4-fold higher than control ($P \leq 0.05$). PAK2 was continuously phosphorylated in parallel with p-Akt in HepG2 cells treated with TGF-β for 6 days (Fig. 2b), indicating that PAK2 is constantly activated in the presence of TGF-β. The level of p-Akt was unchanged in PAK2 siRNA-treated cells in both the serum-containing condition (Fig. 2c) and serum-free condition (Fig. 2c and Supplementary Fig. S1), indicating that Akt is not affected by PAK2 activity. TGF-β significantly induced Akt phosphorylation, which was inhibited by LY294002 and NAC in both control and PAK2 siRNA-transfected cells. These results suggest that TGF-β-Akt signaling is regulated by reactive oxygen species (ROS), but not by PAK2 (Supplementary Fig. S2). The level of p-PAK2 (S141 and S20) was significantly decreased by treatment

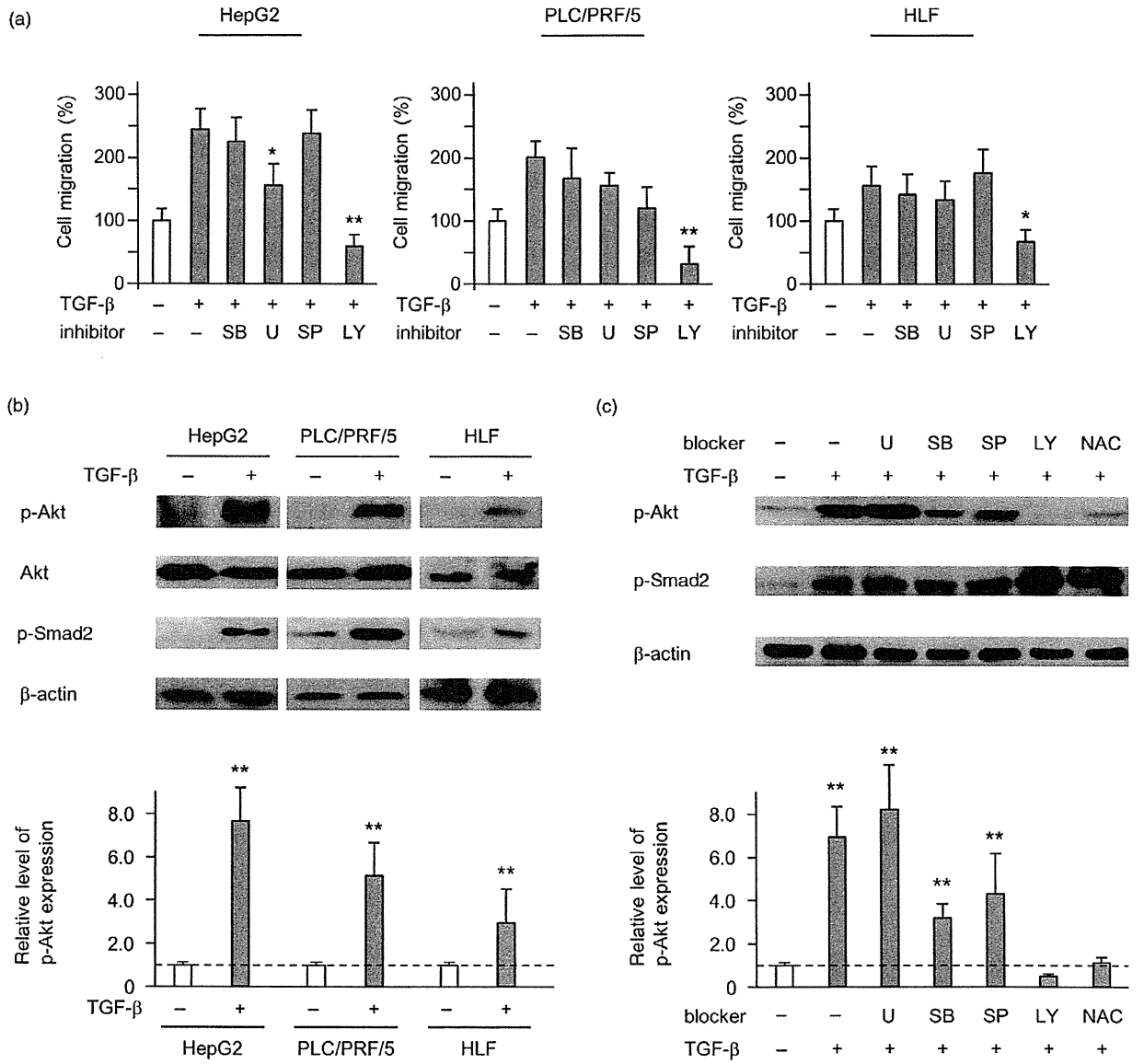

Figure 1 Transforming growth factor-β (TGF-β) induces hepatoma cell migration through Akt signaling. (a) Percentages of migrated cells pretreated with TGF-β for 2 days in serum-containing culture media. Data are means ± SD. $*P < 0.05$ versus TGF-β-treated cells. (b) Western blot analysis of hepatoma cells treated with TGF-β for 2 h in serum-free condition. (C) Western blot analysis of HepG2 cells treated with TGF-β for 2 h in serum-free condition. Columns in b and c show relative band intensities expressed as the fold changes of control cells. Data are expressed as mean ± SD of three independent experiments (b and c, $**P \leq 0.01$ *vs* non-treated cells). LY, LY294002; NAC, N-acetyl-L-cysteine; SB, SB203580; SP, SP600125; U, U0126. p-Smad2, phospho-Smad2. □, non-treated; ▩, TGF-β.

with LY294002 and NAC (Fig. 2d). Immunofluorescence analyses showed that p-PAK2 (S20) was increased in the cytoplasm of TGF-β-treated HepG2 cells, and repressed by treatment with LY294002 and NAC (Fig. 2e).

PAK2 is a critical regulator of TGF-β-mediated hepatoma cell migration. When control siRNA-transfected HepG2 cells were treated with TGF-β, focal adhesion areas accumulated along the leading edges (Fig. 3a). However, when PAK2 siRNA-transfected cells were treated with TGF-β, the formation of the focal adhesion areas was impaired and the number of cell protrusions was decreased (Fig. 3a). Cell migration assays showed that the percentage of migrated control siRNA-transfected cells was increased by 2–2.3-fold ($P < 0.05$) in the presence of TGF-β. In PAK2 siRNA-transfected cells, however, the cell migration was not significantly increased (1.2–1.3-fold; $P > 0.05$) (Fig. 3b). The relative rate of wound closure by the

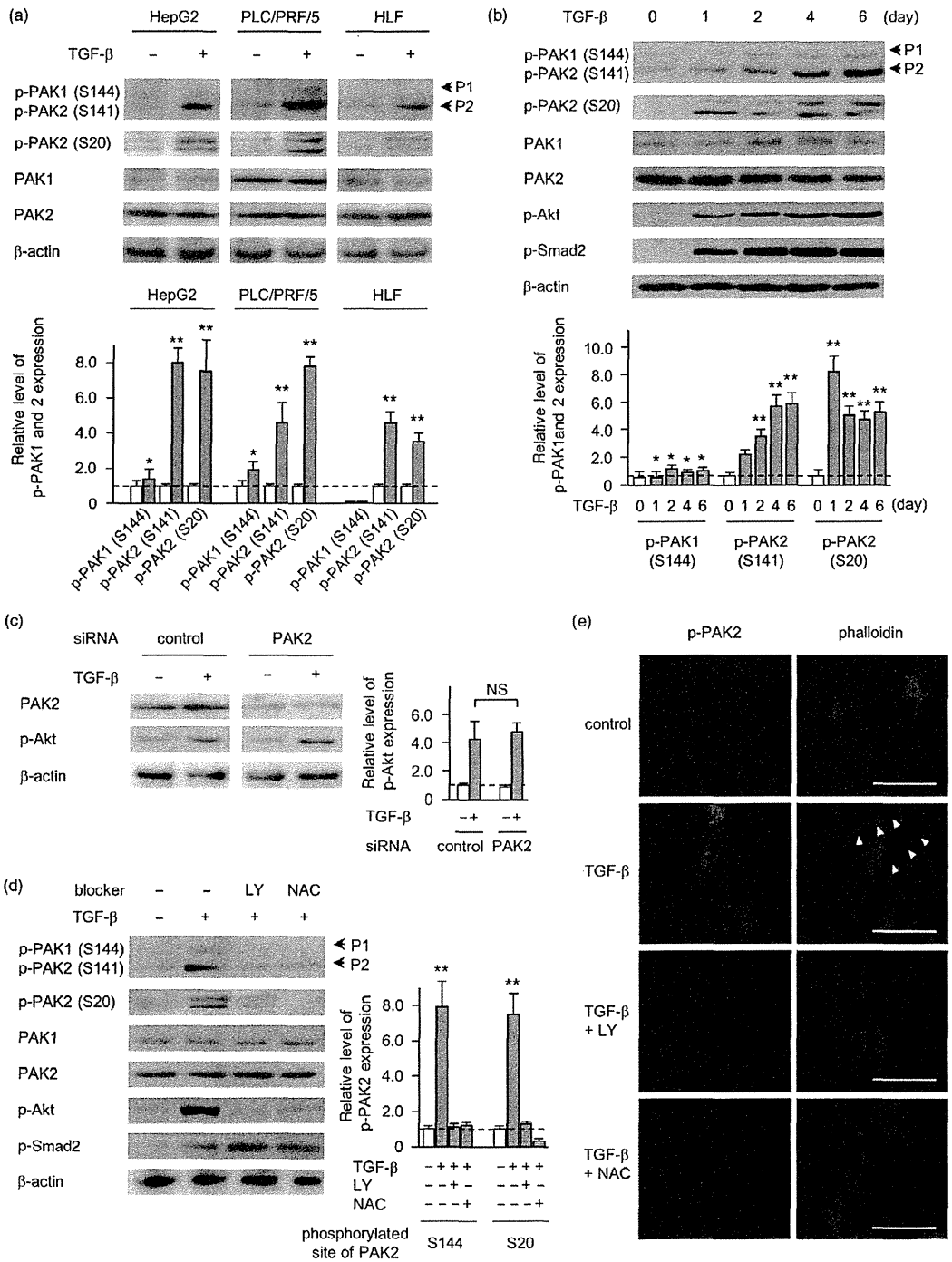

Figure 2 *Transforming growth factor-β (TGF-β) activates p21-activated kinase-2 (PAK2) through reactive oxygen species /Akt signaling.* (a) Western blot analysis of hepatoma cells treated with TGF-β for 2 h in serum-free condition. P1, phospho-PAK1 (p- PAK1, S144); P2, phospho-PAK2 (p- PAK2, S141). (b) Western blot analysis of HepG2 cells treated with TGF-β for 6 days in serum-containing culture media. (c) Western blot analysis of PAK2 small interfering RNA (siRNA)-transfected HepG2 cells treated with TGF-β for 24 h in serum-containing culture media. (d) Western blot analysis of HepG2 cells treated with TGF-β for 2 h in serum-free condition. Relative band intensities of Western blotting were expressed in the columns as the fold changes relative to controls. The levels of p-PAK1 (S144) expression were set as the fold changes of p-PAK2 (S141) in non-treated cells. Data are expressed as mean ± SD of three independent experiments ($*P \leq 0.05$ and $**P \leq 0.01$ *vs* non-treated cells: NS, not significant). (e) Immunofluorescence staining of p-PAK2 (S20) in HepG2 cells (arrowhead, stress fibers). Bar, 20 μm. LY, LY294002; NAC, N-acetyl-L-cysteine; p-Smad2, phospho-Smad2. □, non-treated; ▨, TGF-β.

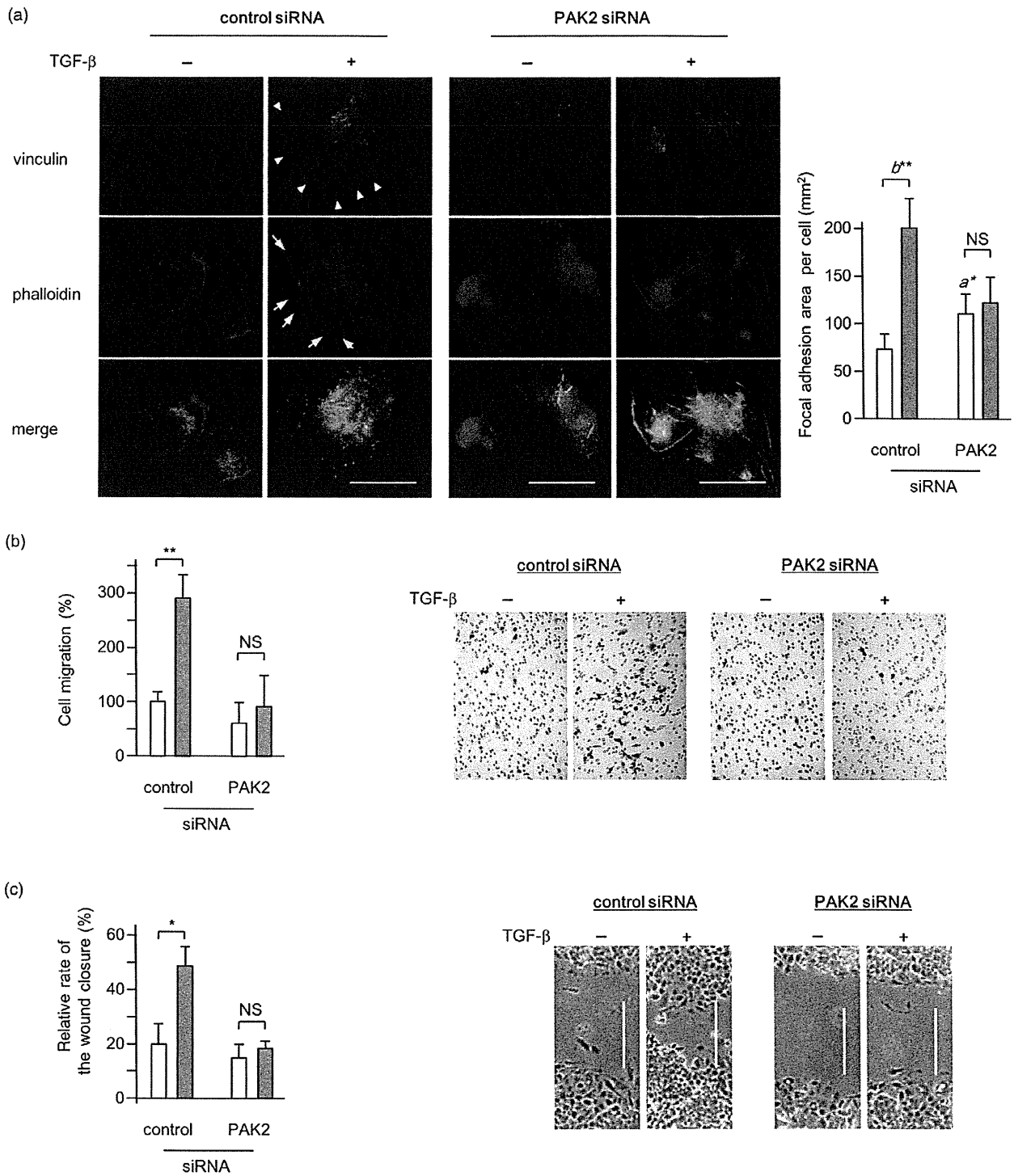

Figure 3 p21-activated kinase-2 (PAK2) induces hepatoma cell migration. (a) Immunofluorescence staining of vinculin (arrowhead, focal adhesion; arrow, cellular protrusion) (Bar, 20 μm; *a**, *P*-value of ≤ 0.05 for control small interfering RNA [siRNA] *vs* PAK2 siRNA cells, *b***, *P*-value of ≤ 0.01 for non-treated *vs* TGF-β-treated control siRNA cells). (b) Cell migration assay in PAK2 siRNA-transfected HepG2 cells (magnification, × 40). (c) Wound healing assay in PAK2 siRNA-transfected HepG2 cells. Data are means ± SD. $*P < 0.05$ and $**P \leq 0.01$ versus non-treated cells. Bars: 100 μm. Data are expressed as mean ± SD of three independent experiments. NS, not significant, □, non-treated; ▨, TGF-β.

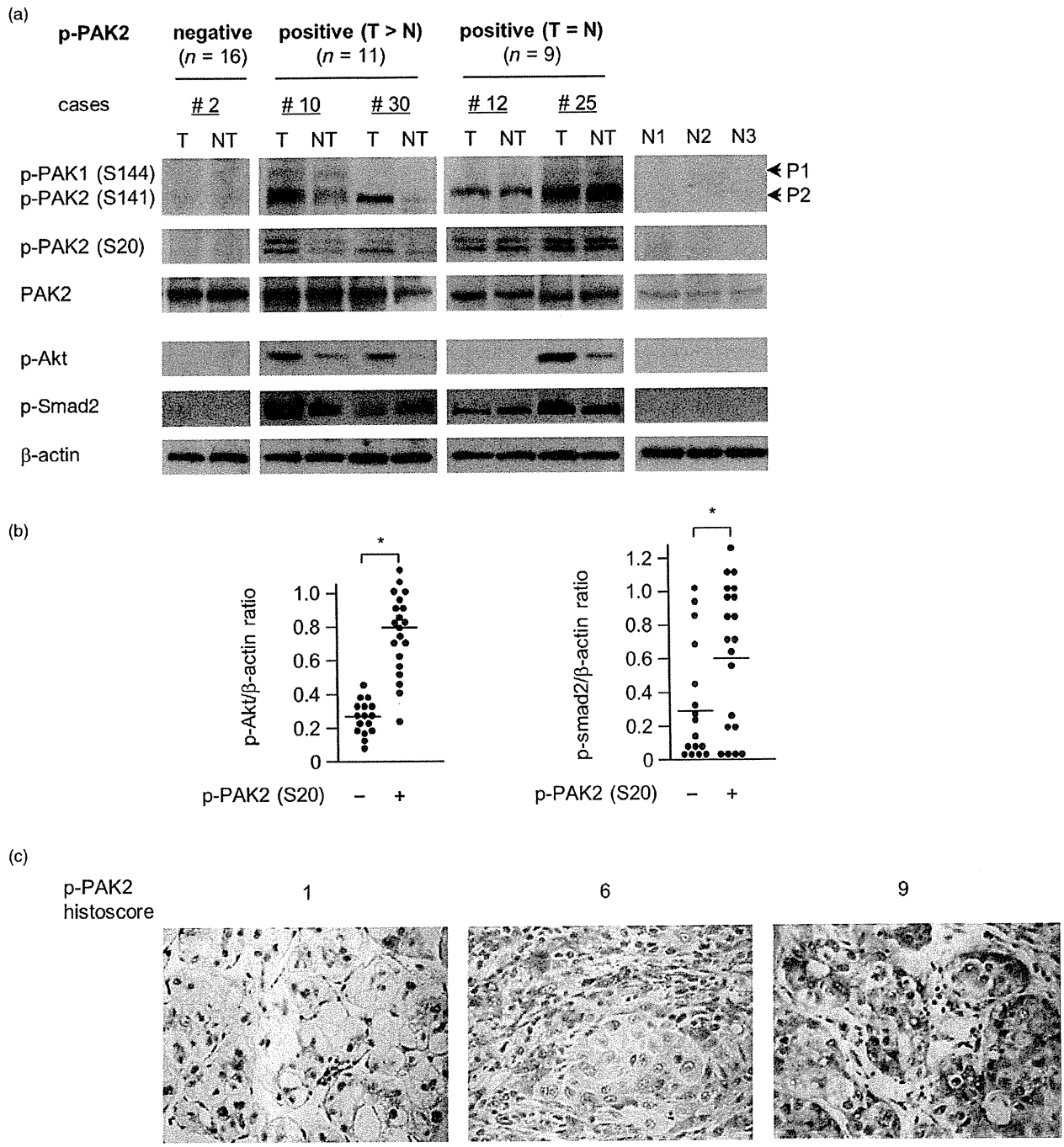

Figure 4 p21-activated kinase-2 (PAK2) is phosphorylated in hepatocellular carcinoma (HCC). (a) Western blot analyses of HCCs. T, tumor; NT, non-tumor; N, normal liver. (b) Columns show the ratio of p-Akt/β-actin and phospho-Smad2/β-actin between the tumors with or without phospho-PAK2 (p-PAK2, S20) expression (*$P \leq 0.05$). (c) Immunohistochemical staining of p-PAK2 (S20) in HCC (magnification, × 40).

control siRNA-transfected cells was increased by TGF-β treatment (non-treated, 18 ± 4% *vs* TGF-β, 54 ± 6%) ($P < 0.01$). In PAK2 siRNA-transfected cells, wound closure was not evidently accelerated by TGF-β treatment (non-treated, 12 ± 4% *vs* TGF-β, 16 ± 2%; $P > 0.05$) (Fig. 3c).

Level of p-PAK2 is associated with a poor prognosis of HCC. Western blot analyses showed that 20 of 36 (56%) HCCs had p-PAK2 (S20) (Fig. 4a). No significant clinicopathological differences were observed between the two groups of patients with and without p-PAK2 in non-tumorous tissues.

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Table 1 Associations of p-PAK2 with clinicopathological features in HCC

Clinicopathological variables		p-PAK2 immunoreactivity		*P*-value
		Low ($n = 36$)	High ($n = 26$)	
Age (years)	< 50	8	5	0.516
	≥ 50	28	21	
Gender	Male	29	22	0.748
	Female	7	4	
Tumor size (cm)	< 3	11	11	0.246
	≥ 3	25	15	
Intrahepatic metastasis	–	24	8	0.005
	+	12	18	
Venous invasion	–	33	13	< 0.001
	+	3	13	
Histological grade†	I/II	34	19	0.023
	III/IV	2	7	

†Histological grade was assessed according to the Edmondson–Steiner grade.
HCC, hepatocellular carcinoma; p-PAK2, phospho-p21-activated kinase-2.

P-PAK2 (Ser141) was detectable in all 20 HCC cases positive for p-PAK2 (Ser20), while p-PAK1 (Ser144) was positive in 18 cases (90%) (Fig. 4a). The ratio of p-PAK1 (Ser144)/β-actin in these cases was significantly less than that of p-PAK2 (Ser141)/β-actin (0.2 ± 0.1 *vs* 1.6 ± 0.5, $p \leq 0.05$). A significant correlation was found between p-PAK2 and the ratio of p-Akt/β-actin ($P = 0.032$) and p-Smad2/β-actin ($P = 0.036$) (Fig. 4b). Immunohistochemical analyses showed that p-PAK2 (S20) was mainly expressed in the cytoplasm, and to a lesser extent in the nucleus, in HCC (Fig. 4c). Of the total of 62 HCCs, 26 cases (42%) were categorized as p-PAK2 high expressors and 36 (58%) were categorized as p-PAK2 low expressors. The p-PAK2 high expressors were significantly associated with the existence of intrahepatic metastasis ($P = 0.005$), venous invasion ($P < 0.001$), and histological grade of the tumor ($P = 0.023$) (Table 1). Kaplan–Meier analyses revealed that the high p-PAK2 expressors were significantly associated with a shorter disease-free survival rate (log-rank, 2.584, $P = 0.009$) and shorter overall survival (log-rank, 2.634, $P = 0.008$) (Fig. 5).

Discussion

In the present study, we found that TGF-β-induced hepatoma cell migration was exclusively regulated by Akt. Our findings appear intriguing, because it was recently shown that TGF-β stimulates Akt-mediated signaling in non-epithelial cell types, but not in epithelial cells.[11,12] Accordingly, PAK2, which is a downstream component of Akt,[10–12] has also been reported to act as a mediator of TGF-β in mesenchymal cells, but not in epithelial cells.[10,19] Moreover, it has been recently found that PAK2 restricts TGF-β-induced Smad2/3 activation and transcriptional responsiveness in epithelial cells.[20] Hence, it is highly likely that PAK2 negatively modulates TGF-β signaling in epithelial cells by attenuating the receptor-Smad interaction, while positively modulating TGF-β-Akt signaling in mesenchymal cells. Why PAK2 mediates TGF-β signaling specifically in mesenchymal cells is unclear. Some studies have reported that the epithelial expression of Erbin, an inhibitor of PAK2 may restrict PAK2 activity to mesenchymal cells.[21] To understand the clinical significance of PAK2, it would be useful to analyze Erbin function in a large quantity of clinical samples. Although the reason for Akt activation in hepatoma cells remains unclear, it should be borne in mind that mammalian livers originate from the mesoderm and endoderm.[22] Even though it is still under debate whether HCC originates from oval cells or hepatocytes,[23] it is not surprising that the mechanism of hepatoma cell invasion is different from that of epithelial type cancer cells. Notably, TGF-β-induced Akt activation in HepG2 cells was inhibited by an antioxidant. Because it is well known that intracellular ROS are overproduced by TGF-β,[24] our results suggest that ROS/Akt signaling may be critical for hepatoma cell migration.

With the aim of dissecting the downstream components of Akt signaling, we set out to assess the serine/threonine kinase PAK2, which is downstream of Akt and involved in cell migration.[10–13] It was recently reported that phosphorylation of PAK2 at Ser141 partially increases the kinase activity, while phosphorylation at Ser20 facilitates cell migration.[16,18,25] In this study, we found that both Ser141 and Ser20 of PAK2 were significantly phosphorylated in TGF-β-treated hepatoma cell lines. Cell migration assays revealed significantly decreased cell migration of PAK2 siRNA-treated cells. TGF-β-induced Akt phosphorylation was significantly inhibited by LY294002 and NAC in both control and PAK2 siRNA-transfected cells, suggesting that TGF-β-Akt signaling is regulated by ROS, but not by PAK2. The level of p-PAK2 was efficiently blocked by an antioxidant, indicating that ROS may be important mediators of TGF-β/Akt/PAK2 signaling. There have been no studies regarding the involvement of PAK2 in HCC, and the results of our *in vitro* experiments led us to assess the clinical significance of PAK2 in HCC tissues. Immunohistochemical staining revealed close associations between the grade of p-PAK2 immunoreactivity and poor disease-free and overall survival in HCC patients, suggesting that PAK2 may be strongly involved in the disease progression of HCC. Very recently, it has been reported that PAK2 might act as a critical paracrine mediator of tumor growth in the liver. In animal models of colorectal liver metastasis, PAK2 is specifically expressed in hepatic stellate cells (HSC), leading to the pronounced proliferation of pro-tumorigenic HSC around the invasive front.[26] Therefore, along with the evidence that HSC is exclusively activated by TGF-β,[7] it would be plausible that TGF-β and PAK2 might cooperate to promote tumor aggressiveness by inducing tumor cell migration and the proliferation of the tumorigenic HSC.

It should be noted that our Western blotting also showed that p21-activated kinase-1 (PAK1), another PAK family member which has been reported to be overexpressed in HCCs,[27] was phosphorylated at Ser144 upon exposure to TGF-β. However, its increased level was significantly less than that of p-PAK2. The increased level of p-PAK1 was efficiently reduced by LY294002 and NAC as was observed for PAK2, suggesting that intracellular ROS-mediated PI3K/Akt signaling might be a common pathway for regulating PAK1 and 2 activities in hepatoma cells. Our results showing that p-PAK1 was detected in 90% of HCCs positive for p-PAK2 support the possibility of concurrent activation of PAK1 and PAK2 in HCC. Recently, some studies have raised the possibility that PAK1 and PAK2 play distinct roles in cell migration.

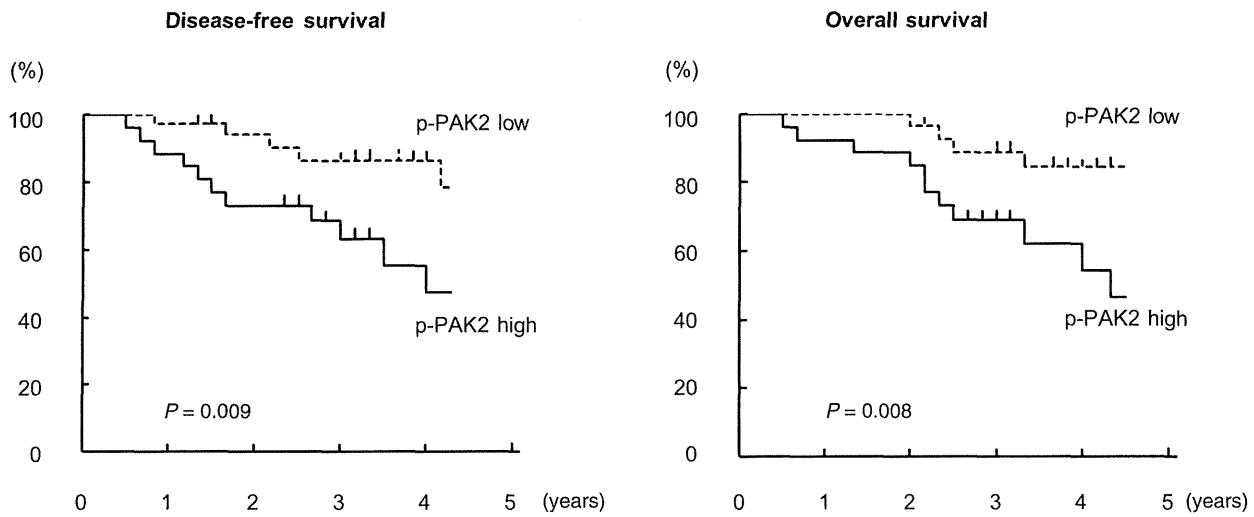

Figure 5 The level of p21-activated kinase-2 (PAK2) phosphorylation is correlated with the prognosis of hepatocellular carcinoma (HCC). Kaplan–Meier analyses of disease-free and overall survival for HCC patients categorized into subgroups of phospho-PAK2 (p-PAK2, S20) low expressors and high expressors.

For example, PAK1 increases the level of phospho-myosin light chain (MLC), while PAK2 decreases the level of p-MLC.[28] Together with our data, it is highly likely that PAK1 and PAK2 synergistically act to promote hepatoma cell migration. Additional study, including comprehensive analysis of PAK1 and PAK2 activities in hepatoma cells, will be required to further delineate the role played by PAK1 in HCC.

In conclusion, we suggest that PAK2 may be a strong mediator of HCC metastasis, and this may be partly owing to the fact that hepatoma cells are unique in their capability for TGF-β-mediated Akt activation through ROS production. To date, several studies have reported that PAK2 plays a significant role in the migration of other cancer cell types. In breast carcinoma, PAK2 has been found to contribute to tumor invasion stimulated by heregulin.[28] In ovarian cancer cells, p-PAK2 is overexpressed and knockdown of PAK2 reduces cell migration and invasion.[29] Taken together, PAK2 may represent a useful target for improving the current poor prognosis of HCC patients.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Western blot analysis of PAK2 siRNA-transfected HepG2 cells. After 24 h of transfection, cells were serum-deprived for 24 h and treated with TGF-β for 2 h. Columns show relative band intensities expressed as the fold changes relative to control cells. Data are expressed as mean ± SD of three independent experiments (NS, not significant).

Figure S2 Western blot analysis of PAK2 siRNA-transfected HepG2 cells. Twenty-four hours after transfection, cells were serum-deprived for 24 h before being treated with TGF-β for 2 h with or without a 1 h pretreatment with either LY294002 or NAC. Columns show relative band intensities expressed as the fold changes compared to control cells. Data are expressed as mean ± SD of three independent experiments ($^{**}P < 0.01$ vs non-treated cells: NS, not significant).

Figure S3 Cell migration assay of PAK2 siRNA-treated cells. Twenty-four hours after transfection, cells were treated with TGF-β for an additional 24 h with or without pretreatment with LY294002 or NAC for 1 h. The cells were suspended in DMEM containing 1% FBS and plated at 1×10^4 cells/mL in the upper chambers of transwell supports with 8-mm pores (BD Biosciences). The lower chambers were filled with medium containing 10% FBS. After 10 h, the cells that had migrated through the pores were counted in five random microscopic fields, and the average number of migrated cells in control siRNA-transfected wells was set at 100%. Data are expressed as mean ± SD of three independent experiments ($^{**}P < 0.01$ vs non-treated cells: NS, not significant).