Prognosis of hepatocellular carcinoma with biliary tumor thrombi after liver surgery

Takehiro Noda, MD,[a] Hiroaki Nagano, MD,[a] Yoshito Tomimaru, MD,[a] Masahiro Murakami, MD,[a] Hiroshi Wada, MD,[a] Shogo Kobayashi, MD,[a] Shigeru Marubashi, MD,[a] Hidetoshi Eguchi, MD,[a] Yutaka Takeda, MD,[a] Masahiro Tanemura, MD,[a] Koji Umeshita, MD,[b] Tonsok Kim, MD,[c] Kenichi Wakasa, MD,[d] Yuichiro Doki, MD,[a] and Masaki Mori, MD,[a] Osaka, Japan

Background. The incidence of biliary tumor thrombi (BTT) in hepatocellular carcinoma (HCC) is very low and operative outcomes are poor. There is little information on the postoperative prognostic factors for patients with HCC with BTT. We analyzed retrospectively the clinicopathologic features of 22 patients with HCC with BTT to identify the prognostic factors associated with operative outcome.
Methods. Of the 551 patients who underwent hepatic resection for HCC between 1988 and 2007, 22 (4.0%) had HCC with BTT. Their clinicopathologic features were compared with those with HCC but no BTT. The survival rates were also calculated for various clinicopathologic factors.
Results. Of the 22 patients, 13 (59%) also had portal or hepatic vein tumor thrombosis. Multiple HCC tumors were identified in 91% of patients and 20 patients were classified as stage IVA. The 1- and 3-year overall survival rates were 89% and 73% for patients with HCC free of BTT, respectively, and 62% and 30%, respectively, for those of HCC with BTT (P < .0001). Portal or hepatic vein tumor thrombosis was the only significant determinant of poor prognosis in 22 patients with HCC with BTT. The 1- and 3-year cumulative survival rates were 89% and 52%, respectively, for patients with only BTT but worse in those of HCC with both BTT and portal or hepatic vein tumor thrombosis (43% and 17%, respectively).
Conclusion. The clinicopathologic features of HCC patients with BTT indicated advanced-stage disease and poor operative outcomes. Portal or hepatic vein tumor thrombosis was the only significant determinant of poor prognosis of HCC patients with BTT. (Surgery 2011;149:371-7.)

From the Departments of Surgery,[a] Health Sciences,[b] and Radiology,[c] Graduate School of Medicine, Osaka University; and the Department of Pathology,[d] Osaka City University Hospital, Osaka, Japan

HEPATOCELLULAR CARCINOMA (HCC) is a common malignancy worldwide and is now the third leading cause of cancer death in Japan.[1] Vascular invasion, especially portal vein thrombi, is a well-documented marker of prognosis.[2,3] On the other hand, the incidence of biliary tumor thrombi (BTT) is very low and has been reported to be 2–9% in surgical cases.[4,5] Extension of tumor thrombi to the major or regional bile ducts often results in obstructive jaundice and subsequent hepatic dysfunction.[6] Operative resection of HCC with obstructive jaundice is rarely indicated, and there is little information on the operative results of hepatectomy in patients

with BTT. However, hepatectomy for patients with BTT can be safely performed at present thanks to recent progress in diagnostic modalities, pre- and postoperative management, and operative techniques.[7-9] Although only a few authors have reported their operative experiences, the outcome of resection has been dismal. Three groups evaluated previously the prognosis of patients with HCC and bile duct tumor thrombosis and the reported 3-year survival rates ranged from 10% to 47%.[9-11] In this regard, there is also little information available at present on the prognostic factors after operative resection of HCC with BTT.

In the present study, we analyzed retrospectively the clinicopathologic features of HCC with BTT in 22 patients who underwent hepatic resection and then analyzed the prognostic factors after operative resection.

PATIENTS AND METHODS

Between 1988 and 2007, 551 patients underwent hepatectomy for HCC in the Department of

Surgery, Osaka University Hospital. Out of these patients, 22 patients (4.0%) had HCC with macroscopic BTT. These patients included 21 men and 1 woman (mean age, 58 years; range, 31–75). Tumor resectability was assessed by ultrasonography (US), computed tomography, and angiography. Intraoperative US was performed in all patients. Preoperatively, all patients underwent endoscopic retrograde cholangiopancreatography or magnetic resonance cholangiopancreatography to diagnose the presence of BTT and evaluate the extension of tumor thrombi. The operative procedure was determined based on extensive preoperative evaluation of the location of the primary tumor and the extent of tumor thrombi in the biliary system. All patients underwent hepatic resection of the primary tumor and removal of BTT through the transected branch of the bile duct or through choledochotomy. Each cut end of the bile duct was examined by frozen section. Furthermore, microscopic examination confirmed the final diagnosis of the surgical margin and the presence of bile duct wall invasion. Surgical curative intent was defined as macroscopic removal of all tumor lesions and lack of no residual tumors in the remnant liver or abdominal cavity after clinical and/or US examination. Histologic differentiation and stage were evaluated by the stage classification of the Liver Cancer Study Group of Japan.[12] All patients were followed closely after discharge. Serum alpha-fetoprotein and concentration of protein-induced vitamin K absence or antagonist II[13] were measured monthly, and US, computed tomography with contrast medium, or both were performed at least every 3 months during the follow-up period.

Clinicopathologic analysis and survival curves. To assess the clinicopathologic features and background of HCC with BTT, univariate analysis of HCC with and without BTT was conducted by using the following 14 clinicopathologic factors: gender, age, hepatitis viral status (hepatitis B or C virus), Child-Pugh classification, Model for End-Stage Liver Disease (MELD) score, alpha-fetoprotein, number of tumors, tumor size, portal vein or hepatic vein tumor thrombi, histologic differentiation, nontumorous tissue, operative procedure, and surgical curative intent. The MELD score was calculated using the previously reported formula.[14]

Among the 22 patients with BTT, the survival curve was calculated by the Kaplan-Meier method according to 16 clinicopathologic factors to examine the prognostic factors in HCC with BTT. These 16 factors included the above 14 factors and 2

additional factors (bile duct resection and the extent of bile duct involvement). The latter 2 factors were included for 2 reasons: (1) There is controversy on whether bile duct resection influences surgical outcome of HCC with BTT, and (2) evidence provided in a previous study suggests the importance of the extent of bile duct involvement on overall survival.[15] The median follow-up period was 35 months. All data were expressed as mean values ± SD. Statistical difference between the groups was analyzed by the Student t test for continuous variables and the χ^2 test for others. Differences in the survival rates between the groups were compared with the log-rank test. All statistical analyses were conducted with StatView (version 5.0; SAS Institute, Inc., Cary, NC). $P < .05$ was considered significant.

RESULTS

Table I lists the clinical and pathologic features of patients with and without BTT. Hepatitis C virus infection was more frequent in patients without BTT than those with BTT. Preoperatively, 8 patients had obstructive jaundice and underwent biliary drainage by percutaneous transhepatic cholangial drainage in 3 cases and endoscopic nasobiliary drainage in 5 cases. Fewer patients with BTT were classified as Child-Pugh A (64%) than patients without BTT (84%). The MELD score was slightly higher in cases of BTT than that of no BTT. As to the extent of bile duct involvement, 5 patients (23%) had tumor thrombi in the common bile duct (CBD), 11 patients (50%) had in the right or left branch, and 6 patients (27%) had in the second branch of the intrahepatic bile duct. Among the 22 patients with BTT, 13 patients (59%) had also portal vein or hepatic vein tumor thrombi. Most patients with BTT had multiple tumors (91%), and 20 patients were classified as stage IVA by the staging system of Liver Cancer Study Group of Japan. Furthermore, 2 patients were classified as stage III. Based on histopathologic examination, 18 patients (82%) had poorly differentiated or undifferentiated HCC, whereas the remaining 4 patients had moderately differentiated HCC. The operative procedure was lobectomy in 16 patients (73%) and surgically noncurative procedure in 6 patients (27%). In the latter group, the reasons for noncurative surgery were resident tumor in the remnant liver ($n = 5$) and microscopically positive margin of the CBD ($n = 1$). Extrahepatic bile duct resection was performed in 2 patients (9%), and removal of BTT through choledochotomy was conducted in

Table I. Characteristics of participating patients

Variables	HCC with BTT (n = 22)	HCC without BTT (n = 529)	P value
Gender			
Male	95%	81%	NS
Female	5%	19%	
Age (y)			
≤60	45%	63%	NS
>60	55%	37%	
HBV			
Positive	68%	48%	NS
Negative	32%	52%	
HCV			
Positive	26%	59%	.0048
Negative	74%	41%	
Child-Pugh classification			
A	64%	84%	.0102
B	36%	16%	
MELD score	7.7 ± 4.8	4.3 ± 2.7	.0005
AFP (ng/mL)			
<400	45%	25%	.0354
≥400	55%	75%	
Number of tumors			
Solitary	9%	64%	<.0001
Multiple	91%	36%	
Tumor size (cm)			
≤5	59%	64%	NS
>5	41%	36%	
Portal vein or hepatic vein tumor thrombi			
Positive	59%	19%	<.0001
Negative	41%	81%	
Histologic differentiation			
Well/moderate	18%	50%	.0039
Poor/undifferentiated	82%	50%	
Nontumorous tissue			
Cirrhosis	27%	52%	.0215
No cirrhosis	73%	48%	
Operative procedure			
Limited resection or segmentectomy	27%	82%	<.0001
Lobectomy	73%	18%	
Curative intent			
Yes	73%	90%	.0090
No	27%	10%	

AFP, Alpha-fetoprotein; *HBV,* hepatitis B virus; *HCV,* hepatitis C virus; *NS,* not significant.

Fig 1. Survival curves for all 551 patients according to the presence or absence of BTT, estimated by the Kaplan-Meier method.
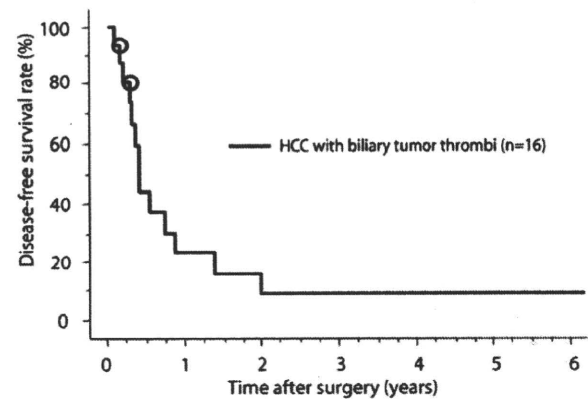
Fig 2. Disease-free survival curves of 16 patients with BTT who underwent curative resection.

multiplicity and other vascular tumor thrombi, and that many patients required major hepatectomy such as lobectomy.

Figure 1 shows the survival curves of all 551 patients according to the presence of BTT. The 1-, 3-, and 5-year overall survival rates of HCC without BTT were 89%, 73%, and 61%, respectively, whereas those with BTT were lower (62%, 30%, and 30%, respectively, P < .0001, each). Among the 16 patients with surgical curative intent, 13 patients developed tumor recurrence. Figure 2 shows the disease-free survival curves of 16 patients with surgical curative intent. The disease-free 1- and 3-year survival rates were 22% and 7%, respectively. The remnant liver after surgery was the first recurrence organ in all patients; 3 patients had both intrahepatic and extrahepatic recurrence. Two cases developed pulmonary recurrence and 1 case had both pulmonary and brain recurrences.

4 patients (18%). Seven patients (32%) developed postoperative complications including biliary leakage (n = 2), intra-abdominal infection (n = 2), acute cholangitis (n = 1), upper gastrointestinal hemorrhage (n = 1), and ascites (n = 1). There were no hospital deaths or operative deaths in this series. Based on these clinicopathologic features, it was concluded that patients with BTT had advanced-stage disease with frequent tumor
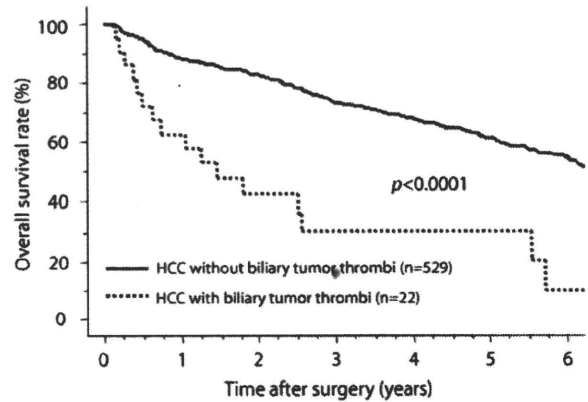
Table II. Survival rates of patients with hepatocellular carcinoma and BTT

		Survival			
Variables	No. of patients	1 year	3 years	5 years	P value
Gender					
Male	21	61	32	32	NS
Female	1	100	—	—	
Age (y)					
≤60	10	78	40	40	NS
>60	12	50	22	22	
HBV					
Positive	15	53	19	19	NS
Negative	7	86	51	51	
HCV					
Positive	5	40	20	—	NS
Negative	14	62	23	23	
Child-Pugh classification					
A	14	64	34	34	NS
B	8	60	23	23	
MELD score					
≤9	11	73	33	33	NS
≥10	7	54	27	27	
AFP (ng/mL)					
<400	10	60	32	32	NS
≥400	12	64	28	28	
Number of tumors					
Solitary	2	100	50	50	NS
Multiple	20	58	28	28	
Tumor size (cm)					
≤5	13	67	30	30	NS
>5	9	56	30	30	
Extent of bile duct involvement					
First branch or second branch	17	65	29	29	NS
CBD	5	53	—	—	
Portal vein or hepatic vein tumor thrombi					
Positive	13	43	17	—	.0156
Negative	9	89	52	52	
Histologic differentiation					
Well/moderate	4	100	50	50	NS
Poor/undifferentiated	18	54	27	27	
Nontumorous tissue					
Cirrhosis	6	67	33	33	NS
No cirrhosis	16	61	30	30	
Operative procedure					
Limited resection or segmentectomy	6	67	22	22	NS
Lobectomy	16	54	24	24	
Bile duct resection					
Present	2	50	—	—	NS
Absent	20	64	33	33	
Surgical curative intent					
Yes	16	61	36	36	NS
No	6	67	17	—	

AFP, Alpha-fetoprotein; *HBV*, hepatitis B virus; *HCV*, hepatitis C virus; *NS*, not significant.

Next, the prognostic significance of 16 clinicopathologic factors was investigated in 22 patients with BTT. The results of survival curve analysis are shown in Table II. Survival was clearly better in the absence of portal vein or hepatic vein tumor thrombi (P = .0156), but was not influenced by any of the other 15 factors. Figure 3 depicts the cumulative survival curves after resection for patients
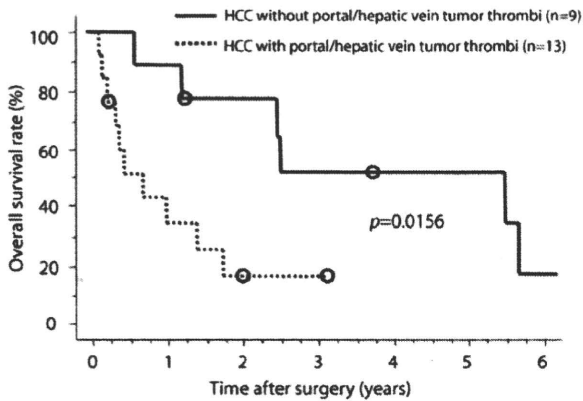
Fig 3. Cumulative survival curves according to the presence or absence of portal or hepatic vein tumor thrombi in patients with HCC and BTT.

with HCC and BTT according to the presence of portal vein or hepatic vein tumor thrombi. The 1- and 3-year cumulative survival rates of patients with HCC and portal vein or hepatic vein tumor thrombi were 43% and 17%, respectively, whereas the 1- and 3-year cumulative survival rates of those without portal vein or hepatic vein tumor thrombi were 89% and 52%, respectively. Out of the 22 patients with BTT, 4 patients received postoperative combination chemotherapy of interferon-α and 5-fluorouracil.[16-18] The 1-year cumulative survival rate of these 4 patients was 75%, which was higher than that of patients without postoperative therapy (60%), although the difference was not significant, probably owing to the small number of patients.

DISCUSSION

Hepatic resection is the only curative treatment available for patients with HCC and BTT. Peng et al[19] reported the survival outcome in 52 patients with bile duct tumor thrombi who underwent operative or interventional treatment (eg, hepatectomy, simple thrombectomy, biliary drainage, and transarterial chemoembolization). They concluded that only hepatic resection was associated with long-term survival.[19] The reported 5-year survival rate after hepatectomy, representing long-term outcome, ranges from 28% to 50%.[9,15] In the present study, the 5-year survival rate was 30% and the long-term prognosis was moderate. The clinicopathologic features of patients with HCC and BTT were those of the advanced stage with frequent tumor multiplicity and other vascular tumor thrombi, and the recurrence rate within 1 year was 78% (ie, very high). Several groups have reported that >50% of patients with HCC and BTT develop

tumor recurrence within 1 year after hepatectomy and the major pattern of recurrence is intrahepatic recurrence.[9,11] These results emphasize the need to identify meaningful prognostic factors after hepatic resection. Several studies have examined the relevance of various clinicopathologic features and/or operative management of HCC with BTT on long-term outcome. However, to our knowledge, there is little information on the prognostic factors after operative resection of HCC with BTT, probably because of the low incidence of HCC with BTT.

In the present study, the prognostic significance of 16 clinicopathologic factors was investigated in 22 patients with BTT. The results identified the absence of portal vein or hepatic vein tumor thrombi as the only significant factor that correlated with increased survival (P = .0156). It is generally accepted that HCC with tumor thrombi is associated with poor prognosis probably because it often reflects early intrahepatic recurrence.[20] Among the HCC patients with advanced stage, invasion of the portal or hepatic veins was an independent marker of poor prognosis, including both overall survival and disease-free survival.[2] Our results are agreement with those of those reports on the importance of portal vein and hepatic vein tumor thrombi on long-term outcome.

Bile duct resection for HCC is not generally conducted because nonoperative therapies such as percutaneous ethanol injection, ablation therapy and transarterial chemoembolization are known to result in serious complications such as liver abscess after bile duct resection and bilioenteric anastomosis.[21,22] Thus, bile duct resection and bilioenteric anastomosis should be avoided in hepatic resection of HCC. However, resection of the bile duct was performed in previous studies in 25–29% of patients with BTT, although there is little information on whether it bile duct resection influenced operative outcome for HCC with BTT.[9,15] In the present study, only 2 patients underwent bile duct resection after intraoperative detection of tumor invasion of the vessels around the bile duct. In both patients, we confirmed by histopathologic examination invasion of the tumor into the veins and/or lymphatic ducts around the bile duct. Analysis of overall survival rate indicated that resection of the bile duct did not improve the operative outcome of hepatic resection for HCC with BTT. On the other hand, it was reported that tumor thrombectomy by choledochotomy might cause peritoneal dissemination.[23] In the present study, 4 patients underwent thrombectomy through choledochotomy but none developed

peritoneal dissemination. Moreover, liver resection and thrombectomy is generally performed for HCC with portal vein tumor thrombi,[24] and there is no evidence that thrombectomy for HCC with portal vein tumor thrombi has increased the incidence of peritoneal dissemination. Thus, bile duct resection might be avoided during the operative treatment of HCC with BTT; the operative technique of thrombectomy for HCC with BTT through choledochotomy is safely conducted.

The extent of bile duct involvement was not a favorable prognostic factor in this study. Esaki et al[15] investigated the operative outcome of patients with biliary tumor involvement based on the extent of biliary invasion. They indicated that tumor spread into the CBD or the main branch was paradoxically a favorable prognostic factor. However, the authors also suggested that patients with HCC invading the large branches of the biliary tree infrequently had coexistent portal vein invasion and maintained preserved liver function. This aspect might contribute to a favorable outcome. Our data also showed that the presence of portal vein or hepatic vein tumor thrombi was a significant prognostic factor. Satoh et al[10] reported the classification of the patients with HCC and BTT into several categories, and they included those with BTT that was separate from the main tumor, which is sometimes confused with necrotic tissue or old clot. The existence of this type of BTT implies that BTT are rarely implanted or invade the bile duct wall, even if tumor thrombi are noticed in CBD or the main branch of the bile duct. These studies and our data suggest that HCC with BTT might have a less aggressive character and the presence of bile duct tumor thrombi might be less important as a prognostic factor than portal vein or hepatic venous tumor thrombi.

In conclusion, the clinicopathologic features of HCC patients with BTT were advanced disease and that the operative outcome of these patients was poor. The only factor that improved the survival rate in HCC patients with BTT was the absence of portal vein or hepatic vein tumor thrombi.

REFERENCES

1. Arii S, Monden K, Niwano M, Furutani M, Mori A, Mizumoto M, et al. Results of surgical treatment for recurrent hepatocellular carcinoma; comparison of outcome among patients with multicentric carcinogenesis, intrahepatic metastasis, and extrahepatic recurrence. J Hepatobiliary Pancreat Surg 1998;5:86-92.
2. Poon RT, Fan ST, Ng IO, Wong J. Prognosis after hepatic resection for stage IVA hepatocellular carcinoma: a need for reclassification. Ann Surg 2003;237:376-83.
3. Ikai I, Arii S, Kojiro M, Ichida T, Makuuchi M, Matsuyama Y, et al. Reevaluation of prognostic factors for survival after liver resection in patients with hepatocellular carcinoma in a Japanese nationwide survey. Cancer 2004;101:796-802.
4. Kojiro M, Kawabata K, Kawano Y, Shirai F, Takemoto N, Nakashima T. Hepatocellular carcinoma presenting as intrabile duct tumor growth: a clinicopathologic study of 24 cases. Cancer 1982;49:2144-7.
5. Ueda M, Takeuchi T, Takayasu T, Takahashi K, Okamoto S, Tanaka A, et al. Classification and surgical treatment of hepatocellular carcinoma (HCC) with bile duct thrombi. Hepatogastroenterology 1994;41:349-54.
6. Lau W, Leung K, Leung TW, Liew CT, Chan MS, Yu SC, et al. A logical approach to hepatocellular carcinoma presenting with jaundice. Ann Surg 1997;225:281-5.
7. Lee YC, Wang HP, Huang SP, Chang YT, Wu CT, Yang CS, et al. Obstructive jaundice caused by hepatocellular carcinoma: detection by endoscopic sonography. J Clin Ultrasound 2001;29:363-6.
8. Qin LX, Tang ZY. Hepatocellular carcinoma with obstructive jaundice: diagnosis, treatment and prognosis. World J Gastroenterol 2003;9:385-91.
9. Shiomi M, Kamiya J, Nagino M, Uesaka K, Sano T, Hayakawa N, et al. Hepatocellular carcinoma with biliary tumor thrombi: aggressive operative approach after appropriate preoperative management. Surgery 2001;129:692-8.
10. Satoh S, Ikai I, Honda G, Okabe H, Takeyama O, Yamamoto Y, et al. Clinicopathologic evaluation of hepatocellular carcinoma with bile duct thrombi. Surgery 2000;128:779-83.
11. Qin LX, Ma ZC, Wu ZQ, Fan J, Zhou XD, Sun HC, et al. Diagnosis and surgical treatments of hepatocellular carcinoma with tumor thrombosis in bile duct: experience of 34 patients. World J Gastroenterol 2004;10:1397-401.
12. Liver Cancer Study Group of Japan. Classification of primary liver cancer. Tokyo: Kanehara & Co, Ltd.; 1997.
13. Suehiro T, Sugimachi K, Matsumata T, Itasaka H, Taketomi A, Maeda T. Protein induced by vitamin K absence or antagonist II as a prognostic marker in hepatocellular carcinoma. Comparison with alpha-fetoprotein. Cancer 1994; 73:2464-71.
14. Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, et al. A model to predict survival in patients with end-stage liver disease. Hepatology 2001; 33:464-70.
15. Esaki M, Shimada K, Sano T, Sakamoto Y, Kosuge T, Ojima H. Surgical results for hepatocellular carcinoma with bile duct invasion: a clinicopathologic comparison between macroscopic and microscopic tumor thrombus. J Surg Oncol 2005;90:226-32.
16. Sakon M, Nagano H, Dono K, Nakamori S, Umeshita K, Yamada A, et al. Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. Cancer 2002;94:435-42.
17. Ota H, Nagano H, Sakon M, Eguchi H, Kondo M, Yamamoto T, et al. Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous interferon-alpha and intra-arterial 5-fluorouracil; role of type 1 interferon receptor expression. Br J Cancer 2005;93:557-64.
18. Nagano H, Miyamoto A, Wada H, Ota H, Marubashi S, Takeda Y, et al. Interferon-alpha and 5-fluorouracil combination therapy after palliative hepatic resection in patients with advanced hepatocellular carcinoma, portal venous tumor thrombus in the major trunk, and multiple nodules. Cancer 2007;110:2493-501.

19. Peng BG, Liang LJ, Li SQ, Zhou F, Hua YP, Luo SM. Surgical treatment of hepatocellular carcinoma with bile duct tumor thrombi. World J Gastroenterol 2005;11:3966-9.

20. Park JH, Koh KC, Choi MS, Lee JH, Yoo BC, Paik SW, et al. Analysis of risk factors associated with early multinodular recurrences after hepatic resection for hepatocellular carcinoma. Am J Surg 2006;192:29-33.

21. Chen C, Chen PJ, Yang PM, Huang GT, Lai MY, Tsang YM, et al. Clinical and microbiological features of liver abscess after transarterial embolization for hepatocellular carcinoma. Am J Gastroenterol 1997;92:2257-9.

22. de Baere T, Risse O, Kuoch V, Dromain C, Sengel C, Smayra T, et al. Adverse events during radiofrequency treatment of 582 hepatic tumors. AJR Am J Roentgenol 2003;181: 695-700.

23. Mok KT, Chang HT, Liu SI, Jou NW, Tsai CC, Wang BW. Surgical treatment of hepatocellular carcinoma with biliary tumor thrombi. Int Surg 1996;81:284-8.

24. Chen XP, Qiu FZ, Wu ZD, Zhang ZW, Huang ZY, Chen YF, et al. Effects of location and extension of portal vein tumor thrombus on long-term outcomes of surgical treatment for hepatocellular carcinoma. Ann Surg Oncol 2006;13:940-6.

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Tyrosine Kinase Inhibitor PTK/ZK Enhances the Antitumor Effects of Interferon-α/5-Fluorouracil Therapy for Hepatocellular Carcinoma Cells

Masahiro Murakami, MD, Shogo Kobayashi, MD, PhD, Shigeru Marubashi, MD, PhD, Yoshito Tomimaru, MD, Takehiro Noda, MD, PhD, Hiroshi Wada, MD, PhD, Hidetoshi Eguchi, MD, PhD, Yutaka Takeda, MD, PhD, Masahiro Tanemura, MD, PhD, Koji Umeshita, MD, PhD, Yuichiro Doki, MD, PhD, Masaki Mori, MD, PhD, and Hiroaki Nagano, MD, PhD

Department of Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan

ABSTRACT

Purpose. There is no standardized treatment for hepatocellular carcinoma (HCC) with portal vein tumor thrombus. We previously reported the efficacy of interferon-alpha and 5-fluorouracil combination (IFN/5-FU) therapy for these patients and the potential mechanism via the regulation of vascular endothelial growth factor (VEGF). In this study, we showed the VEGF-related effects of IFN/5-FU therapy using VEGF-receptor (VEGFR) selective inhibitor, PTK787/ZK222584 (PTK/ZK), in HCC cells.

Methods. Using two VEGF secreting and VEGFR expressing human HCC cell lines, PLC/PRF/5 and HuH7, we performed growth inhibitory assays in vitro and in vivo, apoptosis assay, cell cycle analysis, and Western blot analysis for the mechanism, with or without PTK/ZK in IFN/5-FU therapy.

Results. The combination of PTK/ZK and IFN/5-FU significantly inhibited cell growth in vitro and tended to reduce tumor growth in vivo in a HuH7 xenograft model in nude mice—in both cases without affecting VEGF secretion. PTK/ZK enhanced the IFN/5-FU induced apoptosis, based on increased proteins levels of Bax and reduced Bcl-xL and Bcl-2. Cell cycle analysis showed different results between the HCC cell lines following the combination therapy, possibly due to differences in p21 protein.

Conclusions. VEGF signaling inhibition would support an antitumor effect of IFN/5-FU therapy against HCC cell lines via induction of apoptosis and cell cycle delay.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Recent advances in surgical resection and liver transplantation have improved the local control of HCC; however, there is no standardized treatment for locally advanced HCC, defined by the presence of portal vein tumor thrombus. The prognosis for such patients remains extremely poor with surgery alone, and the reported median survival times are 6–14 months.[1-3] We recently started administering interferon-alpha (IFN-α) and 5-fluorouracil (5-FU) combination (IFN/5-FU) therapy as an adjuvant in cases of advanced HCC, with good clinical efficacy (1-survival rate: 100% vs. 41% in patients without IFN/5-FU historical controls).[4-7] One of our previous biochemical analyses revealed that this therapy controls tumor-associated angiogenesis by regulating endothelial growth factor (VEGF).[8,9] However, VEGF inhibition in this therapy is limited to only 30–40% and control of VEGF may contribute more tumor suppression under the therapy.[8,9]

On the other hand, recent advances in drug development targets angiogenic factors, in the field of HCC, because of its hypervascularity.[10,11] Among these factors, VEGF plays a central role, and VEGF-targeted agents have some clinical benefits for HCC, via tyrosine kinase blocking of VEGF-receptors (VEGFRs; Flt-1, Flk-1/KDR, and Flt-4).[12-16] We thus hypothesized that VEGF inhibition would enhance the VEGF-related antitumor effects of IFN/5-FU therapy. Actually, IFN/5-FU therapy partially inhibits VEGF secretion; therefore, the purpose of this study was

evaluation for an additional benefit under VEGF inhibition in the IFN/5-FU therapy. In this study, we used this VEGFR selective inhibitor, PTK787/ZK22584 (PTK/ZK, Vatalanib®), rather than sorafenib, which is commonly used but targets several tyrosine kinases.[15,16] PTK/ZK is a selective potent inhibitor of all known VEGFR tyrosine kinases, particularly potent against Flt-1 and Flk-1/KDR, and the efficacy against human HCC cell lines in vitro and in vivo was reported.[17] Our results showed that PTK/ZK enhanced the direct effect of IFN/5-FU therapy on human HCC cells both in vitro and in vivo. Furthermore, we investigated the possible additional effects of such therapy on apoptosis and cell cycle, previously reported as the main mechanisms of IFN/5-FU.[18]

MATERIALS AND METHODS

Reagents and Cell Lines

Purified human IFN-α was obtained from Otsuka Pharmaceutical Co. (Tokyo, Japan), 5-FU was obtained from Kyowa Hakko Kirin Co. (Tokyo), and PTK/ZK was obtained from Bayer Schering Pharma (Berlin, Germany). The two human HCC cells lines, PLC/PRF/5 and HuH7, expressing IFN receptor type 2 (IFNAR2), were purchased from the Japanese Cancer Research Resources Bank.[18] Both lines were maintained as adherent monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture at 37°C in a humidified incubator with 5% CO_2 in air.

Growth Inhibitory Assays using PTK/ZK Combined with IFN/5-FU Therapy

The growth inhibitory effects were tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously.[18] The cells were incubated in medium with or without PTK/ZK (5 or 10 μM) and/or IFN (0.5 μg/ml)/5-FU (500 U/ml) for 96 hours, based on previous reports.[17,18] These assays were repeated at least three times, with similar results obtained. The proportion of MTT-positive cells incubated without drugs was denoted as 100% viability.

Flow Cytometric Analysis of Annexin V-FITC Binding

Apoptosis was measured based on the binding of FITC-conjugated annexin, as described previously.[19] Briefly, after treatment with IFN-α and 5-FU with or without various concentrations of PTK/ZK, the cultured cells (1 × 10^6) were incubated with binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) containing

saturating concentrations of annexin V-FITC (BioVision, Mountain View, CA) and propidium iodide (PI) for 15 minutes at room temperature. After incubation, the cells were pelleted and analyzed by flow cytometry on a FACSCalibur (Becton Dickinson Immunocytometry Systems, BD, San Jose, CA), and data were processed using Cell Quest_software (BD).

Cell Cycle Analysis

Flow cytometric analysis was performed to assess the cell cycle, as described previously.[18] Briefly, after treatment with IFN-α and 5-FU with or without PTK/ZK (10 μM), cells were washed twice with phosphate-buffered saline (PBS) and then fixed in 70% cold ethanol for 4 hours before being washed and resuspended in 1 ml of PBS. PI (50 ml of 1 mg/ml solution in PBS) and RNase were added for 30 minutes at 37°C, and data were acquired on the FACSCalibur. Analysis of the cell cycle was performed using ModFIT software (BD).

Concentration of VEGF in Cell Culture Supernatants

After treatment with IFN-α and 5-FU with or without PTK/ZK (15 μM) for 48 hours, this conditioned medium was collected, and VEGF levels were analyzed using the human VEGF enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Camarillo, CA) as recommended by the manufacturer, as described previously.[8]

Western Blot Analysis

Cells were washed twice with ice-cold PBS and harvested from the culture dish. After centrifugation, the cell pellets were resuspended and lysed in RIPA buffer [25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 1 mM phenylmethylsulphonyl fluoride, and 500 KIE/ml aprotinin] containing phosphatase inhibitor. The extracts were centrifuged and the supernatant fractions were collected for Western blot analysis, performed as described previously.[20] The antibodies were used at 1:500 for anti-human Flt-1 antibody, 1:500 for anti-human KDR/Flk-1 antibody, 1:1000 for anti-human Bcl-xL antibody, 1:500 for anti-human Bcl-2 antibody, 1:400 for anti-mouse Bax antibody, 1:500 for anti-human cyclin D1 antibody, 1:300 for anti-human p27 antibody, and 1:500 for anti-human p21 antibody from Santa Cruz Biotechnology (Santa Cruz, CA), and 1:1000 for anti-human β-actin from Sigma (St Louis, MO), and 1:2000 for all secondary antibodies. The protein band intensities were analyzed densitometrically with the values for each protein band expressed relative to the density of the actin band.

Inhibitory Effect on Subcutaneous Xenograft Model in Nude Mice

Four- to 6-week-old female mice (BALB/c nu/nu, CLEA Japan, Tokyo) were used for subcutaneous xenograft models as described previously, under specific pathogen-free conditions in accordance with the institutional guidelines for animal care.[9] The doses and schedules of PTK/ZK and IFN/5-FU therapy were based on the results of previous studies.[9,17] Mice were assigned at random to one of the following four groups (5 mice per group): a) mice of the PTK/ZK group were administered PTK/ZK as an oral instillation (PO) at 20 mg/kg daily; b) each mouse of the IFN/5-FU group was injected subcutaneously (SC) 20,000U of IFN-α, three times per week, and an injected intraperitoneally (IP) 30 mg/kg 5-FU three times per week; c) mice of the IFN/5-FU+PTK/ZK group were administered PTK/ZK PO combined with IFN/5-FU SC/IP; and d) mice of the control group were administered SC/IP and PO injections of PBS. Tumor volume (TV) and body weight were measured twice per week, and TV was calculated using the following formula: (longest diameter) x (shortest diameter)^2 x 0.5. Four weeks after the initial treatment, all mice from each group were killed and tumors were harvested for examination.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). The unpaired Student's t test was used to examine differences in growth inhibitory effects in vitro. $P < 0.05$ was considered statistically significant.

RESULTS

VEGFR Expression and VEGF Secretion in Human HCC Cells

Flt-1 (VEGFR1) and KDR/Flk-1 (VEGFR2) were both expressed and in similar amounts in the two human HCC cells, PLC/PRF/5 and HuH7 (Fig. 1a). Incubation of both cells with IFN/5-FU alone and IFN/5-FU plus PTK/ZK, resulted in significant reductions of supernatant VEGF to 68.5% and 65.8%, respectively (Fig. 1b). These results indicated that PTK/ZK did not enhance the effect of IFN/5-FU on VEGF secretion through VEGFRs.

Inhibitory Effects of PTK/ZK and IFN/5-FU on Human HCC Cells In Vitro

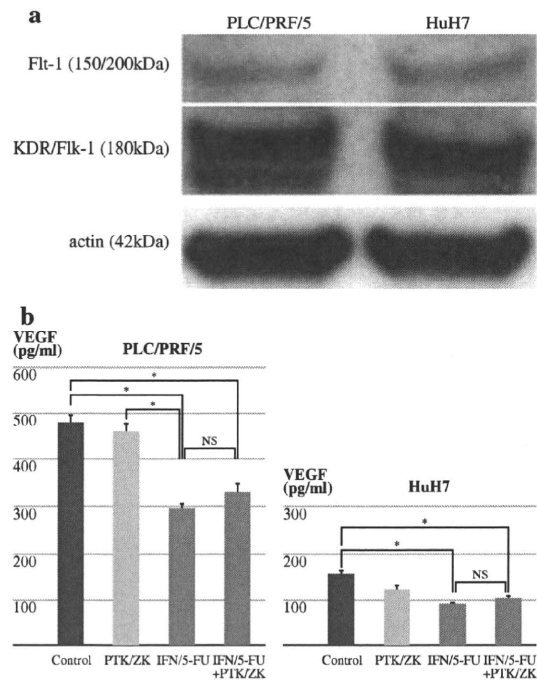To evaluate whether the combination of PTK/ZK and IFN/5-FU has an antiproliferative effect on human HCC

FIG. 1 VEGFRs expression and VEGF secretion in human HCC cells. a Expression of Flt-1 (VEGFR1) and KDR/Flk-1 (VEGFR2) in human HCC cells. b Secretion of VEGF from human HCC cells. PTK/ZK combined with IFN/5-FU significantly reduced the concentration of secreted VEGF in culture supernatants compared with the control group, whereas IFN/5-FU alone had no such effect. Data are mean ± SEM of triplicate assays; * $P < 0.05$

cells, we measured first the growth inhibition by the MTT assay. The data showed PTK/ZK concentration-dependent inhibition of cell growth, and PTK/ZK augments the inhibitory effect of IFN/5-FU; the addition of PTK/ZK (10 μM) to IFN/5-FU reduced the percentage of viable cells by 22.2% and 45.9% in PLC/PRF/5 and HuH7 cells, respectively (Fig. 2). The cooperative effect was statistically significant ($P < 0.05$).

PTK/ZK and IFN/5-FU Therapy Enhances Apoptosis of Human HCC Cells

To determine the mechanism of the antiproliferative effects of PTK/ZK combined with IFN/5-FU therapy on human HCC cells and whether it is related to the induction of apoptosis, we evaluated the extent of apoptosis using the annexin V assay to detect pre-apoptotic cells. The combination of PTK/ZK and IFN/5-FU increased the rate of apoptotic PLC/PRF/5 cells in a concentration-dependent manner and to a greater extent than IFN/5-FU alone (Fig. 3a, b; $P <0.05$). The HuH7 cells showed similar results (data not shown).

Next, we assessed the expression levels of Bcl-xL and Bcl-2 proteins for anti-apoptosis and Bax for pro-apoptosis
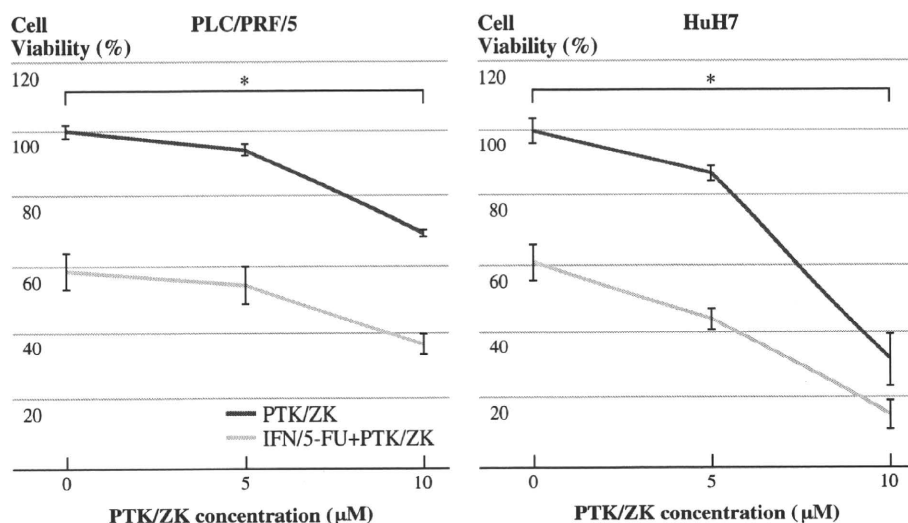
(Fig. 3c), which were reported previously to be associated with the apoptotic effect of IFN/5-FU therapy.[20] Figure 3d shows the relative expression levels of each protein compared with actin. PTK/ZK combined with IFN/5-FU decreased the expression levels of Bcl-xL and Bcl-2 to half of those in control, although there was no statistical difference in Bcl-2 expression of PLC/PRF/5 cells. In Bcl-2 expression, the addition of PTK/ZK to IFN/5-FU alone attenuated 10–30%, although there was no statistical difference. On the other hand, PTK/ZK combined with IFN/5-FU upregulated the expression of Bax compared with control by 1.5–3.4 times. The effect of the additional PTK/ZK to IFN/5-FU was 10–30% increments, although there was no statistical difference.

Effects of PTK/ZK and IFN/5-FU on the Cell Cycle in Human HCC Cells

Flow cytometric analysis was used to examine cell cycle progression of treated and untreated human HCC cells. Before any treatment, all cells were synchronized in G0-G1 phase by serum starvation for 72 hours. The cells were then put back in the growth medium with 10% FBS and the treatments were started. Cells were collected 12, 24, 48, and 72 hours later. Flow cytometric data confirmed that after serum starvation, the majority of cells (PLC/PRF/5, 77.9%; HuH7, 50.3%) were in G0-G1 phase. At 24 hours after the addition of either PTK/ZK combined with IFN/5-FU or IFN/5-FU alone, the PLC/PRF/5 cells showed increased S-phase-DNA content. This S phase accumulation and G0/G1 phase degradation was maintained at 48 and 72 hours (Fig. 4a). In contrast, HuH7 cells treated with PTK/ZK combined with IFN/5-FU showed more cells with G0/G1-phase-DNA content at 24, 48, and 72 hours; a

similar pattern was seen with PTK/ZK alone (Fig. 4b). We evaluated the cell cycle-related protein expression levels: cyclin D1 for promotion of cell cycle, p27 and p21 for delay (Fig. 4c). PTK/ZK combined with IFN/5-FU decreased the expression level of cyclin D1 in PLC/PRF/5 cells by 0.84 times that in control cells and increased the expression of p27 by 0.65 times than in control HuH7 cells; however, the differences were not significant. On the other hand, the expression of p21 protein was significantly different between PLC/PRF/5 and HuH7 cells. PTK/ZK combined with IFN/5-FU significantly decreased the expression of p21 by 0.71 times than in control PLC/PRF/5 cells, whereas the relative decrease was only small in HuH7 cells.

Inhibitory Effects of PTK/ZK and IFN/5-FU on Human HCC Xenografts In Vivo

The serial changes in implanted tumor volume in each treatment group are shown in Fig. 5. HuH7 cells were injected SC into nude mice, which were then treated for 4 weeks according to their group (n = 5 each). On day 30, the mean TV in the control group was 4.8 ± 1.1 cm^3, whereas TVs in the single-treatment groups were 3.2 ± 0.1 cm^3 (PTK/ZK group) and 2.0 ± 0.4 cm^3 (IFN/5-FU group). PTK/ZK combined with IFN/5-FU therapy significantly reduced the mean TV to 1.3 ± 0.3 cm^3 at 30 days. There were no significant differences in body weights among the different mice groups after removing the xenografts on the 27th day in each group compared with the respective pretreatment weight. Considered together, the above findings indicate that the combination of PTK/ZK and IFN/5-FU therapy inhibited the growth of human HCC cells both in vitro and in vivo, and that PTK/ZK enhanced the inadequate effect of IFN/5-FU therapy.

FIG. 3 Effects of PTK/ZK combined with IFN/5-FU on cell apoptosis, using the annexin V assay. Cells were harvested and double stained for annexin V-FITC and PI, with apoptosis defined by annexin V-positive/PI-negative cells. **a** Representative figure of flow cytometry. **b** Percentage of apoptosis at the indicated PTK/ZK concentration with or without IFN/5-FU. Data are mean ± SEM of triplicate assays. PTK/ZK combined with IFN/5-FU increased apoptosis in a concentration-dependent manner in PLC/PRF/5 cells to a greater extent than IFN/5-FU alone (P <0.05). **c** Expression of apoptosis-related proteins (Bcl-xL, Bcl-2, Bax) in PLC/PRF/5 and HuH7 cells treated for 6 hours, assessed by Western blot analysis. **d** Relative expression levels of apoptosis-related proteins. The band intensities were analyzed by densitometry and expressed relative to actin. Data are mean ± SEM of triplicate assays. * $P < 0.05$
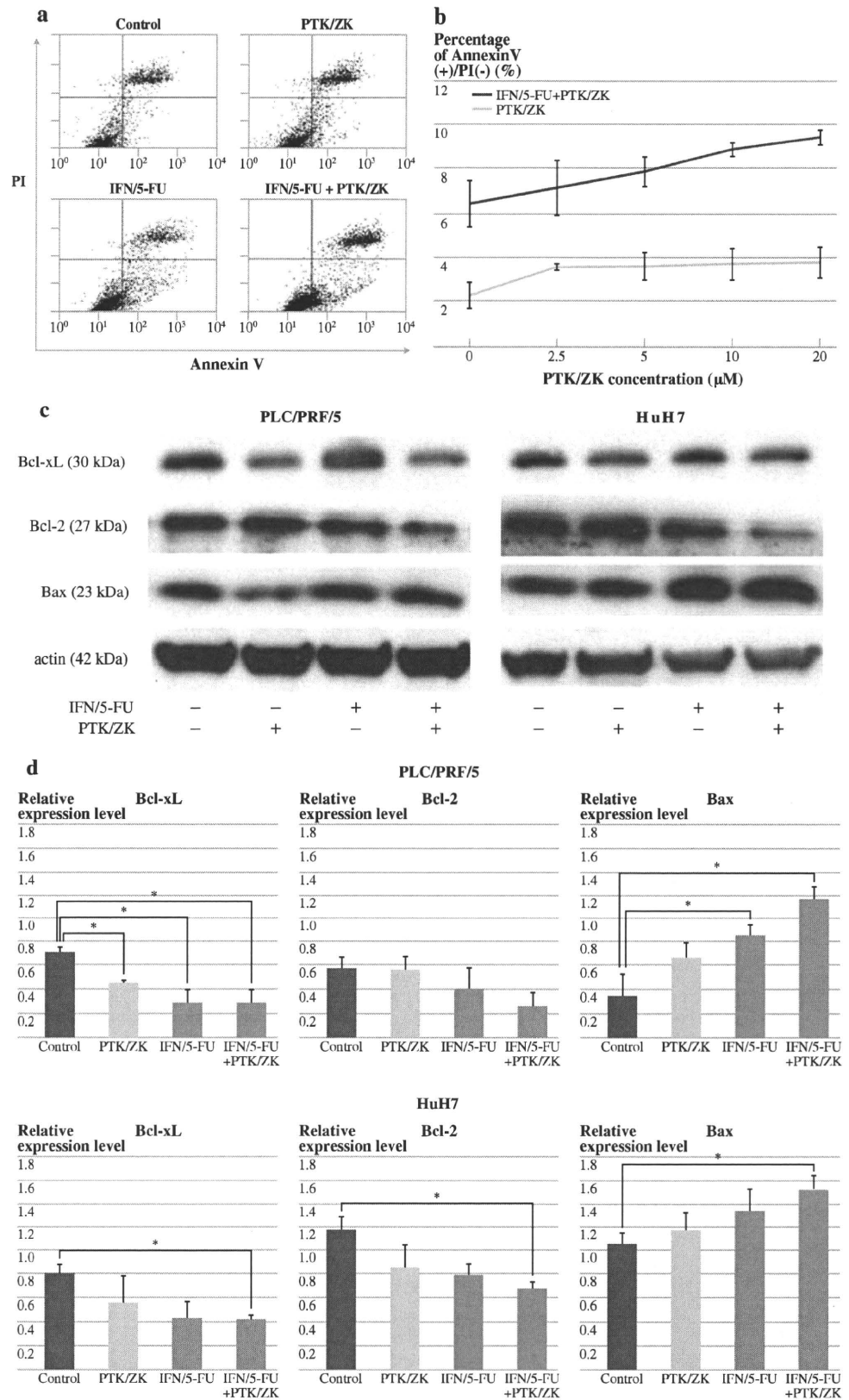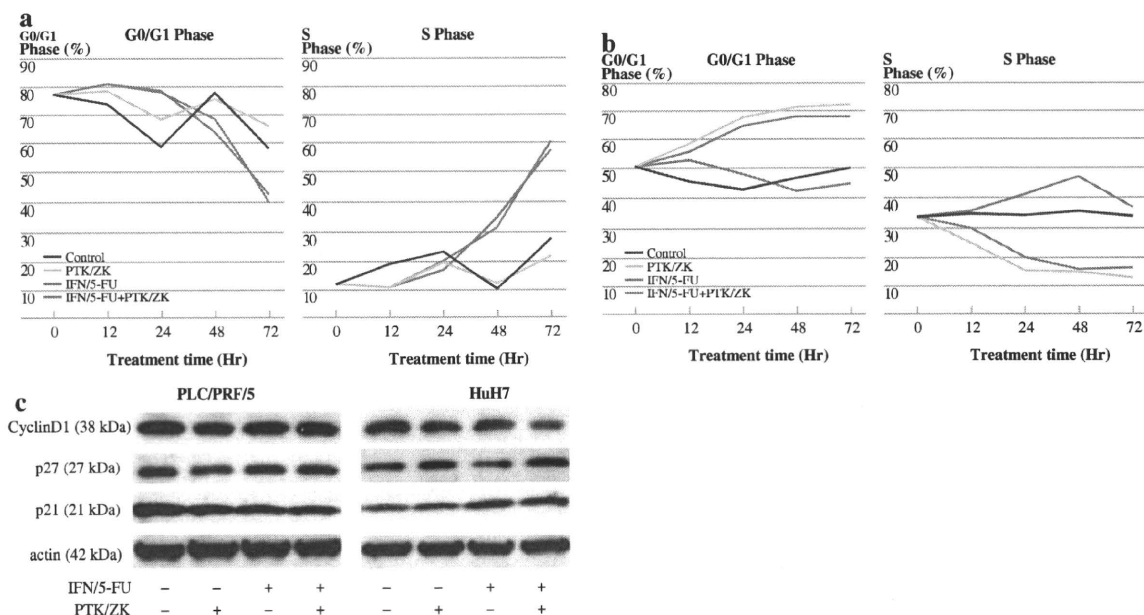
FIG. 4 Effects of PTK/ZK combined with IFN/5-FU on cell cycle in PLC/PRF/5 (**a**) and HuH7 (**b**) cells. The percentages of cells in G0-G1 phase (*left panel*) and S phase (*right panel*) are indicated for each

treatment time course. **c** Expression of cell cycle-related proteins (cyclin D1, p27, p21) in PLC/PRF/5 and HuH7 cells treated for 12 h, assessed by Western blot analysis

FIG. 5 Effect of PTK/ZK combined with IFN/5-FU therapy on tumor volume in xenografted nude mice. **a** Representative figure. **b** Change of tumor volume in each treatment. Data are mean volume of tumors $\pm$ SEM. * $P < 0.05$. Tumor volume of the IFN/5-FU+PTK/ZK therapy group was significantly decreased compared with the other two groups (control and PTK/ZK group); however, there was no significant difference between the IFN/5-FU and IFN/5-FU+PTK/ZK groups

DISCUSSION

We reported previously the potential mechanisms of the antitumor effects of IFN/5-FU therapy, both in vitro and in vivo, namely, the synergistic inhibition of cell proliferation and induction of apoptosis. The cell growth inhibition, including regulation of cell cycle progression, was orchestrated by increasing the S-phase fraction and thereby cell cycle arrest, whereas the apoptotic effect was mediated via IFNAR2 signaling to regulate the expression of apoptosis-related molecules.[18,20] Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and/or the Fas/Fas-L pathway also seemed to partially mediate the antitumor effects of IFN/5-FU therapy.[19,21] In addition, this therapy showed significant antitumor activity through the inhibition of angiogenesis in vitro and in vivo.[8,9] On the other hand, IFNAR2 protein is expressed on the cell surface in human HCC cell lines, although it is relatively weak in HuH7 cells and no cooperative effects were seen in these cells.[18] There are some nonresponders to IFN/5-FU therapy clinically, possibly due to activation of Wnt/β-catenin signaling pathway inducing chemoresistance to the IFN/5-FU therapy.[22] Thus, it is desirable to further examine the antitumor effects of this therapy to identify different potential targets.

Our previous study also showed that IFN/5-FU therapy inhibits VEGF secretion by tumor cells.[8] In the current study, we anticipated a stronger inhibitory effect on VEGF signaling with supplemented IFN/5-FU therapy, therefore we chose PTK/ZK because it potently inhibits all known VEGFR tyrosine kinases (including Flt-4, which is
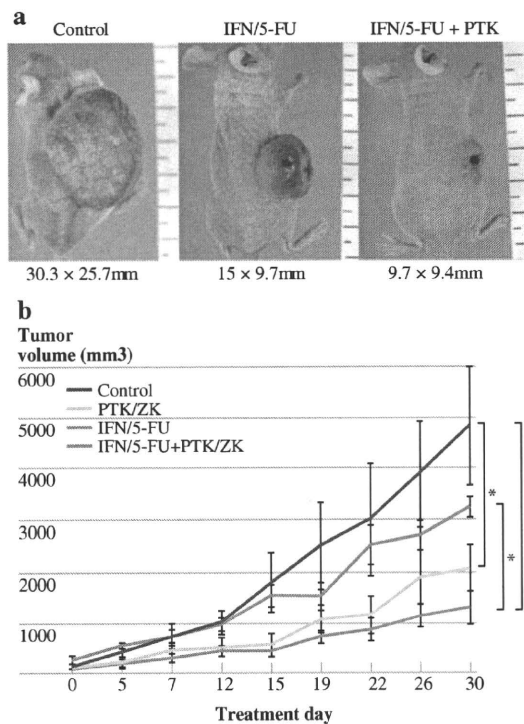
associated with the lymph system), selectively.[23] PTK/ZK works by binding to the ATP-binding sites of VEGFRs inhibiting tyrosine kinase phosphorylation.[23]

The major findings of the present study were as follows: a) PTK/ZK did not enhance the effect of IFN/5-FU therapy on secreted VEGF; b) PTK/ZK combined with IFN/5-FU inhibited cell growth in vitro and in vivo; c) the combination of PTK/ZK and IFN/5-FU enhanced the induction of apoptosis, but had different effects on cell cycle between two cell lines; and d) Bcl-2 family protein-related apoptosis plays a key role in the effect of these therapies, and the expression of p21 protein related to the cell cycle changed adversely in cells incubated with PTK/ZK and IFN/5-FU.

The findings suggested that pathways other than the VEGF secretion could be involved in the antitumor effects of PTK/ZK combined with IFN/5-FU. In considering the mechanisms of this effect related to apoptosis and cell cycle, we found that the addition of PTK/ZK increased the apoptotic effect of IFN/5-FU therapy dose-dependently. To elucidate the molecular mechanisms underlying this additional effect in human HCC cells, we examined the expression of key apoptotic regulators, Bcl-xL, Bcl-2, and Bax, which were regulated by IFN/5-FU therapy in the previous report.[20] Our findings suggested that VEGF binding to VEGFR activates a phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway leading to the upregulation of Bcl-2 protein.[24,25] On the other hand, evidence suggests that IFN induces apoptosis with activation of the Bcl-2-family members Bak and Bax, and that it activates several signaling pathways, including mainly the canonical Janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, but also the p38 mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways.[26] In addition, since PTK/ZK potently inhibits the activities of all known VEGFR tyrosine kinases, it also is active against other receptors, such as platelet-derived growth factor receptor beta and c-kit, although at higher concentrations.[23] It is therefore probable that PTK/ZK acts in different apoptotic pathways following IFN/5-FU treatment and that the combined therapy tested herein enhanced such effect.

Second, we evaluated the effect of PTK/ZK combined with IFN/5-FU on the cell cycle. Interestingly, we obtained different results on cell cycle analysis between the two cell lines tested, with the findings suggesting that IFN/5-FU has a stronger effect on PLC/PRF/5 cells than on HuH7 cells, whereas PTK/ZK works the other way. This phenomenon could be related to p21 protein in the cells, which reacted adversely in the present study. This protein is a cyclin-dependent kinase inhibitor acting mainly to induce G1 arrest,[27–29] thus our results could be understood within this functional context. A number of studies showed that p21 plays a role in cell cycle progression and growth via

p53-dependent and -independent pathways.[27,28,30] The two cell lines used in the present study harbor p53 mutations, and therefore such effects must be p53-independent via stimuli, such as IFNs and transforming growth factor-β (TGF-β).[31–33] However, HuH7 cells are associated with a p53 point mutation, which results in mutated proteins expressed with a prolonged half-life and thus protracted effects on apoptosis and cell cycle arrest.[31,34,35] Such properties could explain the observed changes in cell response and action site among the different drug treatment of the two cell lines. Thus, combining several drugs that act by different mechanisms should enhance these effects, and expand the possibility of potent therapy against HCC.

In these results, PTK/ZK could synergistically enhance the antitumor effects of IFN/5-FU, particularly in nonresponders, but the potential side effects must be managed. In our in vivo experiments, we used PTK/ZK alone at 20 mg/kg. Although this dose was lower than the effective doses used previously [50 or 100 mg/kg; effective concentration for human HCC cells xenografts as a single agent[17]], IFN/5-FU therapy combined with low-dose PTK/ZK therapy would sufficiently inhibit tumor growth and had no obvious adverse effects on the mice. Such combination therapy for advanced HCC might provide sufficient antitumor effects with fewer side effects. Sorafenib, multikinase inhibitor including VEGF, is widely used against HCC, and this also may be useful in this combination therapy.

In conclusion, we showed that PTK/ZK combined with IFN/5-FU therapy had antitumor effects on human HCC cells in vitro and in vivo, and that these effects were related to upregulated apoptosis and the complementary effects on cell cycle delay, without any change on VEGF secretion.

REFERENCES

1. Ikai I, Hatano E, Hasegawa S, et al. Prognostic index for patients with hepatocellular carcinoma combined with tumor thrombosis in the major portal vein. *J Am Coll Surg.* 2006;202:431–8.
2. Le Treut YP, Hardwigsen J, Ananian P, et al. Resection of hepatocellular carcinoma with tumor thrombus in the major vasculature. A European case-control series. *J Gastrointest Surg.* 2006;10:855–62.
3. Minagawa M, Makuuchi M. Treatment of hepatocellular carcinoma accompanied by portal vein tumor thrombus. *World J Gastroenterol.* 2006;12:7561–7.
4. Sakon M, Nagano H, Dono K, et al. Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. *Cancer.* 2002;94:435–42.
5. Ota H, Nagano H, Sakon M, et al. Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous interferon-alpha and intra-arterial 5-fluorouracil; role of type 1 interferon receptor expression. *Br J Cancer.* 2005;93:557–64.
6. Nagano H, Sakon M, Eguchi H, et al. Hepatic resection followed by IFN-alpha and 5-FU for advanced hepatocellular carcinoma

with tumor thrombus in the major portal branch. *Hepatogastroenterology*. 2007;54:172–9.

7. Nagano H, Miyamoto A, Wada H, et al. Interferon-alpha and 5-fluorouracil combination therapy after palliative hepatic resection in patients with advanced hepatocellular carcinoma, portal venous tumor thrombus in the major trunk, and multiple nodules. *Cancer*. 2007;110:2493–501.

8. Wada H, Nagano H, Yamamoto H, et al. Combination of interferon-alpha and 5-fluorouracil inhibits endothelial cell growth directly and by regulation of angiogenic factors released by tumor cells. *BMC Cancer*. 2009;9:361.

9. Wada H, Nagano H, Yamamoto H, et al. Combination therapy of interferon-alpha and 5-fluorouracil inhibits tumor angiogenesis in human hepatocellular carcinoma cells by regulating vascular endothelial growth factor and angiopoietins. *Oncol Rep*. 2007;18:801–9.

10. Ribatti D, Vacca A, Nico B, Sansonno D, Dammacco F. Angiogenesis and anti-angiogenesis in hepatocellular carcinoma. *Cancer Treat Rev*. 2006;32:437–44.

11. Sun HC, Tang ZY. Angiogenesis in hepatocellular carcinoma: the retrospectives and perspectives. *J Cancer Res Clin Oncol*. 2004;130:307–19.

12. Moon WS, Rhyu KH, Kang MJ, et al. Overexpression of VEGF and angiopoietin 2: a key to high vascularity of hepatocellular carcinoma? *Mod Pathol*. 2003;16:552–7.

13. Yoshiji H, Noguchi R, Kuriyama S, et al. Different cascades in the signaling pathway of two vascular endothelial growth factor (VEGF) receptors for the VEGF-mediated murine hepatocellular carcinoma development. *Oncol Rep*. 2005;13:853–7.

14. Wada H, Nagano H, Yamamoto H, et al. Expression pattern of angiogenic factors and prognosis after hepatic resection in hepatocellular carcinoma: importance of angiopoietin-2 and hypoxia-induced factor-1 alpha. *Liver Int*. 2006;26:414–23.

15. Tanaka S, Arii S. Molecularly targeted therapy for hepatocellular carcinoma. *Cancer Sci*. 2009;100:1–8.

16. Marijon H, Faivre S, Raymond E. Targeted therapies in hepatocellular carcinomas: recent results and future development. *Bull Cancer*. 2009;96:553–61.

17. Liu Y, Poon RT, Li Q, Kok TW, Lau C, Fan ST. Both antiangiogenesis- and angiogenesis-independent effects are responsible for hepatocellular carcinoma growth arrest by tyrosine kinase inhibitor PTK787/ZK222584. *Cancer Res*. 2005;65:3691–9.

18. Eguchi H, Nagano H, Yamamoto H, et al. Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells. *Clin Cancer Res*. 2000;6:2881–90.

19. Nakamura M, Nagano H, Sakon M, et al. Role of the Fas/FasL pathway in combination therapy with interferon-alpha and fluorouracil against hepatocellular carcinoma in vitro. *J Hepatol*. 2007;46:77–88.

20. Kondo M, Nagano H, Wada H, et al. Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells. *Clin Cancer Res*. 2005;11:1277–86.

21. Yamamoto T, Nagano H, Sakon M, et al. Partial contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway to antitumor effects of interferon-alpha/5-fluorouracil against Hepatocellular Carcinoma. *Clin Cancer Res*. 2004;10:7884–95.

22. Noda T, Nagano H, Takemasa I, et al. Activation of Wnt/beta-catenin signalling pathway induces chemoresistance to interferon-alpha/5-fluorouracil combination therapy for hepatocellular carcinoma. *Br J Cancer*. 2009;100:1647–58.

23. Wood JM, Bold G, Buchdunger E, et al. PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res*. 2000;60:2178–89.

24. Gerber HP, McMurtrey A, Kowalski J, et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem*. 1998;273:30336–43.

25. Nor JE, Christensen J, Liu J, et al. Up-Regulation of Bcl-2 in microvascular endothelial cells enhances intratumoral angiogenesis and accelerates tumor growth. *Cancer Res*. 2001;61:2183–8.

26. Platanias LC. The p38 mitogen-activated protein kinase pathway and its role in interferon signaling. *Pharmacol Ther*. 2003;98:129–42.

27. Hata T, Yamamoto H, Ngan CY, et al. Role of p21wafl/cip1 in effects of oxaliplatin in colorectal cancer cells. *Mol Cancer Ther*. 2005;4:1585–94.

28. Lee TK, Man K, Poon RT, Lo CM, Ng IO, Fan ST. Disruption of p53-p21/WAF1 cell cycle pathway contributes to progression and worse clinical outcome of hepatocellular carcinoma. *Oncol Rep*. 2004;12:25–31.

29. Martin J, Dufour JF. Tumor suppressor and hepatocellular carcinoma. *World J Gastroenterol*. 2008;14:1720–33.

30. Qin LF, Ng IO, Fan ST, Ng M. p21/WAF1, p53 and PCNA expression and p53 mutation status in hepatocellular carcinoma. *Int J Cancer*. 1998;79:424–8.

31. Hsu IC, Tokiwa T, Bennett W, et al. p53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines. *Carcinogenesis*. 1993;14:987–92.

32. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res*. 1994;54:3391–5.

33. Nakaji M, Yano Y, Ninomiya T, et al. IFN-alpha prevents the growth of pre-neoplastic lesions and inhibits the development of hepatocellular carcinoma in the rat. *Carcinogenesis*. 2004;25:389–97.

34. Puisieux A, Galvin K, Troalen F, et al. Retinoblastoma and p53 tumor suppressor genes in human hepatoma cell lines. *FASEB J*. 1993;7:1407–13.

35. Fan G, Ma X, Wong PY, Rodrigues CM, Steer CJ. p53 dephosphorylation and p21(Cip1/Wafl) translocation correlate with caspase-3 activation in TGF-beta1-induced apoptosis of HuH-7 cells. *Apoptosis*. 2004;9:211–21.

Resistance to cisplatin-induced apoptosis via PI3K-dependent survivin expression in a rat hepatoma cell line

HIROYUKI ASECHI, ETSURO HATANO, TAKASHI NITTA, MASAHARU TADA,
KEIKO IWAISAKO, NOBUYUKI TAMAKI, HIROMITSU NAGATA, MASATO NARITA,
ATSUKO YANAGIDA, IWAO IKAI and SHINJI UEMOTO

Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Abstract. Hepatocellular carcinoma (HCC) is known to be resistant to chemotherapy. Survivin, a member of the inhibitor of apoptosis proteins, is overexpressed in most cancers but is absent in most normal adult tissue. The aim of this study was to investigate whether expression of survivin contributes to resistance to cisplatin-induced apoptosis. We confirmed induction of survivin expression in hepatoma in the N-diethylnitrosamine (DEN) induced rat and in the rat hepatoma cell line (K-251). We examined cell proliferation after treatment with cisplatin (CDDP) in the presence and absence of siRNA or the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 to suppress survivin or PI3K/Akt, respectively. Survivin was expressed in DEN-induced rat HCC with RT-PCR and Western blotting. Expression of survivin was observed primary in the nuclei and in the cytoplasm with immunohistochemistry. However, survivin was not detected in non-tumor tissues. Expression of survivin was also observed primarily in the nuclei and in the cytoplasm of the K-251 rat hepatoma cell line. CDDP induced survivin expression, which was blocked by siRNA. LY294002 also attenuated survivin expression induced by CDDP. Our results indicate that survivin expression via PI3K contributes to resistance to CDDP-induced apoptosis in a rat hepatoma cell line.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, accounting for approximately 6% of all human cancers and 1 million deaths annually, with an estimated number of new cases of over 500,000 per year (1,2). Although the clinical management for early-stage HCC has

improved significantly, the prognosis of HCC is still extremely poor and is the third highest cause of cancer-related mortality. HCC exhibits resistance to chemotherapy, such as cisplatin (CDDP). Therefore, investigating and finding new treatment for HCC is important.

Cell proliferation is determined not only by the rate of cell growth but also by the rate of cell loss. Apoptosis is a major component of this regulatory process, which consists of 2 converging cascades 'death-receptor pathway' and 'mitochondrial pathway' (3,4). The death-receptor pathway is triggered by the interaction of death ligands of the tumor necrosis factor family with their cognate receptors that assemble the death-inducing signaling complex, resulting in activation of caspase-8. The mitochondrial pathway involves mitochondria that release apoptogenic factors, such as cytochrome c and Smac/DIABLO, in response to noxious stimuli.

Members of the IAP family are known to inhibit death receptors and the mitochondrial pathway by acting as endogenous inhibitors of caspases (4,5). Survivin is a bifunctional protein that controls cell division and inhibits apoptosis (6). The mechanism by which survivin inhibits apoptosis remains in dispute. While it is capable of binding to effector caspases under cell-free conditions, under more physiological conditions it inhibits apoptosis by binding to the second mitochondrial activator of caspases (Smac) (7). Survivin is present in only small amounts in terminally-differentiated normal cells, but is found to be prominently overexpressed in a variety of tumors, including cancers of the lung, stomach, breast, melanoma, pancreas, esophagus and colon (8-15). Overexpression of survivin has been identified as a negative prognostic factor in various cancer types (8,14,16), and is implicated in resistance to the induction of apoptosis by anti-cancer agents (17).

Despite increasing evidence in support of survivin as a promising target for molecular intervention, the mechanism of survivin overexpression in HCC and its implication in drug resistance remain to be investigated. Recent reports have demonstrated that survivin was up-regulated via the PI3K/Akt pathway in myeloid leukemia cells (18), endothelial cells (19), and prostate cancer cells (20). Furthermore, Akt is also activated in response to stress by UV irradiation and chemotherapy (21-23). Whether activation of Akt constitutes a physiological stress response that enables cancer cells to evade apoptosis by up-regulation of survivin remains to be demonstrated.

Correspondence to: Dr Etsuro Hatano, Department of Surgery, Graduate School of Medicine Kyoto University, 54 Kawahara-cho Shogoin Sakyo-ku Kyoto, 606-8507, Japan
E-mail: etsu@kuhp.kyoto-u.ac.jp

The purpose of this study was to clarify whether survivin is overexpressed in rat HCCs and if expression of survivin contributes to resistance to cisplatin-induced apoptosis in HCC cells. We investigated alterations in cell morphology and proliferation in HCC cells in the presence and absence of siRNA against survivin and a PI3K inhibitor.

Materials and methods

Rat DEN-induced HCC and HCC cell lines. Rat HCC was induced with N-diethylnitrosamine (DEN, Sigma, St. Louis, MO, USA). SD rats (4-week-old males, weighing 100 g; SLC, Hamamatsu, Japan) were provided with 100 ppm DEN *ad libitum* in drinking water for 8 weeks and were maintained for an additional 4 weeks without administration of DEN (24-26).

The DEN-induced rat HCC cell line K-251 was obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University. The cell line K-251 was grown in RPMI-1640 with L-glutamine (Invitrogen Corporation, Gibco 21875-034) supplemented with 10% FBS and 1% (w/v) penicillin/streptomycin (PC/SM) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were treated with CDDP (Nippon Kayaku, Tokyo, Japan) and LY294002 (Calbiochem-Novabiochem. Corp.). Cells were treated with the PI3K inhibitor LY294002 dissolved in DMSO.

Quantitative real-time RT-PCR. Total RNA was extracted from the liver tissues or cultured cells with TRIzol (Invitrogen, Tokyo, Japan). Total RNA (2 μg) was reverse transcribed to cDNA with an Omniscript RT kit (Qiagen, Tokyo, Japan) with oligo $(dT)_{8-12}$ primers (Invitrogen). Real-time PCR was performed with a QuantiTect SYBR Green PCR kit (Qiagen) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Tokyo, Japan). RT-PCR was performed with an initial step at 95°C for 15 min, followed by 40 cycles at 95°C for 20 sec, 59°C for 30 sec, and 72°C for 1 min with a final step at 72°C for 10 min. Two specific primers were used to identify survivin (Forward: 5'-TAAGCCACTTGTC CCAGCTT-3'; Reverse: 5'-CTCATCCACTCCCTTCCTC A-3') and G3PDH (Forward: 5'-ACCACAGTCCATGCCAT CAC-3'; Reverse: 5'-TCCACCACCCTGTTGCTGTA-3') The expression levels were calculated after conversion to numerical values by ABI PRISM 7700 SDS software and are expressed as ratios relative to the expression of G3PDH.

Western blot analysis. Frozen liver tissues or cultured hepatocytes were homogenized in lysis buffer containing 50 mM Tris-HCl, 2% sodium dodecylsulfate and 10% glycerol, and then boiled for 2 min. After the protein concentration in the sample was determined, 0.1% bromophenol blue and 6% 2-mercaptoethanol were added. Subsequently, 20 μg of each sample was electrophoresed and transferred onto PVDF membranes (Immobilon, Millipore, Billerica, MA). The membranes were blocked with 5% skim milk in PBS and incubated at 4°C overnight with the following primary antibodies and dilutions: anti-survivin antibody (no. sc-10811; Santa Cruz Biotechnology, Santa Cruz, CA; 1:200); anti-cleaved caspase-3 antibody (no. 9661; Cell Signaling Technology; 1:1000); anti-PARP antibody (no. 9542; Cell Signaling

Technology; 1:1000); anti-phospho Akt antibody (no. 9271; Cell Signaling Technology; 1:500); anti-XIAP antibody (no. 2042; Cell Signaling Technology; 1:1000); anti-α-tubulin antibody (no. CP06; Calbiochem; 1:1000); and anti-GAPDH antibody (no. sc-20357; Santa Cruz Biotechnology; 1:200). After washing, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase. Chemiluminescence detection was performed using ECL Western Blotting Detection Regents (Amersham, Buckinghamshire, UK), according to the manufacturer's instructions. The intensity of the band was quantified with National Institutes of Health Image (NIH-Image) and normalized to GAPDH as an internal control.

Immunohistochemistry. The specimens were fixed in 10% formalin and embedded in paraffin. They were subsequently deparaffinized and endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol at room temperature for 15 min. The antigen was retrieved by incubation in citric acid buffer at 90°C for 20 min. After blocking, the sections were incubated with primary antibody recognizing survivin (no. sc-10811; Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 dilution) overnight at 4°C and then with labeled polymer in an Envision+ System HRP (Dako, Tokyo, Japan) at room temperature for 1 h. The sections were examined after incubation with a Liquid DAB Substrate Chromogen System (Dako) for 2 sec.

siRNA for survivin transfection. siRNA with the sequence GGACUUAAGCACUGAGGAA targeting the survivin mRNA and the negative control sequence ATCCGCGCGAT AGTACGTA were purchased from B-Bridge International, Inc. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the reverse transfection method (27) using Opti-MEM (Invitrogen Corp., Gibco 31985-070) and Insulin-Transferrin-Selenium (ITS; Invitrogen Corp., Gibco 41400-045). Briefly, cells were plated at 20×10^4 cells/well in 6-well plates. Cells were transfected for 6 h, respectively. After transfection, cells were grown in RPMI-1640 with L-glutamine supplemented with ITS and 1% (w/v) penicillin/ streptomycin. Samples were collected 24 h after transfection. Experiments were repeated at least 3 times.

Cell proliferation assay. Cell proliferation was determined using the Cell Counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) to count living cells. Cells were cultured and treated in 24-well plates. At 0, 12, 24, 48 and 72 h, the CCK-8 was used to determine cell proliferation according to the manufacturer's instruction.

Statistical analysis. All data are expressed as the means ± SEM, and the statistical significance of differences between groups was assessed by Mann-Whitney U-test. P<0.05 were regarded as statistically significant.

Results

Expression of survivin in DEN-induced hepatoma and in the rat hepatoma cell line. We first used RT-PCR to investigate the level of survivin messenger RNA (mRNA) in DEN-induced
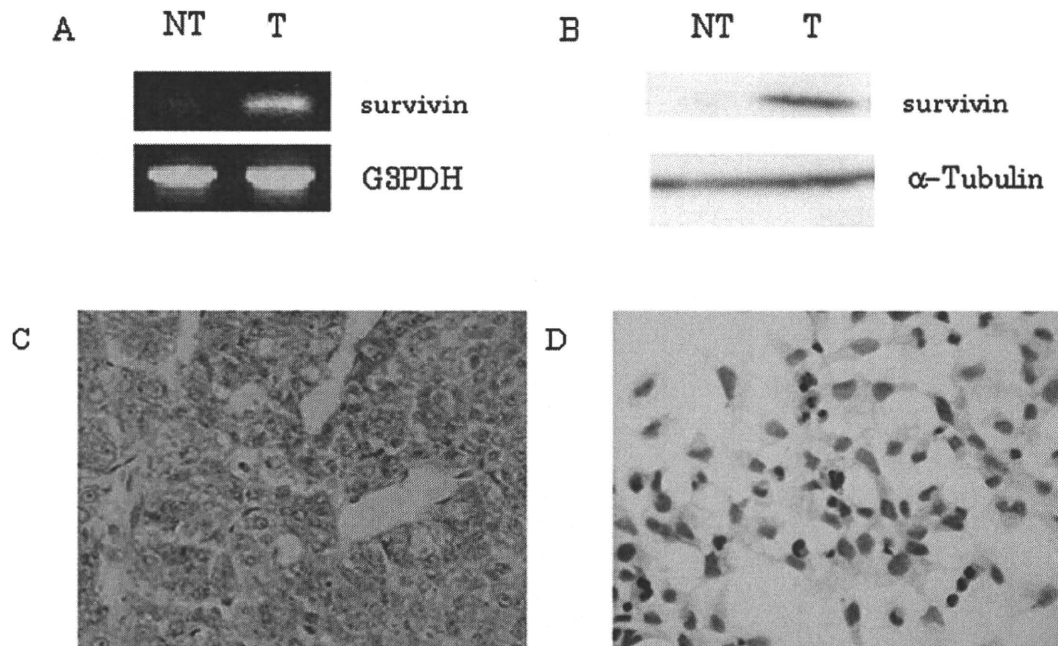
Figure 1. Expression of survivin in DEN-induced rat HCC. (A) RT-PCR of survivin mRNA in the non-tumor area of the rat liver and in rat HCC. (B) Western blot analysis of survivin protein in the non-tumor area of the rat liver and in rat HCC. (C) Immunohistochemical staining of survivin in rat HCC. Original magnification, x200. (D) Immunocytochemical staining of survivin in the DEN-induced rat hepatoma cell line K-251. Original magnification, x200.

rat HCC. A 75-bp fragment of survivin was detected in rat HCC (Fig. 1A). However, survivin mRNA was faintly detected in non-tumor tissues. Furthermore, a 16.5 kDa fragment of survivin protein was detected in rat HCC with Western blotting (Fig. 1B), while survivin was not detected in non-tumor tissues. We performed immunohistochemical staining to determine the cellular localization of survivin. Expression of survivin was observed primarily in the nuclei and in the cytoplasm of HCC cells. In contrast, survivin was not expressed in the adjacent non-cancer tissues (Fig. 1C). Furthermore, we examined the survivin expression in rat hepatoma cell line (K-251) derived from DEN-induced HCC using immuno-cytochemical staining. Expression of survivin was observed primarily in the nuclei and in the cytoplasm (Fig. 1D).

Down-regulation of survivin by siRNA in the rat hepatoma cell line. In rat HCC cells, the efficiency of transfection was about 80-90%, using red-labeled double-stranded RNA (dsRNA) oligomer by Lipofectamine 2000 (data not shown). Real-time PCR demonstrated that transfection with siRNA targeting survivin attenuated the expression of survivin mRNA to 68.1±8.5% of the expression level in cells transfected with negative control siRNA (Fig. 2A). Similarly, Western blotting showed that survivin expression was inhibited to 34.5±7.5% of control cells (Fig. 2B). The CCK-8 assay showed that the cell number decreased to 10.6±7.8% and 62.7±6.1% at 24 and 48 h after transfection, respectively, compared with control cells (Fig. 2C). These results indicate that transfection with siRNA significantly inhibited cell proliferation.

CDDP treatment up-regulates survivin expression and the silencing of RNA for survivin sensitizes CDDP-induced apoptosis in cancer cells. Treatment with CDDP induced over-expression of survivin protein in K-251 cells (189.6±26.1%

expression relative to cells without CDDP). Similarly, the induction of survivin was observed in cells transfected with negative control siRNA and siRNA against survivin (167.7±26.9%, 149.5±23.9%, respectively) (Fig. 3).

We examined fluorescence staining of nuclei using Hoechst 33342 and propidium iodide (PI) at 12 h after transfection with siRNA and treatment with CDDP. Irregular staining of the nuclei, nuclear fragmentation, and blebbing phenomenon were observed in cells treated with siRNA for survivin and CDDP, indicating apoptotic cell death (Fig. 4A). Percentage of apoptotic cells treated with negative control siRNA, negative control siRNA + CDDP, siRNA for survivin, siRNA for survivin + CDDP were 2.70±0.91%, 8.47±0.77%, 25.74±6.57%, 33.83±5.88%, respectively (Fig. 4B). The CCK-8 assay showed that 48 h after transfection with siRNA for survivin and treatment with CDDP cell proliferation was attenuated to 85.69±1.80% compared with cells transfected with negative control siRNA (Fig. 4C). Similarly, 48 h after transfection with siRNA for survivin and treatment with CDDP cell proliferation was attenuated to 78.15±1.12% compared with CDDP alone (data not shown).

siRNA for survivin induces caspase activation. Western blot analysis demonstrated that expression of cleaved caspase-3 was up-regulated in cells treated with siRNA for survivin compared to those treated with negative control siRNA. Similarly, siRNA for survivin increased the expression of cleaved caspase-3 in cells treated with CDDP, compared with those treated with negative control siRNA (Fig. 5A). Furthermore, expression of cleaved PARP was also up-regulated in cells treated with siRNA for survivin compared to those treated with negative control siRNA, in cells treated with/without CDDP (Fig. 5B). CDDP up-regulated the expression of phospho-Akt in cells treated with negative
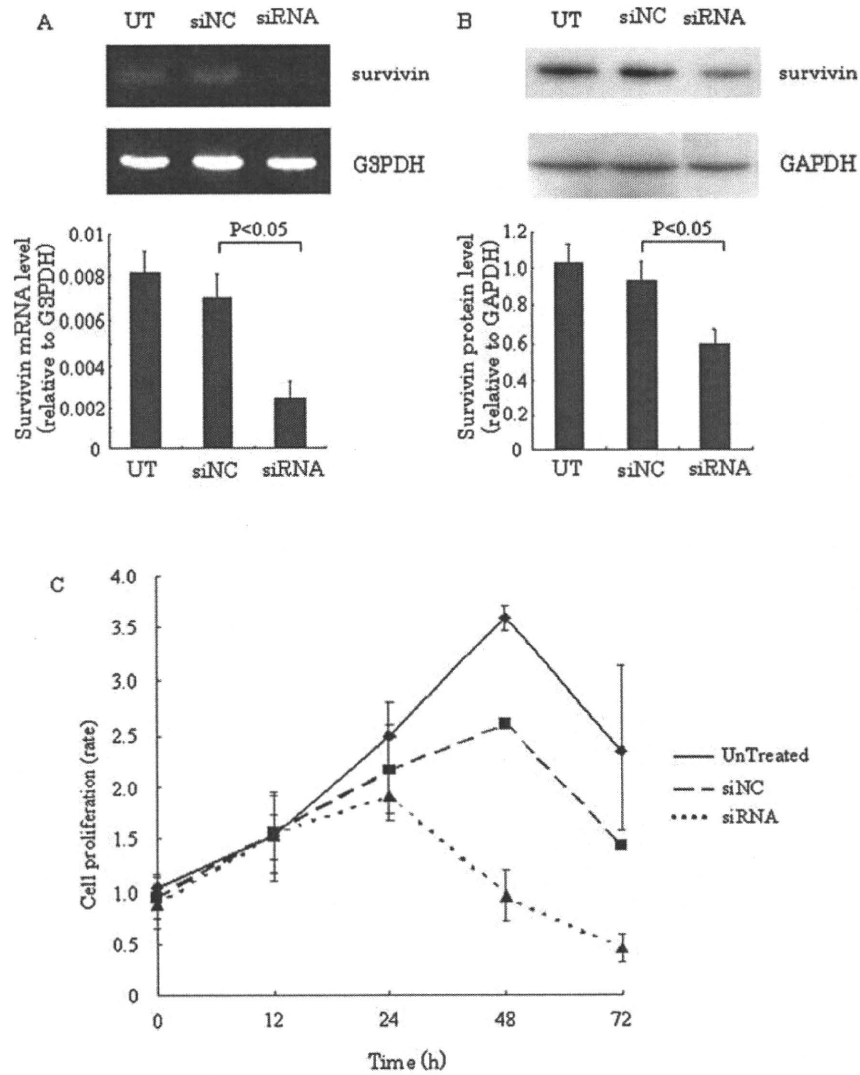
Figure 2. Down-regulation of survivin by siRNA in the DEN-induced rat hepatoma cell line. (A) Quantitative real-time PCR of survivin mRNA in un-treated HCC cells, in HCC cells treated with negative control siRNA and in HCC cells treated with siRNA for survivin. Data are expressed as the mean ± SEM (n=5). (B) Western blot analysis of survivin protein in un-treated HCC cells, in HCC cells treated with negative control siRNA and in HCC cells treated with siRNA for survivin. Data are expressed as the mean ± SEM (n=4). (C) CCK-8 cell proliferation assay of un-treated HCC cells, HCC cells treated with negative control siRNA, and HCC cells treated with siRNA for survivin. Data are expressed as the mean ± SEM (n=3).
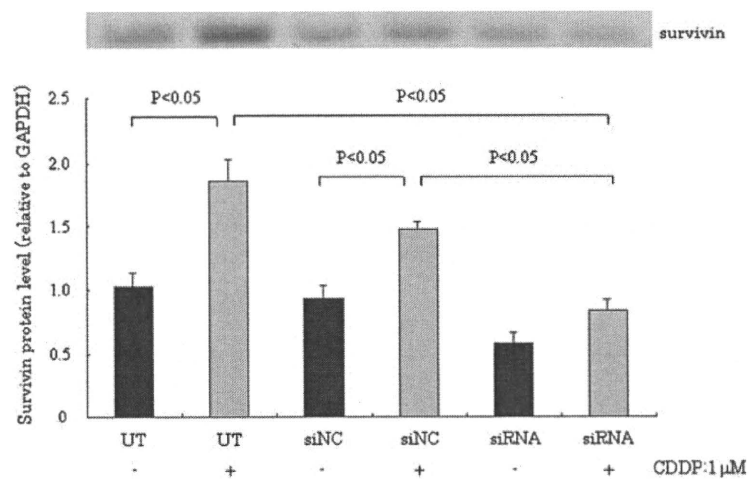

Figure 3. CDDP up-regulates survivin expression. Western blot analysis of survivin protein in untreated HCC cells, HCC cells treated with CDDP (1 μM), HCC cells treated with negative control siRNA (50 μM), HCC cells treated with negative control siRNA (50 μM) + CDDP (1 μM), HCC cells treated with siRNA for survivin (50 μM), and HCC cells treated with siRNA for survivin (50 μM) + CDDP (1 μM). Data are expressed as the mean ± SEM (n=4).
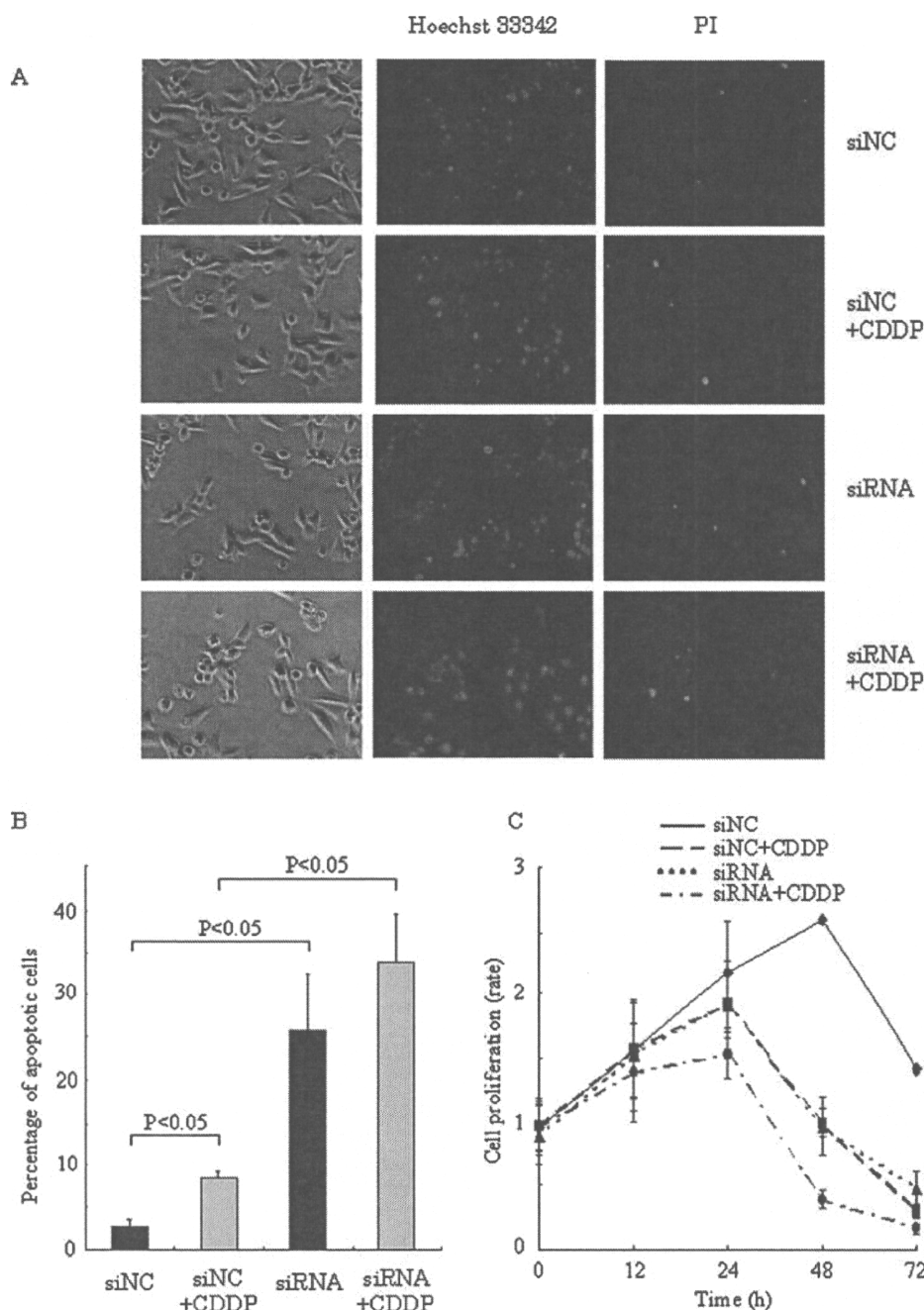
Figure 4. Survivin silencing and CDDP induce apoptosis. (A) Fluorescence staining of nuclei using Hoechst 33342 and propidium iodide (PI), 12 h after treatment with negative control siRNA (50 μM), negative control siRNA (50 μM) + CDDP (1 μM), siRNA for survivin (50 μM), and siRNA for survivin (50 μM) + CDDP (1 μM). (B) Percentage of apoptotic cells, 12 h after treatment. Data are expressed as the mean ± SEM (n=4). (C) CCK-8 cell proliferation assay of HCC cells. Data are expressed as the mean ± SEM (n=3).

control siRNA or siRNA for survivin. However, the expression level was similar between cells treated with negative control siRNA and siRNA for survivin, indicating that survivin did not affect phosphorylation of Akt in cells in the absence of treatment with CDDP (Fig. 5C). There was no significant difference of XIAP expression between cells treated with negative control siRNA or siRNA for survivin (Fig. 5D).

Regulation of survivin by PI3K/Akt. Rat HCC cells were treated with 25 μM LY294002, phosphoinositide-3 kinase inhibitor, to elucidate the relationship between survivin and PI3K. LY294002 blocked the expression of survivin (Fig. 6A).

Similarly, expression of phospho-Akt was down-regulated in cells treated with LY294002 compared to those with treated with DMSO, in cells treated with/without CDDP (Fig. 6B). On the other hand, expression of XIAP was up-regulated in cells treated with LY294002 compared to those with treated with DMSO, and LY294002 increased expression of XIAP in cells treated with CDDP (Fig. 6C).

Discussion

We confirmed overexpression of survivin in DEN-induced rat HCC *in vivo* and in DEN-induced rat HCC cells *in vitro*.