[21]. According to our findings, the washout pattern can be useful for identifying p-HCC or ICC. However, distinguishing p-HCC from some ICCs showing diffuse hyperenhancement in the arterial phase and subsequent washout is difficult.

In our present study, 15 (78.9%) of the 19 ICCs showed an intratumoral artery in the arterial phase. Although we occasionally recognized vessels running into the tumor, to the best of our knowledge, no previous reports have described the presence of intratumoral arteries in ICC and p-HCC. In our study, ICCs were able to be differentiated from p-HCCs based on the finding of an intratumoral artery ($p = 0.037$), according to multivariate binary logistic regression analysis. Based on our results, the presence of an intratumoral artery in the arterial phase on contrast-enhanced CT could be a predictive finding for reliable differentiation of ICC from p-HCC. Few reports have described intratumoral arteries of ICC being demonstrated on contrast-enhanced CT. One study showed intratumoral arteries of the ICC identified immediately after the injection of contrast material for CT during hepatic arteriography [22]. Furthermore, that study indicated that tumor enhancement gradually spreads from each intratumoral artery [22]. Infiltrating replacement is an inherent growth feature of ICC, with the surrounding liver gradually incorporated into the tumor as it grows [23]. In this process, the blood vessel is not destroyed by tumor cells and is retained inside. By contrast, HCC shows fibrous encapsulation or compressive growth [24]. With such growth, blood vessels are pressed to the outside of the tumor. Our cases also showed these features (Fig. 2). Such differences in growth type may be related to differences in intratumoral arteries between ICC and HCC. No significant difference was seen between ICC and p-HCC in regard to intratumoral portal veins, intratumoral hepatic veins, or portal vein tumor thrombus. We supposed that intratumoral artery was retained within the ICC rather than portal or hepatic veins because of the stiffness of the arterial wall.

The results of this study have revealed features that allow ICC and p-HCC to be distinguished based on findings from contrast-enhanced CT. In clinical practice, contrast-enhanced CT is a useful diagnostic method to distinguish ICC from p-HCC, since results of tumor marker levels and tissue biopsy are difficult and often indeterminate. The optimal treatment for ICC is complete tumor resection, including lymph node removal [25–27]. In cases of HCC, the treatment modality of choice depending on the degree of cirrhosis is complete resection, topical therapy including radiofrequency ablation or liver transplantation. If the patient has advanced cirrhosis or advanced HCC, then treatments such as transarterial chemoembolization hepatic arterial infusion chemotherapy and systemic chemotherapy are applicable [28, 29]. Because misdiagnosis of ICC as HCC can lead to inadequate medical care, our identification of characteristic findings for ICC may have important practical value in attaining a correct diagnosis.

This study has several limitations that must be considered when interpreting the results. First, our study might have included some degree of selection bias, as we retrospectively analyzed only those patients with ICC or p-HCC who underwent contrast-enhanced CT and hepatic surgery. The absence of the well- and moderately differentiated subtypes of HCC in this study is an important limitation in interpreting our results. Additionally, the numbers of ICCs and p-HCCs were relatively small, because the patient group was limited to those with a pathologic diagnosis determined by surgery. Finally, most tumors were relatively large, and the findings in our results may not be observed in smaller sized tumors.

In conclusion, the presence of an intratumoral artery during arterial phase on enhanced CT is valuable in differentiating between ICC and p-HCC, as is the washout pattern. This new finding may facilitate correct diagnosis and more timely selection of appropriate treatment strategies.

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Conflict of interest. The authors declare that they have no conflicts of interest.

References

1. Kham SA, Thomas HC, Davidson BR, et al. (2005) Cholangiocarcinoma. Lancet 366:1303–1314
2. Patel T (2001) Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. Hepatology 33:1353–1357
3. Singh P, Patel T (2006) Advances in the diagnosis, evaluation and management of cholangiocarcinoma. Curr Opin Gastroenterol 22:294–299
4. Shaib Y, El-Serag HB (2004) The epidemiology of cholangiocarcinoma. Semin Liver Dis 24:115–125
5. Valls C, Guma A, Puig I, et al. (2000) Intrahepatic peripheral cholangiocarcinoma: CT evaluation. Abdom Imaging 25:490–496
6. Bruix J, Sherman M (2005) Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. Hepatology 42:1208–1236
7. Khan SA, Davidson BR, Goldin R, et al. (2002) Guidelines for the diagnosis and management of cholangiocarcinoma. Gut 61:1657–1669
8. Choi BI, Han JK, Shin YM, et al. (1995) Peripheral cholangiocarcinoma: comparison of MRI with CT. Abdom Imaging 20:357–360
9. Fan ZM, Yamashita Y, Harada M, et al. (1993) Intrahepatic cholangiocarcinoma: spin-echo and contrast-enhanced dynamic MR imaging. Am J Roentgenol 161:313–317
10. Hirohashi S, Ishak KG, Kojiro M, et al. (2000) Hepatocellular carcinoma. In: Hamilton SRAL (ed) *World Health Organization classification of tumours pathology and genetics of tumours of the digestive system.* Lyon: IARC Press, pp 159–172
11. Nakanuma Y, Leong AS-Y, Sripa B, et al. (2000) Intrahepatic cholangiocarcinoma. In: Hamilton SR (ed) *World Health Organization classification of tumours Pathology and genetics of tumours of the digestive system.* Aaltonen: IARC Press, pp 173–180

12. Lee WJ, Lim HK, Jang KM, et al. (2001) Radiologic spectrum of cholangiocarcinoma: emphasis on unusual manifestations and differential diagnosis. Radiographics 21:97–116
13. Han JK, Choi BI, Kim AY, et al. (2002) Cholangiocarcinoma: pictorial essay of CT and cholangiographic findings. Radiographics 22:173–187
14. Baheti AD, Tirumani SH, Rosenthal MH, Shinagare AB, Ramaiya NH (2014) Diagnosis and management of intrahepatic cholangiocarcinoma: a comprehensive update for the radiologist. Clin Radiol 69:e463–e470
15. Kim TK, Choi BI, Han JK, et al. (1997) Peripheral cholangiocarcinoma of the liver: two-phase helical CT findings. Radiology 204:539–543
16. Olnes MJ, Erlich R (2004) A review and update on cholangiocarcinoma. Oncology 66:167–179
17. Choi BI, Han JK, Shin YM, et al. (1995) Peripheral cholangiocarcinoma: comparison of MRI with CT. Abdom Imaging 20:357–360
18. Valls C, Guma A, Puig I, et al. (2000) Intrahepatic peripheral cholangiocarcinoma: CT evaluation. Abdom Imaging 25:490–496
19. Lim JH (2003) Cholangiocarcinoma: morphologic classification according to growth pattern and imaging findings. Am J Roentgenol 181:819–827
20. Ros PR, Buck JL, Goodman ZD, et al. (1988) Intrahepatic cholangiocarcinoma: radiologic-pathologic correlation. Radiology 167:689–693
21. Bruix J, Sherman M (2011) American association for the study of liver diseases. Management of hepatocellular carcinoma: an update. Hepatology 53:1020–1022
22. Miura F, Okazumi S, Takayama W, et al. (2004) Hemodynamics of intrahepatic cholangiocarcinoma: evaluation with single-level dynamic CT during hepatic arteriography. Abdom Imaging 29:467–471
23. Kozaka K, Sasaki M, Fujii T, et al. (2007) A subgroup of intrahepatic cholangiocarcinoma with an infiltrating replacement growth pattern and a resemblance to reactive proliferating bile ductules: bile ductular carcinoma. Histopathology 51:390–400
24. Ueda K, Terada T, Nakanuma Y, et al. (1992) Vascular supply in adenomatous hyperplasia of the liver and hepatocellular carcinoma: a morphometric study. Hum. Pathol 23:619–626
25. Singh P, Patel T (2006) Advances in the diagnosis, evaluation and management of cholangiocarcinoma. Curr Opin Gastroenterol 22:294–299
26. Jarnagin WR, Shoup M (2004) Surgical management of cholangiocarcinoma. Semin Liver Dis 24:189–199
27. Nagorney DM, Donohue JH, Farnell MB, et al. (1993) Outcomes after curative resections of cholangiocarcinoma. Arch Surg 128:871–879
28. Bruix J, Sherman M, Llovet JM, et al. (2001) Clinical management of hepatocellular carcinoma: conclusions of the Barcelona-2000 EASL conference. J Hepatol 35:421–430
29. Hertl M, Cosimi AB (2005) Liver transplantation for malignancy. Oncologist 10:269–281

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Original Article

Synthetic lethal interaction of combined CD26 and Bcl-xL inhibition is a powerful anticancer therapy against hepatocellular carcinoma

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Aim: CD26 is a membrane glycoprotein that has multiple functions, including dipeptidyl peptidase IV activity. CD26 expression varies in different tumor types, and its role in tumor growth in hepatocellular carcinoma (HCC) remains unclear.

Methods: CD26 expression levels were examined in resected HCC and surrounding non-cancerous lesions. The effect of CD26 knockdown on the cellular proliferation of HepG2 or Huh7 cells, both of which highly express CD26, was studied *in vitro*.

Results: CD26 mRNA expression levels were significantly increased in HCC compared with their surrounding non-cancerous lesions. We confirmed that various HCC cell lines, especially HepG2 and Huh7 cells, showed high expression levels of CD26. siRNA-mediated knockdown of CD26 suppressed hepatoma cell growth *in vitro*. CD26 knockdown induced cell cycle arrest through the upregulation of Cip/Kip family proteins, p21 in HepG2 cells and p27 in Huh7 cells. CD26 knockdown did not affect apoptosis, but it increased expressions of the pro-apoptotic proteins Bim and Bak and the anti-apoptotic protein Bcl-xL, suggesting an addiction of CD26 knockdown cells to Bcl-xL for survival. We thus treated CD26 knockdown cells with ABT-737, a Bcl-xL/-2/-w inhibitor, and observed that the synthetic lethal interaction of combined Bcl-xL and CD26 inhibition induced significant apoptosis and impaired cellular viability.

Conclusion: CD26 mRNA was overexpressed in HCC, and its inhibition suppressed cellular proliferation through cell cycle arrest. The combined use of CD26 knockdown with a Bcl-xL inhibitor further elicited substantial apoptosis and therefore may serve as a powerful anticancer combination therapy against HCC.

Key words: ABT-737, apoptosis, CD26, cell cycle, hepatocellular carcinoma

INTRODUCTION

CD26 IS A membrane glycoprotein widely expressed in various tissues, such as T lymphocytes and epithelial and endothelial cells.[1,2] The CD26 molecule consists of a cytoplasmic domain, transmembrane domain and extracellular domain, which contains dipeptidyl peptidase-4 (DPPIV). Currently, DPPIV activity is one of the most well-known functions of CD26 because DPPIV degrades glucagon-like peptide-1 and many DPPIV inhibitors are used as drugs against type 2 diabetes.[3] However, in addition to DPPIV activity, CD26 has other functions, such as a receptor, co-stimulatory protein and adhesion molecule.[4] CD26 expression levels are altered in various types of cancers. CD26 overexpression is observed in prostate cancer,[5] brain glioma,[6] thyroid carcinoma[7] and malignant mesothelioma.[8] In contrast, CD26 is downregulated in various cancers, including ovarian cancer[9] and melanoma.[10] The role of CD26 in cancer biology also varies and appears to be tumor type dependent.[2] Although CD26

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expression is localized to the bile canalicular plasma membrane of the normal liver, its distribution pattern is altered in hepatocellular carcinoma (HCC). In addition, some HCC cases display an aberrant increase in DPPIV activity.[11] However, the role of CD26 in liver carcinogenesis remains unclear.

Hepatocellular carcinoma is the third leading cause of cancer mortality worldwide. However, few therapeutic options against advanced HCC exist, especially for patients with metastasis outside the liver. Sorafenib is the only US Food and Drug Administration-approved molecularly targeted drug against HCC that has demonstrated survival prolongation in clinical trials.[12,13] To date, various types of molecularly targeted drugs, including vascular endothelial growth factor, vascular endothelial growth factor receptor, epidermal growth factor receptor, mammalian target of rapamycin, DR5 and XIAP, were investigated but none of them prolonged survival.[14] In this aspect, new therapeutic targets are needed to conquer HCC.

In this study, we found that CD26 mRNA levels were frequently increased in HCC and that their levels were positively correlated with tumor size. CD26 inhibition decreased hepatoma cell growth through the induction of cell cycle arrest but not apoptosis. Although CD26 inhibition increased the expression of pro-apoptotic proteins, their pro-apoptotic effect was not exerted due to the counteracting increase in the anti-apoptotic protein Bcl-xL. The combined inhibition of CD26 and Bcl-xL caused a synthetic lethal pro-apoptotic effect in hepatoma cells. This is the first report to reveal the therapeutic potential of CD26 inhibition in HCC, and our current results propose a novel potent combination therapy against HCC.

METHODS

Human samples

HEPATOCELLULAR CARCINOMA SAMPLES and surrounding non-cancerous liver samples were obtained from 71 patients undergoing surgical resection for HCC at Osaka University Hospital. The average patient age was 62.7 ± 10.7 years old, and 56 patients were male. Among the 71 patients, 17 were positive for hepatitis B surface antigen (HBsAg) and negative for hepatitis C virus (HCV) antibody, 33 were negative for HBsAg and positive for HCV antibody, and three were positive for both. The average maximum diameter of HCC was 53.0 ± 37.8 mm. For immunohistochemistry using anti-CD26 antibody (Novus Biologicals, Littleton, CO, USA), formaldehyde-fixed HCC were obtained from 12 patients undergoing surgical resection at Osaka University Hospital. Detection of immunolabeled proteins was performed using an avidin–biotin complex of Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Written informed consent was obtained from all patients according to a protocol approved by the Institutional Research Board of Osaka University Hospital.

Real-time reverse transcription polymerase chain reaction (RT–PCR)

Total RNA isolated from liver tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was reverse transcribed and subjected to real-time RT–PCR as previously described.[15] The mRNA expression levels of the following genes were quantified using TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA): human CD26 (assay ID: Hs00175210_m1), human p21 (assay ID: Hs00355782_m1), human β-actin (assay ID: Hs99999903_m1) and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; assay ID: Hs02758991_g1). The transcript levels are presented as fold change relative to GAPDH levels unless otherwise indicated.

Western blot analysis

Liver tissue was lysed with a lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [SDS], protease inhibitor cocktail [Nacalai Tesque, Kyoto, Japan], phosphatase inhibitor cocktail [Nacalai Tesque], phosphate-buffered saline, pH 7.4). Equal amounts of protein were electrophoretically separated using SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membrane. For immunodetection, the following antibodies were used: anti-CD26 (R&D Systems, Minneapolis, MN, USA), anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA), anti-p15 (Cell Signaling Technology), anti-p16 (Becton Dickinson, San Jose, CA, USA), anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p27 (Cell Signaling Technology), anti-Bak (Millipore, Billerica, MA, USA), anti-Bax (Cell Signaling Technology), anti-Bim (Cell Signaling Technology), anti-Bid (Cell Signaling Technology), anti-Mcl-1 (Cell Signaling Technology), anti-Bcl-xL (Santa Cruz Biotechnology) and cleaved caspase-3 (Cell Signaling Technology). Detection of immunolabeled proteins was performed using a chemiluminescent substrate (Thermo Fisher Scientific). Protein expression levels were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized by expression levels of GAPDH.

Cell cultures

Cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Sigma-Aldrich, St Louis, MO, USA). ABT-737, which inhibits Bcl-xL, Bcl-2 and Bcl-w, was purchased from Selleckchem (Houston, TX, USA) and used to treat Huh7 cells for 24 h as described previously.[16] Sitagliptin and vildagliptin were purchased from Viovision (Milpitas, CA, USA) and Santa Cruz Biotechnology, respectively. Measurements of caspase-3 and -7 activity and determination of cell viability by WST-1 assay were also described previously.[17] Lactate dehydrogenase (LDH) activity was measured by LDH-Cytotoxic Test (Wako, Osaka, Japan) according to the manufacturer's instructions. In some experiments, cells were transfected Silencer Select siRNA (Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's protocol.

Flow cytometry analysis

For the detection of surface CD26, cells were incubated with antigen-presenting cell-conjugated human anti-CD26 antibody (Miltenyi Biotec, Auburn, CA, USA) and then subjected to flow cytometric analysis. Flow cytometric analysis was performed using a FACS Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

To detect apoptotic cells, the cells were suspended in annexin V binding buffer (Becton Dickinson). Next, the cells were stained with annexin V and propidium iodide (PI; Becton Dickinson) and subjected to flow cytometric analysis. Annexin V^+ PI^- cells were regarded as apoptotic. Cell cycle assay was examined by CycleTest (Becton Dickinson) according to the manufacturer's protocol.

Statistics

Data are expressed as the mean ± standard deviation. Statistical analyses were performed using Student's unpaired *t*-test unless otherwise indicated. $P < 0.05$ was considered statistically significant.

RESULTS

CD26 mRNA levels are increased in HCC

WE FIRST EXAMINED the levels of CD26 mRNA in tumor tissue and surrounding non-cancerous lesions in HCC patients who underwent surgical resection. CD26 mRNA levels in tumor tissues were significantly increased compared with their adjacent non-cancerous lesions (Fig. 1a). To examine the significance of CD26 upregulation in HCC, tumor specimens were divided into two groups based on CD26 mRNA expression levels, and several clinical parameters were compared between the two groups (Table S1). Tumor size in the high CD26 mRNA expression group was signifi-

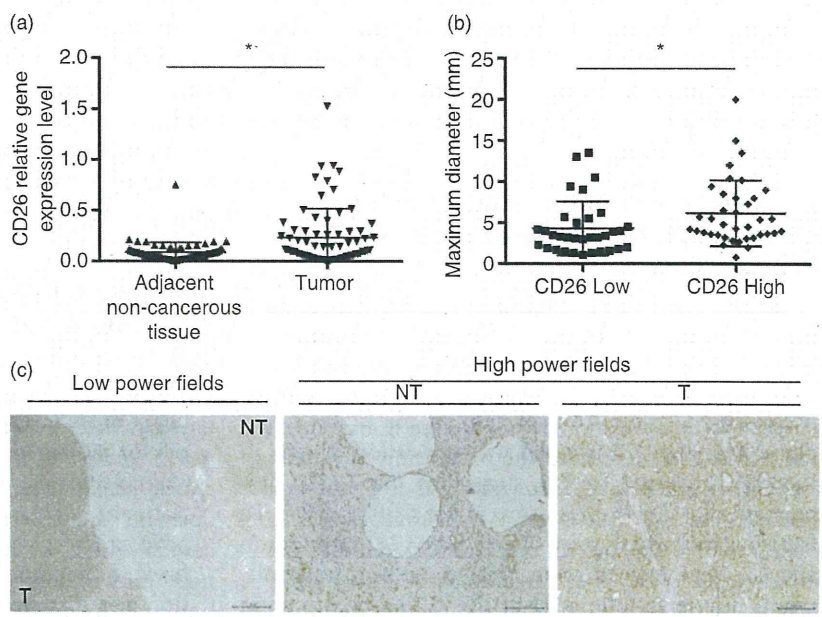

Figure 1 CD26 expression is frequently upregulated in hepatocellular carcinomas (HCC) and positively correlated with tumor size. (a) CD26 mRNA levels were examined in HCC and adjacent non-cancerous liver tissues. Relative CD26 mRNA levels normalized to β-actin mRNA expression (*$P < 0.05$ by Student's paired *t*-test). (b) Maximum diameter of HCC (*$P < 0.05$). HCC were divided into two groups based on the CD26 mRNA levels: CD26 high group and CD26 low group. (c) CD26 was labeled in the paraffin-embedded liver sections with HCC. Representative pictures of stained a section are shown. T and NT stand for tumor and non-tumor, respectively.

cantly larger than the low CD26 mRNA expression group (Fig. 1b), suggesting that CD26 overexpression may be involved in HCC cell growth. To evaluate the protein expression of CD26 in HCC, we stained 12 sections of formaldehyde-fixed HCC with anti-CD26 antibody. All examined HCC were stained with anti-CD26 antibody to varying degrees (Fig. 1c).

CD26 inhibition suppresses the growth of hepatoma cell lines

Then, we evaluated CD26 expression levels in a variety of hepatoma cell lines (Hep3B, HepG2, HLE, Huh7 and PLC/PRF/5). Based on the gene expression data from the Cancer Cell Line Encyclopedia (CCLE), HepG2 and Huh7 cells have the highest expression of CD26 among these hepatoma cell lines.[18] Consistent with these data, we used flow cytometry to confirm that CD26 was expressed on the surface of these two hepatoma cell lines (Fig. 2a). Based on these data, we selected them for further *in vitro* analysis and studied the role of CD26 in their cellular proliferation using negative control or two different CD26 siRNA oligos. The transfection of each CD26 siRNA oligo efficiently reduced CD26 expression at the mRNA and protein levels in both HepG2 (Fig. 2b,c) and Huh7 cells (Fig. 2d,e). Upon CD26 knockdown, cellular proliferation, as assessed by WST-1 assay, was significantly suppressed in both HepG2 (Fig. 2f) and Huh7 cells (Fig. 2g), indicating that CD26 was required for hepatoma cell growth and therefore can serve as a therapeutic target. To investigate whether decreased DPPIV activity is responsible for the observed anticancer effects of CD26 inhibition, we treated hepatoma cells with DPPIV inhibitors, sitagliptin or vildagliptin, instead of CD26 knockdown. However, inhibition of DPPIV activity failed to suppress hepatoma cell growth (Fig. 2h).

CD26 knockdown induces G0/G1 cell cycle arrest through the upregulation of Cip/Kip family proteins

To elucidate how CD26 inhibition impairs hepatoma cell growth, we studied the effect of CD26 knockdown on the cell cycle. Flow cytometric analysis revealed that siRNA-mediated CD26 knockdown in HepG2 cells decreased the proportion of cells in the S and G2/M phase and slightly increased the proportion of cells in the G0/G1 phase (Fig. 3a), suggesting that CD26 suppression causes cell cycle arrest at the G0/G1 phase. To further clarify which stage of cell cycle CD26 inhibition affects, HepG2 cells were treated with nocodazole, which arrests mitotic cells at the G2/M phase, upon transfection with the negative control or CD26 siRNA oligos. While nocodazole treatment dramatically increased the number of G2/M phase cells in control siRNA-transfected cells (Fig. 3a), this effect was greatly attenuated in CD26 siRNA-transfected cells, which maintained an increased number of G0/G1 phase cells (Fig. 3a). Similar observations were obtained using CD26 siRNA-transfected Huh7 cells treated with nocodazole (Fig. 3b). Taken together, these findings indicated that CD26 inhibition induced cell cycle arrest at the G0/G1 phase, leading to the suppression of cell growth. To address the mechanism of CD26 inhibition-mediated cell cycle arrest, we examined the change in expression levels of Ink4 family proteins, p15 and p16, and Cip/Kip family proteins, p21 and p27, upon CD26 knockdown because these proteins negatively control the G1/S checkpoint. Two different siRNA oligos targeting the CD26 gene individually increased p21 expression in HepG2 cells (Fig. 3c), suggesting its potential involvement in G0/G1 cell cycle arrest. To pursue this possibility, we co-transfected CD26 and p21 siRNA oligos and examined their effect on the cell cycle. We first confirmed that their co-transfection simultaneously reduced CD26 and p21 expression (Fig. 3d). Although CD26 knockdown caused cell cycle arrest at the G0/G1 phase, CD26 and p21 knockdown restored the S and G2/M cell populations to approximately the same level observed with p21 knockdown alone (Fig. 3e). Importantly, in accordance with this finding, cell growth impairment upon CD26 knockdown was completely rescued by additional p21 knockdown (Fig. 3e). On the other hand, our Western blot analysis showed that p21 protein expression was not detected in Huh7 cells but CD26 knockdown increased p27 expression (Fig. 3f). Furthermore, p27 knockdown rescued impaired cellular proliferation induced by CD26 inhibition (Fig. 3g). Collectively, CD26 knockdown induced cell cycle arrest at the G0/G1 phase through the upregulation of Cip/Kip family proteins.

Synthetic lethal interaction of combined CD26 and Bcl-xL inhibition induces substantial hepatoma cell apoptosis

Although CD26 appears to be a promising therapeutic target in HCC, CD26 inhibition may carry the potential risk of aiding in the transition of cancer cells from a chemo-sensitive replicative status to a chemo-resistant dormant status. To compensate for this potential adverse effect, we attempted to identify the "Achilles' heel" for hepatoma cells in the context of CD26 inhibition and discovered that CD26 inhibition upregulated

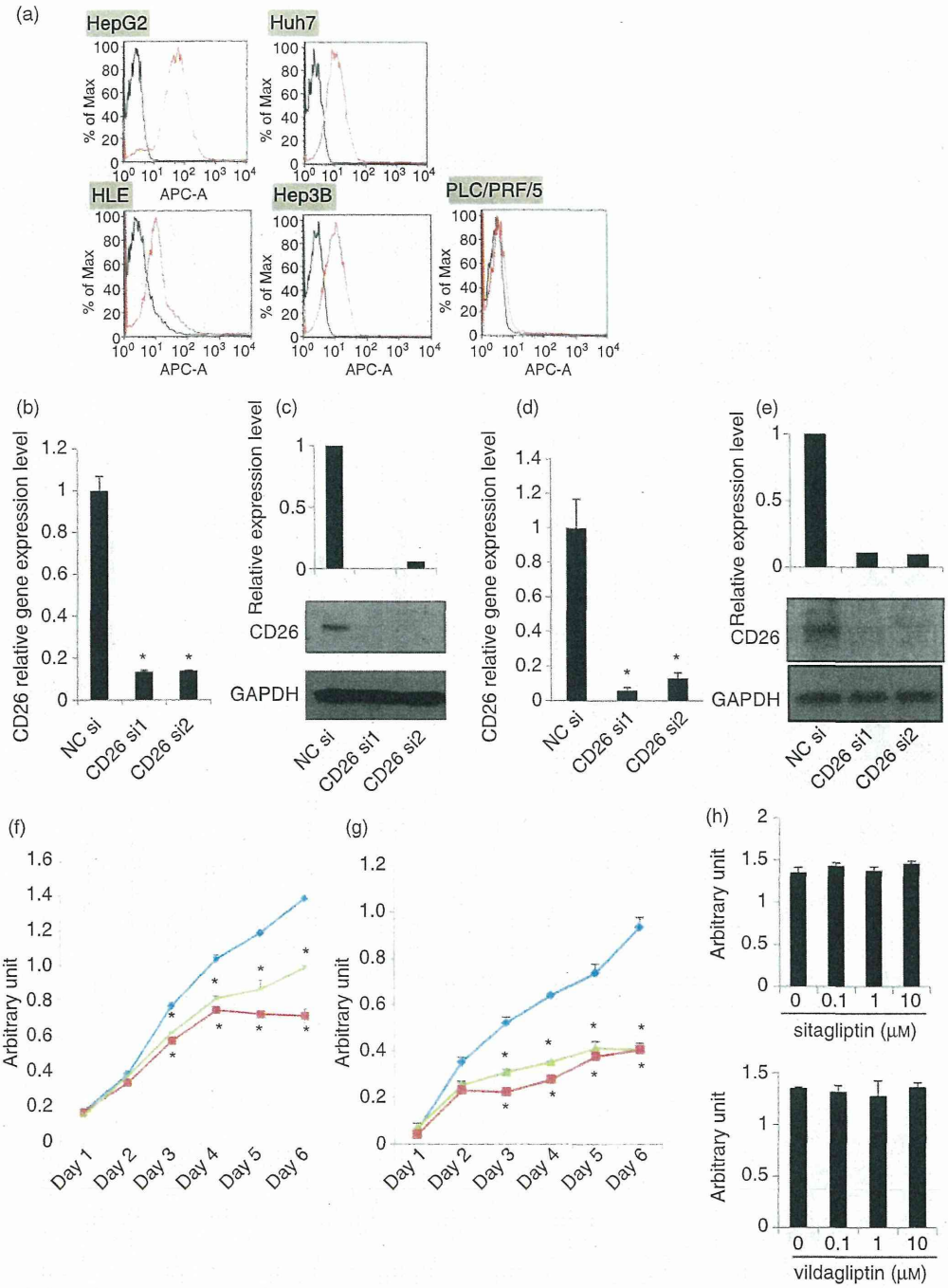

Figure 2 CD26 knockdown inhibits hepatoma cell growth. (a) CD26 expression levels were examined by flow cytometry in various hepatoma cell lines. (b–g) Two different siRNA oligos targeting CD26 or a negative control siRNA oligo were individually transfected into (b,c,f) HepG2 or (d,e,g) Huh7 cells. (b,d) CD26 mRNA (*$P < 0.05$ vs negative control siRNA). Western blotting of CD26 protein (lower panels of [c,e]) and bar charts showing its protein levels normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels (uppers panels of [c,e]). (f,g) Cell proliferation examined by WST-1 assay. NC and si indicate negative control and siRNA, respectively. (h) HepG2 cells were treated with sitagliptin or vildagliptin for 72 h and cell proliferation examined by WST-1 assay. (a) —, Iso type; —, anti-CD26Ab. (f,g) —, NC si; , CD26 si1; —, CD26 si2.

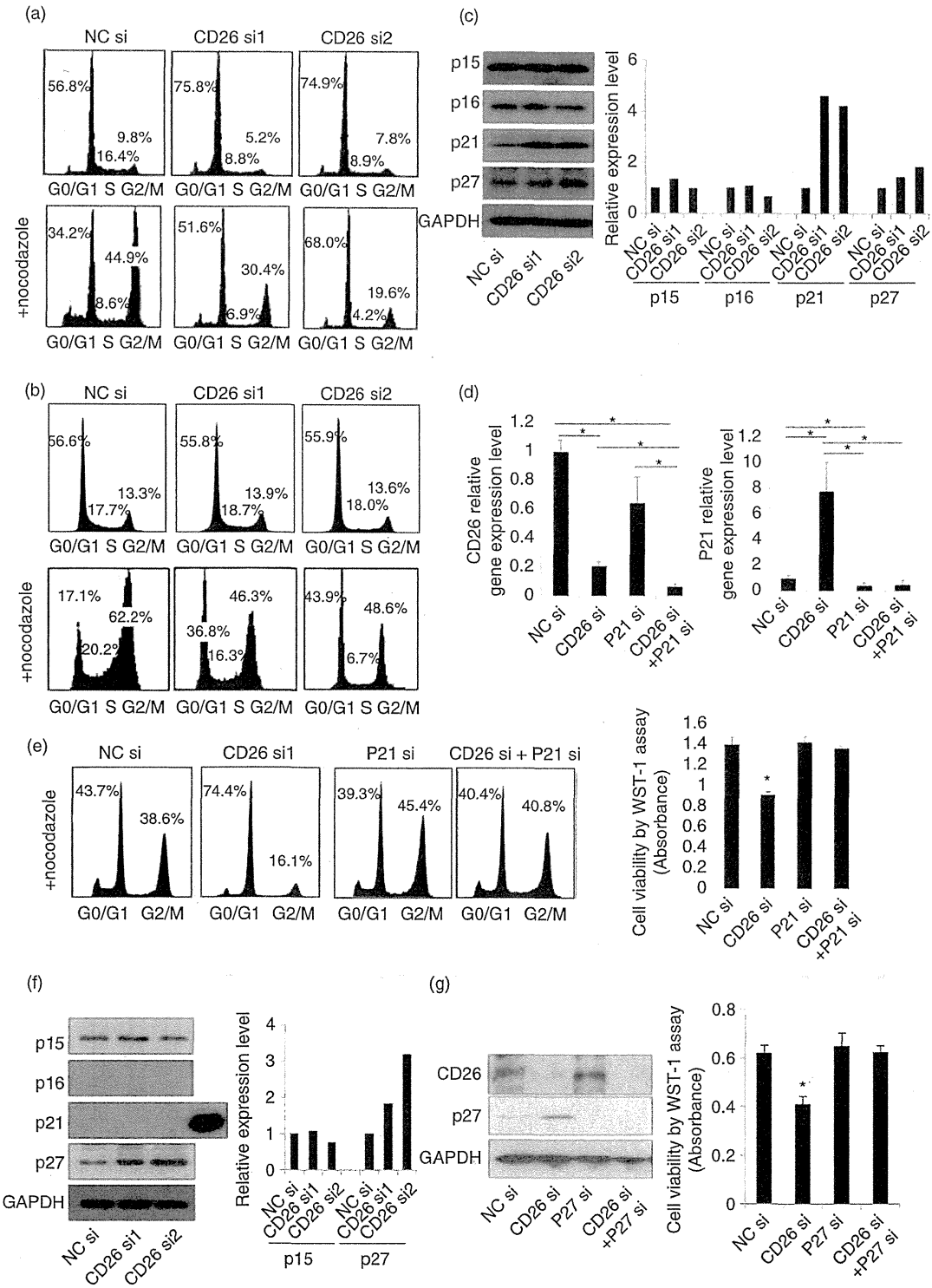

Figure 3 CD26 knockdown induces cell cycle arrest at the G0/G1 phase via upregulation of Cip/Kip family proteins. (a–c) Two different siRNA oligos targeting CD26 or a negative control siRNA oligo were individually transfected into (a,c) HepG2 or (b) Huh7 cells. The cell cycle was analyzed by flow cytometry 24 h after incubation with or without 500 ng/mL nocodazole in (a) HepG2 and (b) Huh7 cells. Western blotting of cell cycle-related proteins in HepG2 cells (left panel of [c]) and bar charts showing the protein levels normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels (right panel of [c]). (d,e) HepG2 cells were co-transfected with two different siRNA oligos targeting CD26 and p21. Relative mRNA levels of CD26 (left panel of [d]) and P21 (right panel of [d]) (*$P < 0.05$). Cell cycle analysis 24 h after incubation with 500 ng/mL nocodazole (left panel of [e]). Cell proliferation as measured by WST-1 assay (right panel of [e]) (*$P < 0.05$ vs all). (f) Two different siRNA oligos targeting CD26 or a negative control siRNA oligo were individually transfected into Huh7 cells. Western blotting of cell cycle-related proteins (left panel) and bar charts showing the protein levels normalized by GAPDH protein levels (right panel). HepG2 cells were used as a positive control for p21 in the right-hand end. (g) Huh7 cells were co-transfected with two different siRNA oligos targeting CD26 and p27. CD26 and p27 protein levels as determined by western blotting (left panel of [g]). Cell proliferation as determined by WST-1 assay (left panel of [g]) (*$P < 0.05$ vs all). NC and si indicate negative control and siRNA, respectively.

the expression of pro-apoptotic proteins Bak and Bim and the anti-apoptotic protein Bcl-xL (Fig. 4a). We then examined the effect of CD26 inhibition on apoptosis as assessed by annexin V positivity. siRNA-mediated knockdown of CD26 did not result in an increase in the number of annexin V^+ PI^- cells, which are considered to be apoptotic cells (Fig. 4b). These data indicate that CD26 knockdown did not cause apoptosis despite increasing pro-apoptotic stress, suggesting that counteract increases in anti-apoptotic Bcl-xL protein play an important pro-survival role of hepatoma cells under CD26 inhibition. To target this propensity, we treated CD26 knockdown cells with ABT-737, a specific small molecule inhibitor of Bcl-xL/Bcl-2/Bcl-w. Using caspase-3 and -7 activity, a mild induction of apoptosis was observed in negative control siRNA-transfected cells treated with ABT-737; in contrast, substantial apoptosis was observed in CD26 siRNA-transfected cells after ABT-737 treatment (Fig. 4c). Expression levels of cleaved caspase-3 and LDH activity also showed similar results with caspase-3 and -7 activity (Fig. 4d,e). Consistent with these observations, ABT-737 and CD26 knockdown synergistically decreased cellular viability (Fig. 4e). These findings suggested that the synthetic lethal interaction of combined CD26 and Bcl-xL inhibition may serve as a novel powerful anticancer therapy against HCC.

DISCUSSION

HERE, WE SHOWED that CD26 mRNA levels were increased in HCC and that CD26 inhibition can serve as a therapeutic option in HCC primarily through the induction of cell cycle arrest and potential modulation of apoptosis-related proteins. CD26 is a 110-kDa surface glycoprotein that was originally characterized as a T-cell differentiation antigen. This protein has multiple functions; most importantly, CD26 exerts its biological function through DPPIV activity via cleavage of a variety of peptides involved in glucose metabolism (GLP-1 and GIP) as well as chemokines (CCL5 and CXCL12) and other proteins.[19] Indeed, a previous report demonstrated that a CD26 antibody provoked cell cycle arrest in human T cells, and this action was dependent on DPPIV enzymatic activity.[20] However, in our current study, inhibition of DPPIV activity did not suppress hepatoma cell growth (Fig. 2h). CD26 also exerts pleiotropic effects by binding to the extracellular matrix or functioning as a T-cell co-stimulatory factor.[21–23] However, these CD26 interactions may not explain our current *in vitro* findings. Further investigation is required to understand the precise molecular mechanism of action of CD26 inhibition.

In the liver tissue, CD26 expression was also reported to be upregulated in HCV infection and non-alcoholic fatty liver disease,[19,24] which are common pre-existing diseases in HCC patients. Besides, Stecca *et al.*[11] have reported that cell distribution pattern of CD26 was altered in HCC. Although our current study focused on mRNA levels of CD26 in human HCC, these reports suggested the importance to assess CD26 protein expression and distribution in HCC and their surrounding liver tissues as well as its gene expression levels. However, we cannot address the relationship among protein expression levels, distribution and gene expression levels because of the small number of cases. They need to be addressed in our future study.

We showed that CD26 inhibition decelerated hepatoma cell growth through the induction of cell cycle arrest at the G0/G1 phase. The cell cycle is controlled by

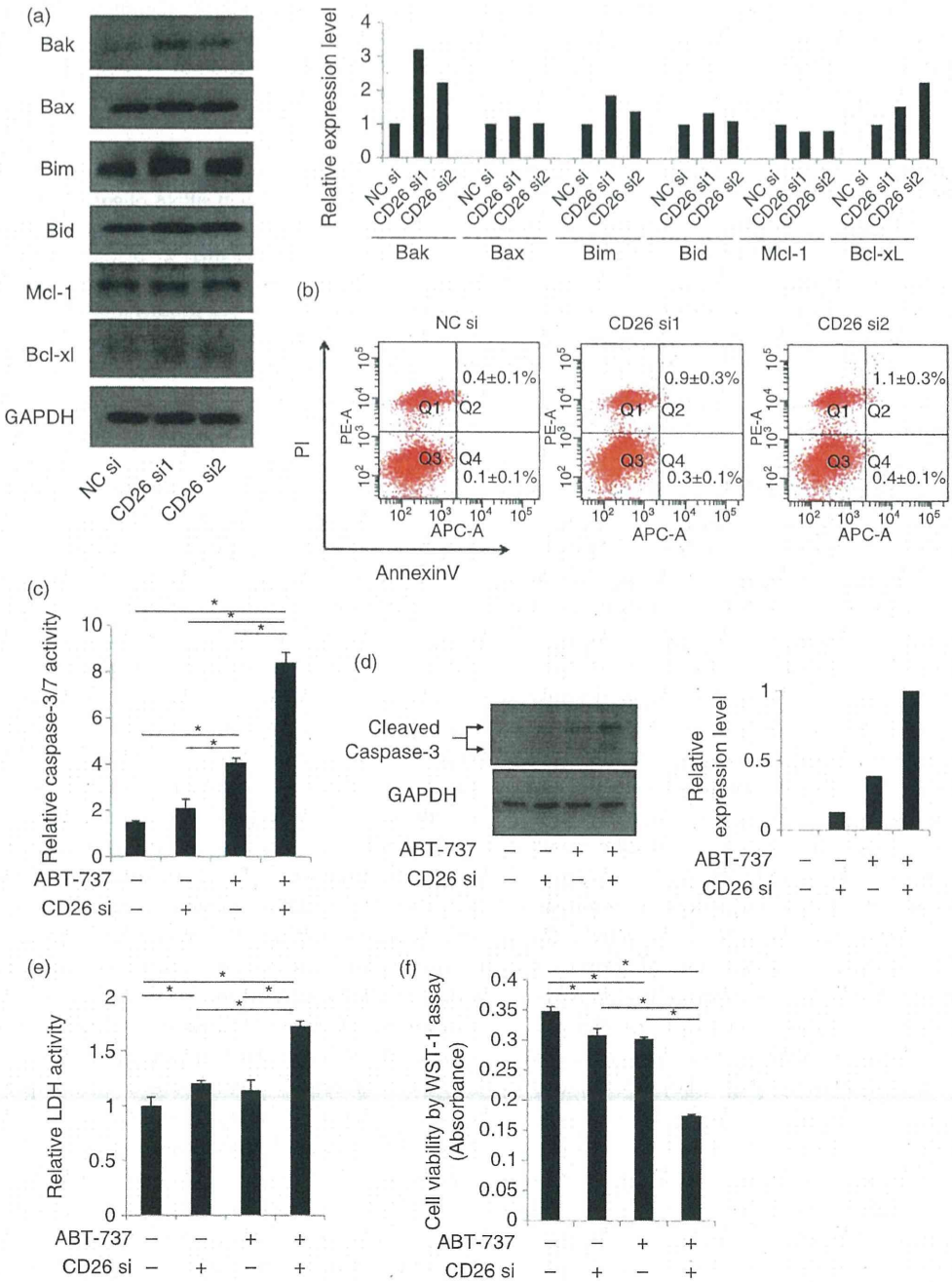

Figure 4 Synthetic lethal interaction of combined CD26 and Bcl-xL inhibition induces substantial apoptosis in hepatoma cells. Two different siRNA oligos targeting CD26 or a negative control siRNA oligo were individually transfected into Huh7 cells. (a) Western blotting of several Bcl-2 family proteins (left panel) and bar charts showing the protein levels normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels (right panel). (b) Flow cytometric analysis detecting apoptotic cell proportion by propidium iodide and annexin V staining. (c–e) After CD26 knockdown, cells are treated with or without 2 μM ABT-737. (c) Caspase-3 and -7 activity. Expression levels of cleaved caspase-3 (left panel of [d]) and bar chart showing the protein levels normalized by GAPDH protein levels (right panel of d). (e) Lactate dehydrogenase (LDH) activity and (f) cell viability as determined by WST assay (*$P < 0.05$).

several cyclins and cyclin-dependent kinase (CDK) complexes at each cell cycle checkpoint.[25] Whereas cyclins promote CDK activity to allow entry into the next cell cycle phase, CDK inhibitors (CKI) block CDK activity to halt the cell cycle.[26] CKI are divided into two groups based on their structure and CDK specificity: Ink4 family members and Cip/Kip family members. Ink4 family members, including p15 and p16, primarily target Cdk4 and Cdk6, which are important for cell cycle progression from the G1 to S phase. On the other hand, Cip/Kip family members, including p21 and p27, more broadly interfere with several CDK activities, thus regulating multiple stages of the cell cycle.[27] In this study, we revealed that CD26 knockdown caused cell cycle arrest at the G0/G1 phase via the upregulation of p21 in HepG2 cells and p27 but not p21 in Huh7 cells. According to the somatic mutation data by hybrid capture sequencing in CCLE,[18] Huh7 cells do not have a mutation in the *cdkn1a* gene. However, p21 mRNA expression levels in Huh7 cells are the second lowest among 28 human liver cancer cell lines tested in CCLE. In addition, Koga *et al.*[28] have previously reported that p21 expression in Huh7 cells was not detected by western blotting and only detected by quantitative PCR at lower levels than the other four human liver cancer cell lines. These findings suggested that p21 expression may be strongly suppressed in Huh7 cells by an unrevealed mechanism, which may generate alternative interaction between p27 and CD26. Previously, Ohnuma *et al.*[20] reported that anti-CD26 monoclonal antibody treatment induces cell cycle arrest in human T cells through p21 upregulation; however, this antibody did not affect p27. Meanwhile, Inamoto *et al.*[29] reported that another anti-CD26 monoclonal antibody elicited cell cycle arrest in a human renal clear cell carcinoma cell line through the upregulation of p27, not p21. Although these antibodies are different, they recognize the same cell membrane-proximal glycosylated region. These data suggest that the interaction between CD26 and Cip/Kip family proteins may be highly cell context-dependent.

Apoptosis is regulated by a fine balance between anti-apoptotic and pro-apoptotic proteins. We have reported that increases in anti-apoptotic proteins promote accelerated cell growth, and conversely their inhibition impairs hepatoma cell survival.[16] These results indicate the important contribution of this apoptosis pathway in hepatoma cell homeostasis. In this study, CD26 inhibition itself did not appear to alter this balance because we did not observe a change in the apoptotic cell population upon CD26 knockdown. However, we discovered that CD26 inhibition increased both anti-apoptotic and pro-apoptotic proteins. Under this condition, elevated level of the anti-apoptotic proteins may be indispensable for the survival of hepatoma cells, because they restrain increased levels of pro-apoptotic stress. In fact, combination treatment with CD26 knockdown and ABT-737, a Bcl-xL/-2/-W inhibitor, synergistically induced substantial apoptosis, leading to a significant decrease in hepatoma cell viability. Therefore, combined inhibition of CD26 and Bcl-xL may serve as a promising powerful therapy against HCC. In terms of a clinical perspective, navitoclax, a pro-drug of ABT-737, is currently available for clinical use in a trial.[30,31] Regarding a drug manipulating CD26, several anti-CD26 monoclonal antibodies are under investigation.[32,33] These antibodies displayed promising antitumor effects in lymphoma,[34] mesothelioma[33] and renal cell carcinoma.[29] In HCC, Gaetaniello *et al.*[35] have previously reported that anti-CD26 monoclonal antibody itself triggered an apoptotic signal in PLC/PRF/5 and HepG2 cell lines. In addition, a humanized anti-CD26 monoclonal antibody is currently being evaluated in a phase I clinical trial targeting CD26-expressing tumors.[36] Taken together, although the mechanisms of action of antibody and siRNA are different, combination of these drugs with a Bcl-xL inhibitor may serve as a feasible option for HCC treatment.

In conclusion, we demonstrated that CD26 was frequently overexpressed in HCC and that CD26 inhibition suppressed cell growth through the induction of cell cycle arrest. Although CD26 inhibitor monotherapy potentially carries the risk of promoting cancer cell survival in a dormant state, CD26 inhibition primes these cells to become susceptible to anti-apoptotic protein inhibitors via the increase of pro-apoptotic stress. Combined inhibition of CD26 and Bcl-xL may serve as a powerful potential therapy against HCC.

REFERENCES

1 Mentzel S, Dijkman HB, Van Son JP, Koene RA, Assmann KJ. Organ distribution of aminopeptidase A and dipeptidyl peptidase IV in normal mice. *J Histochem Cytochem* 1996; 44: 445–61.
2 Pro B, Dang NH. CD26/dipeptidyl peptidase IV and its role in cancer. *Histol Histopathol* 2004; 19: 1345–51.
3 Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* 2006; 368: 1696–705.
4 Boonacker E, Van Noorden CJ. The multifunctional or moonlighting protein CD26/DPPIV. *Eur J Cell Biol* 2003; 82: 53–73.

5 Wilson MJ, Ruhland AR, Quast BJ, Reddy PK, Ewing SL, Sinha AA. Dipeptidylpeptidase IV activities are elevated in prostate cancers and adjacent benign hyperplastic glands. *J Androl* 2000; **21**: 220–6.
6 Mareš V, Stremeňová J, Lisá V *et al.* Compartment- and malignance-dependent up-regulation of γ-glutamyltranspeptidase and dipetidylpeptidase-IV activity in human brain gliomas. *Histol Histopathol* 2012; **27**: 931–40.
7 Hirai K, Kotani T, Aratake Y, Ohtaki S, Kuma K. Dipeptidyl peptidase IV (DPP IV/CD26) staining predicts distant metastasis of "benign" thyroid tumor. *Pathol Int* 1999; **49**: 264–5.
8 Aoe K, Amatya VJ, Fujimoto N *et al.* CD26 overexpression is associated with prolonged survival and enhanced chemosensitivity in malignant pleural mesothelioma. *Clin Cancer Res* 2012; **18**: 1447–56.
9 Kajiyama H, Kikkawa F, Suzuki T, Shibata K, Ino K, Mizutani S. Prolonged survival and decreased invasive activity attributable to dipeptidyl peptidase IV overexpression in ovarian carcinoma. *Cancer Res* 2002; **15** (62): 2753–7.
10 Pethiyagoda CL, Welch DR, Fleming TP. Dipeptidyl peptidase IV (DPPIV) inhibits cellular invasion of melanoma cells. *Clin Exp Metastasis* 2000; **18**: 391–400.
11 Stecca BA, Nardo B, Chieco P, Mazziotti A, Bolondi L, Cavallari A. Aberrant dipeptidyl peptidase IV (DPP IV/CD26) expression in human hepatocellular carcinoma. *J Hepatol* 1997; **27**: 337–45.
12 Llovet JM, Ricci S, Mazzaferro V *et al.* Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378–90.
13 Cheng AL, Kang YK, Chen Z *et al.* Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25–34.
14 Shen YC, Lin ZZ, Hsu CH, Hsu C, Shao YY, Cheng AL. Clinical trials in hepatocellular carcinoma: an update. *Liver Cancer* 2013; **2**: 345–64.
15 Kodama T, Takehara T, Hikita H *et al.* Increases in p53 expression induce CTGF synthesis by mouse and human hepatocytes and result in liver fibrosis in mice. *J Clin Invest* 2011; **121**: 3343–56.
16 Hikita H, Takehara T, Shimizu S *et al.* The Bcl-xL inhibitor, ABT-737, efficiently induces apoptosis and suppresses growth of hepatoma cells in combination with sorafenib. *Hepatology* 2010; **52**: 1310–21.
17 Shimizu S, Takehara T, Hikita H *et al.* The let-7 family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma. *J Hepatol* 2010; **52**: 698–704.
18 Barretina J, Caponigro G, Stransky N *et al.* The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* 2012; **483**: 603–7.
19 Itou M, Kawaguchi T, Taniguchi E, Sata M. Dipeptidyl peptidase-4: a key player in chronic liver disease. *World J Gastroenterol* 2013; **19**: 2298–306.
20 Ohnuma K, Ishii T, Iwata S *et al.* G1/S cell cycle arrest provoked in human T cells by antibody to CD26. *Immunology* 2002; **107**: 325–33.
21 Cheng HC, Abdel-Ghany M, Pauli BU. A novel consensus motif in fibronectin mediates dipeptidyl peptidase IV adhesion and metastasis. *J Biol Chem* 2003; **278**: 24600–7.
22 Ishii T, Ohnuma K, Murakami A *et al.* CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO. *Proc Natl Acad Sci U S A* 2001; **9** (98): 12138–43.
23 Johnson RC, Zhu D, Augustin-Voss HG, Pauli BU. Lung endothelial dipeptidyl peptidase IV is an adhesion molecule for lung-metastatic rat breast and prostate carcinoma cells. *J Cell Biol* 1993; **121**: 1423–32.
24 Harada T, Kim DW, Sagawa K *et al.* Characterization of an established human hepatoma cell line constitutively expressing non-structural proteins of hepatitis C virus by transfection of viral cDNA. *J Gen Virol* 1995; **76** (Pt 5): 1215–21.
25 Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* 2009; **9**: 153–66.
26 Lim S, Kaldis P. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Development* 2013; **140**: 3079–93.
27 Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999; **13**: 1501–12.
28 Koga H, Sakisaka S, Harada M *et al.* Involvement of p21(WAF1/Cip1), p27(Kip1), and p18(INK4c) in troglitazone-induced cell-cycle arrest in human hepatoma cell lines. *Hepatology* 2001; **33**: 1087–97.
29 Inamoto T, Yamochi T, Ohnuma K *et al.* Anti-CD26 monoclonal antibody-mediated G1-S arrest of human renal clear cell carcinoma Caki-2 is associated with retinoblastoma substrate dephosphorylation, cyclin-dependent kinase 2 reduction, p27(kip1) enhancement, and disruption of binding to the extracellular matrix. *Clin Cancer Res* 2006; **1** (12): 3470–7.
30 Rudin CM, Hann CL, Garon EB *et al.* Phase II study of single-agent navitoclax (ABT-263) and biomarker correlates in patients with relapsed small cell lung cancer. *Clin Cancer Res* 2012; **18**: 3163–9.
31 Roberts AW, Seymour JF, Brown JR *et al.* Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol* 2012; **30**: 488–96.
32 Thompson MA, Ohnuma K, Abe M, Morimoto C, Dang NH. CD26/dipeptidyl peptidase IV as a novel therapeutic target for cancer and immune disorders. *Mini Rev Med Chem* 2007; **7**: 253–73.
33 Inamoto T, Yamada T, Ohnuma K *et al.* Humanized anti-CD26 monoclonal antibody as a treatment for malignant

mesothelioma tumors. *Clin Cancer Res* 2007; **13**: 4191–200.
34 Ho L, Aytac U, Stephens LC *et al.* In vitro and in vivo antitumor effect of the anti-CD26 monoclonal antibody 1F7 on human CD30+ anaplastic large cell T-cell lymphoma Karpas 299. *Clin Cancer Res* 2001; **7**: 2031–40.
35 Gaetaniello L, Fiore M, de Filippo S, Pozzi N, Tamasi S, Pignata C. Occupancy of dipeptidyl peptidase IV activates an associated tyrosine kinase and triggers an apoptotic signal in human hepatocarcinoma cells. *Hepatology* 1998; **27**: 934–42.
36 Hatano R, Yamada T, Matsuoka S *et al.* Establishment of monoclonal anti-human CD26 antibodies suitable for immunostaining of formalin-fixed tissue. *Diagn Pathol* 2014; **9**: 30.

SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

Table S1 Relationships between the expression levels of CD26 and the clinical parameters of hepatocellular carcinoma (HCC) patients.

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Post-treatment Levels of α-Fetoprotein Predict Incidence of Hepatocellular Carcinoma After Interferon Therapy

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BACKGROUND & AIMS: **In patients with chronic hepatitis C virus (HCV) infection, lack of sustained virologic response (SVR) 24 weeks after the end of interferon therapy is a significant risk factor for hepatocellular carcinoma (HCC). Although many pretreatment factors are known to affect HCC incidence, less is known about post-treatment factors—many change during the course of interferon therapy.**

METHODS: **We performed a prospective study, collecting data from 2659 patients with chronic hepatitis C without a history of HCC who had been treated with pegylated interferon (Peg-IFN) plus ribavirin from 2002 through 2008 at hospitals in Japan. Biopsy specimens were collected before treatment; all patients received Peg-IFN plus ribavirin for 48 to 72 weeks (HCV genotype 1) or 24 weeks (HCV genotype 2). Hematologic, biochemical, and virologic data were collected every 4 weeks during treatment and every 6 months after treatment. HCC was diagnosed based on angiography, computed tomography, and/or magnetic resonance imaging findings.**

RESULTS: **HCC developed in 104 patients during a mean observation period of 40 months. Older age, male sex, lower platelet counts and higher levels of α-fetoprotein at baseline, and lack of an SVR were significant risk factors for HCC. The cumulative incidence of HCC was significantly lower in patients without SVRs who relapsed than those with no response to treatment. Levels of α-fetoprotein 24 weeks after the end of treatment (AFP24) were significantly lower than levels of α-fetoprotein at baseline in patients with SVRs and those who relapsed, but not in nonresponders. Post-treatment risk factors for HCC among patients with SVRs included higher AFP24 level and older age; among those without SVRs, risk factors included higher AFP24 level, integrated level of alanine aminotransferase, older age, and male sex. AFP24 (≥10 ng/mL, 10–5 ng/mL, and then <5 ng/mL) was a better predictor of HCC incidence than pretreatment level of AFP among patients with and without SVRs.**

[a]Authors share co-first authorship.

Abbreviations used in this paper: AFP, α-fetoprotein; AFP24, α-fetoprotein levels at 24 weeks after the end of treatment; ALT, alanine aminotransferase; ALT24, alanine aminotransferase levels at 24 weeks after the end of treatment; CH-C, chronic hepatitis C; CT, computed tomography; EOT, end of treatment; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; i-ALT, integrated alanine aminotransferase value after the end of treatment; IFN, interferon; NR, nonresponse; Peg-IFN, pegylated interferon; PreAFP, α-fetoprotein levels at baseline; PreALT, alanine aminotransferase levels at baseline; SVR, sustained virologic response.

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CONCLUSIONS: **In patients with chronic HCV infection, levels of α-fetoprotein decrease during interferon therapy. High post-treatment levels of α-fetoprotein predict HCC, regardless of whether patients achieve an SVR. University Hospital Medical Information Network Clinical Trials Registry: C000000196, C000000197.**

Keywords: ALT; Liver Cancer; Risk Factor; Response to Therapy; Outcome.

Many reports have shown that hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC) was suppressed by interferon (IFN) therapy in patients who attained HCV eradication.[1,2] Generally, for patients showing HCV eradication by IFN therapy, the risk for HCC incidence has been shown to be low, but 1.3% to 4.7% of patients developed HCC at 5 years.[3–5] Conversely, for patients without HCV eradication by IFN therapy, although the risk for HCC incidence is high, many patients remain free of HCC incidence for long periods.[2] Therefore, the risk factors for HCC incidence should be evaluated separately between the 2 groups with distinctly different risk levels for HCC incidence, that is, patients who attained HCV eradication and those who did not.

Currently, many studies have assessed factors associated with HCC incidence among pretreatment factors but not post-treatment factors. However, IFN therapy for patients with chronic HCV infection mainly aims for HCV eradication but also may have immunologic and anti-inflammatory effects and antineoplastic activity. Therefore, there is the potential for a change in biochemical parameters. Notably, serum alanine aminotransferase (ALT) or α-fetoprotein (AFP) levels and liver fibrosis have been reported to change after IFN therapy.[5–8] Such synthetic effects can be involved in the suppression of HCC incidence. However, the relationship among the factors changed by IFN therapy and HCC incidence has not been fully examined.

In the present study, the changes in factors caused by pegylated IFN (Peg-IFN) plus ribavirin therapy were analyzed, and the relationship between post-treatment factors and HCC incidence among the 2 patient groups, those who attained HCV eradication and those who did not, was examined in a large-scale cohort of patients with chronic hepatitis C (CH-C).

Patients and Methods

Patients

The current study was a prospective multicenter study conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A ztotal of 2659 CH-C patients without a history of HCC who had been treated with Peg-IFN plus ribavirin therapy between December 2002 and December 2008 were enrolled in this study. Eligible patients did not have decompensated cirrhosis or other forms of liver disease (alcohol liver disease, autoimmune hepatitis), co-infection with hepatitis B, or human immunodeficiency virus. After enrollment, 26 patients who developed HCC within the first 12 months from the start of therapy were excluded because of the possibility of microscopic HCC having been present before treatment. In addition, 33 patients were excluded because their virologic response to Peg-IFN plus ribavirin therapy was not assessed. Finally, 2600 CH-C patients were assessed for HCC incidence. This study was conducted according to the ethical guidelines of the Declaration of Helsinki amended in 2002, and was approved by the ethics commission of Osaka University Hospital (University Hospital Medical Information Network Clinical Trials Registry: C000000196, C000000197).

Histologic Evaluation

Pretreatment liver biopsies were performed within 6 months before the start of therapy. Experienced liver pathologists who had no clinical, biochemical, or virologic information about the samples performed the histopathologic interpretation of the specimens. The histologic appearances, activity, and fibrosis were evaluated according to METAVIR histologic scores.[9]

Treatment and Definition of Virologic Response

All patients received Peg-IFN alpha-2b (Pegintron; Merck & Co, Inc, Whitehouse Station, NJ) plus ribavirin (Rebetol; Merck & Co, Inc). Peg-IFN was administered once a week at a dose of 1.5 μg/kg, and ribavirin was administered at a total dose of 600 to 1000 mg/d based on body weight, according to the standard treatment protocol for Japanese patients. In principle, treatment duration was 48 to 72 weeks for HCV genotype 1, and 24 weeks for HCV genotype 2. The serum HCV RNA level was analyzed qualitatively using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection, 50 IU/L; Roche Diagnostics, Branchburg, NJ). A sustained virologic response (SVR) was defined as an undetectable serum HCV RNA level at 24 weeks after the end of treatment (EOT). Relapse was defined as an undetectable serum HCV RNA level at the EOT but a detectable level after the EOT. Nonresponse (NR) was defined as a detectable HCV RNA level during therapy; the treatment generally was stopped at 24 weeks. The patients who discontinued the treatment because of an adverse event also were assessed in the same way.

Hepatocellular Carcinoma Surveillance and Data Collection

At the start of Peg-IFN plus ribavirin therapy, all patients were assessed by hepatic ultrasonography and/or computed tomography (CT) to confirm the absence of HCC. Hematologic, biochemical, and virologic data were collected every 4 weeks during treatment and every 6 months after treatment. Serum ALT levels after completion of the therapy were indicated as the average integrated values, which were calculated from the area of a trapezoid, with the ALT value divided by the observation period. For HCC surveillance, hepatic ultrasonography, CT, and/or magnetic resonance imaging was performed every 3 to 6 months during the follow-up period. When new space-occupying lesions were detected or suspected, they were examined by hepatic angiography. HCC was diagnosed by the presence of typical hypervascular characteristics on the angiography, in addition to CT and/or magnetic resonance imaging findings. If no typical image of HCC was observed, a fine-needle aspiration biopsy was performed with the patient's consent, or the patient was followed up carefully until a diagnosis was possible by definite observation using CT or angiography.

Follow-up Period

The follow-up period started from the date of the start of Peg-IFN plus ribavirin therapy. The end points were the date when new HCC developed or that of the last follow-up imaging test. For patients who did not attain SVR by Peg-IFN plus ribavirin therapy and had to be re-treated with another antiviral therapy, observation was discontinued at the date of the start of re-treatment. After completion of the Peg-IFN plus ribavirin therapy, liver-supporting therapy using ursodeoxycholic acid or glycyrrhizinate was allowed. The mean observation period was 40.0 ± 16.3 months. The cumulative incidence of HCC was assessed from the date of the start of Peg-IFN plus ribavirin therapy for the pretreatment analysis and from the date of the end of this therapy for the post-treatment analysis.

Statistical Analysis

Baseline continuous variables were expressed as means ± standard deviation and categoric variables were expressed as frequencies. Differences between the 2 groups (SVR vs non-SVR) were assessed by the chi-square test or the Mann–Whitney *U* test, and differences among 3 groups (SVR vs relapse vs NR) were assessed by analysis of variance and the Tukey post hoc test. The paired *t* test was used to analyze the difference between continuous variables before and after treatment. The variables of age, sex, white blood cells, hemoglobin levels, platelet counts, total bilirubin levels, albumin levels, ALT levels, AFP levels, and virologic response to the therapy were examined as correlates of HCC development. The Kaplan–Meier method was used to assess the cumulative incidence of HCC, and the groups were compared using the log-rank test. The Cox proportional-hazards model was used to identify the significant risk factors associated with HCC development. The factors selected as significant by simple Cox regression were evaluated by multiple Cox regression. The likelihood ratio test was used to compare the fitness of model for HCC incidence. A P value less than .05 was considered significant. Statistical analysis was conducted with SPSS version 19.0J (IBM, Armonk, NY).

All authors had access to the study data and reviewed and approved the final manuscript.

Results

Patient Characteristics at Baseline and 24 Weeks After the End of Treatment According to Antiviral Effect

The characteristics of the patients at baseline and 24 weeks after the EOT of Peg-IFN plus ribavirin therapy are summarized in Table 1. Of the 2600 patients, 1425 (55%) attained SVR, whereas 1175 showed non-SVR (relapse, n = 607; NR, n = 558) with Peg-IFN plus ribavirin therapy. The patients with METAVIR fibrosis stages 3 to 4 were grouped as advanced liver fibrosis because those with cirrhosis (METAVIR fibrosis stage 4) were in a minority in this study (2%, 47 of 1852 patients who received liver biopsy). The factors at baseline with a significant difference between the SVR and non-SVR groups are shown in Table 1.

The changes of the continuous hematologic and biochemical parameters between baseline and 24 weeks after the EOT were analyzed among the patients with corresponding continuous variables by paired *t* test. The mean AFP levels at 24 weeks after the EOT (AFP24) were significantly lower compared with AFP levels at baseline (PreAFP) in SVR patients, but not for non-SVR patients. After dividing non-SVR patients into relapse and NR groups, the mean AFP24 level was significantly lower compared with PreAFP in relapsers, but not for NR patients. The mean AFP24 levels were significantly lower in SVR patients and relapsers than in NR patients ($P < .001$ and $P < .001$, respectively), and the percentages of patients with AFP24 less than 5 ng/mL, which is the upper limit of normal range, were higher in the order of SVR, relapse, and NR. Alternatively, the mean ALT levels at 24 weeks after the EOT (ALT24) were significantly lower compared with ALT levels at baseline (PreALT) irrespective of the virologic response. The mean ALT24 levels were significantly lower in the order of SVR, relapse, and NR (SVR and relapse compared with NR, $P < .001$; SVR compared with relapse, $P < .001$), and the respective percentages of patients with ALT24 of 30 IU/L or less were higher in the same manner.

Table 1. Patients' Characteristics at Baseline and 24 Weeks After the Antiviral Treatment According to Antiviral Effect

Factor	SVR		Non-SVR		Non-SVR			
					Relapse		NR	
Factor	Baseline	24 wks after EOT	Baseline	24 wks after EOT	Baseline	24 wks after EOT	Baseline	24 wks after EOT
Age, *y*	54.5 ± 11.5[a]		58.8 ± 9.4		58.6 ± 9.0		59.0 ± 9.8	
Sex, male/female	727/698[b]		519/656		261/346		254/304	
HCV serotype, 1/2/unknown	955/451/19[a]		1049/110/16		512/86/9		529/23/6	
Liver histology[c]								
Activity, A0–1/2–3	573/446[d]		426/407		229/207		194/196	
Fibrosis, F0–2/3–4	902/118[a]		659/174		353/81		299/93	
White blood cells/mm^3	5317 ± 1626[a]	5251 ± 1614	4922 ± 1503	4613 ± 1535[e]	4994 ± 1446	4731 ± 1593[e]	4849 ± 1566	4489 ± 1470[e]
Hemoglobin level, *g/dL*	14.0 ± 1.4[a]	13.8 ± 1.4[e]	13.8 ± 1.4	13.4 ± 1.5[e]	13.8 ± 1.4	13.4 ± 1.5[e]	13.7 ± 1.4	13.5 ± 1.5[e]
Platelet level, $\times 10^4/mm^3$	17.7 ± 5.6[a]	19.0 ± 5.7[e]	15.6 ± 5.7	15.7 ± 5.9	16.2 ± 5.8	16.7 ± 6.0[f]	14.8 ± 5.5	14.6 ± 5.6
Total bilirubin level, *mg/dL*	0.81 ± 0.32[b]	0.74 ± 0.31[e]	0.86 ± 0.34	0.78 ± 0.34[e]	0.86 ± 0.32	0.76 ± 0.33[e]	0.85 ± 0.36	0.80 ± 0.35[e]
Serum albumin level, *g/dL*	4.1 ± 0.4[a]	4.4 ± 0.3[e]	4.0 ± 0.4	4.1 ± 0.4[e]	4.0 ± 0.4	4.2 ± 0.3[e]	3.9 ± 0.4	4.1 ± 0.4[e]
ALT level, *IU/L*	79 ± 78	20 ± 17[e]	75 ± 65	50 ± 39[e]	70 ± 56	44 ± 36[e]	78 ± 61	56 ± 40[e]
ALT ≤30 IU/L	19%	89%	17%	34%	20%	44%	14%	24%
ALT level >30 to ≤60 IU/L	34%	9%	38%	41%	37%	39%	38%	44%
ALT level >60 IU/L	47%	2%	45%	25%	43%	17%	48%	32%
AFP, *ng/mL*	8.4 ± 13.7[a]	3.7 ± 3.1[e]	21.0 ± 82.9	17.5 ± 119.6	11.3 ± 24.0	6.1 ± 14.7[e]	30.8 ± 114.6	29.3 ± 168.7
AFP level <5 ng/mL	51%	79%	34%	49%	47%	67%	21%	32%
AFP level ≥5 to <10 ng/mL	31%	19%	31%	29%	31%	24%	31%	32%
AFP level ≥10 ng/mL	18%	2%	35%	22%	22%	9%	48%	36%

[c]METAVIR, 748 missing.
The values at baseline were compared between SVR and non-SVR by the chi-square test or the Mann–Whitney *U* test: [a]$P < .001$, [b]$P < .01$, [d]$P = .03$.
The values were compared between 24 weeks after EOT and at baseline by paired *t* test: [e]$P < .001$, [f]$P < .01$.

Risk Factors for Hepatocellular Carcinoma Incidence Before Interferon Therapy and the Cumulative Incidence of Hepatocellular Carcinoma According to Antiviral Effects

HCC developed in 104 patients during the follow-up period (SVR, n = 23; non-SVR, n = 81). The significant risk factors of HCC incidence were older age, being male, lower platelet counts at baseline, higher PreAFP levels, and non-SVR to Peg-IFN plus ribavirin therapy according to multiple Cox regression analysis (Table 2). The cumulative incidence of HCC was significantly lower in SVR patients than in non-SVR patients (Figure 1*A*), and in SVR patients and relapsers than in NR patients (Figure 1*B*).

Hepatocellular Carcinoma Incidence According to α-Fetoprotein and Alanine Aminotransferase Levels at 24 Weeks After the End of Treatment

Because the AFP and ALT levels have been reported to be associated with the risk of HCC incidence,[10,11] HCC incidence was assessed according to AFP24 levels and ALT24 levels (Supplementary Table 1). Among SVR patients, HCC incidence was significantly higher with a higher level of AFP24 ($P < .001$). Among non-SVR patients, HCC incidence was significantly higher with a higher level of AFP24 ($P < .001$) and ALT24 ($P = .002$). After dividing non-SVR patients into relapse and NR groups, the same tendency of HCC incidence increasing with AFP24 and ALT24 increases was observed. HCC incidence was less than 1% in the group with an AFP24 less than 5 ng/mL and an ALT24 of 30 IU/L or less, irrespective of the virologic response (SVR, 0.7%; relapse, 0.8%; NR, 0%).

Risk Factors for Hepatocellular Carcinoma Incidence After Interferon Therapy in Sustained Virologic Response Patients and Non–Sustained Virologic Response Patients

The significant risk factors of HCC incidence were analyzed for patients with and without SVR using host factors and biomarkers at 24 weeks after the EOT using multiple Cox regression analysis (Tables 3 and 4). For ALT, integrated ALT values after the EOT (i-ALT) were used for this analysis because ALT levels can change in response to liver-supporting therapy. The SVR patients showed higher AFP24 levels and older age as the factors associated with HCC incidence (Table 3). Among non-SVR patients, significant risk factors of HCC incidence were older age, being male, higher i-ALT levels, and

Table 2. Risk Factors for HCC Incidence Among the Pretreatment Factors Plus Antiviral Effect (Cox Proportional-Hazards Model)

		Univariate analysis			Multivariate analysis		
Factor	Category	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
Age, *y*	0: <55	1			1		
	1: 55–64	5.162	2.331–11.434	<.001	4.898	1.437–16.694	.011
	2: ≥65	9.798	4.446–21.590	<.001	9.286	2.765–31.182	<.001
Sex	0: female	1	1.383–3.104	<.001	1	2.335–8.802	<.001
	1: male	2.072			4.534		
Liver fibrosis[a]	0: F0–F2	1	2.037–5.080	<.001	1	0.592–2.147	.716
	1: F3–F4	3.217			1.127		
White blood cells at baseline	0: ≥5000/mm^3	1	0.788–1.711	.450			
	1: <5000/mm^3	1.161					
Hemoglobin at baseline	0: ≥14 g/dL	1	0.554–1.196	.295			
	1: <14 g/dL	0.814					
Platelets at baseline	0: ≥15 × 10^4/mm^3	1	2.003–4.729	<.001	1	1.180–5.105	.016
	1: <15 × 10^4/mm^3	3.078			2.454		
Total bilirubin at baseline	0: <0.8 mg/dL	1	1.409–4.107	.001	1	0.770–2.725	.251
	1: ≥0.8 mg/dL	2.406			1.448		
Serum albumin at baseline	0: ≥4.0 g/dL	1	1.164–3.003	.010	1	0.368–1.228	.196
	1: <4.0 g/dL	1.870			0.672		
PreALT	0: ≤30 IU/L	1			1		
	1: 31–60 IU/L	3.318	1.171–9.404	.024	4.419	0.581–33.577	.151
	2: >60 IU/L	5.564	2.027–15.271	.001	2.845	0.371–21.782	.314
PreAFP	0: <5 ng/mL	1			1		
	1: AFP, ≥5 to <10 ng/mL	3.412	1.434–8.118	.006	2.089	0.761–5.730	.153
	2: ≥10 ng/mL	16.324	7.491–35.574	<.001	5.473	2.102–14.252	<.001
PEG-IFN/RBV antiviral effect	0: non-SVR	1	0.163–0.412	<.001	1	0.183–0.737	.005
	1: SVR	0.259			0.368		

CI, confidence interval; RBV, ribavirin.
[a]METAVIR.

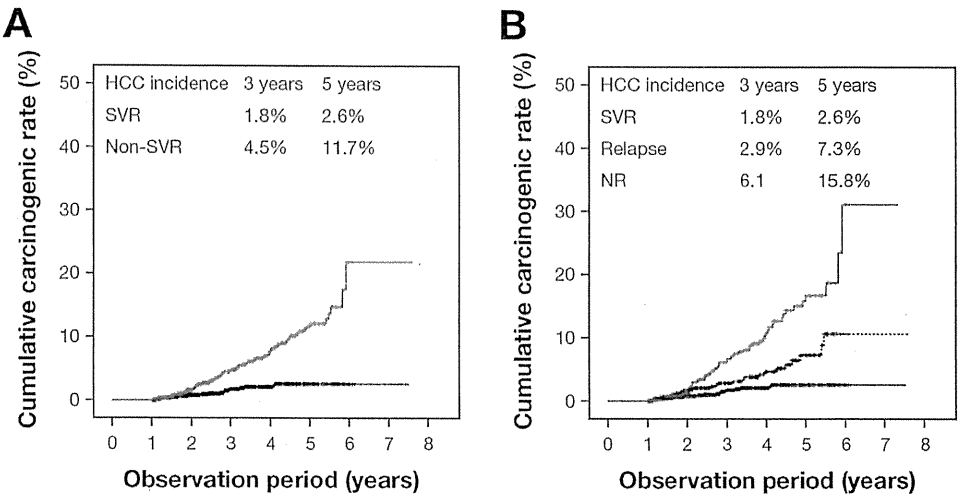

Figure 1. Cumulative incidence of HCC according to the antiviral effect of Peg-IFN plus ribavirin combination therapy. (*A*) The cumulative incidence of HCC was significantly lower in SVR patients (*black line*) than that in non-SVR patients (*gray line*). $P < .001$, SVR vs non-SVR. (*B*) The cumulative incidence of HCC was significantly lower in SVR patients (*black line*) and relapsers (*black dashed line*) than that in NR patients (*gray line*). $P < .001$, SVR vs NR; $P = .002$, SVR vs relapse; $P = .001$, relapse vs NR.

higher AFP24 levels (Table 4). As for stratified analysis for HCC incidence, the cumulative incidence of HCC was higher with higher AFP24 levels in both SVR (Figure 2*A*) and non-SVR patients (Figure 2*B*), and with higher i-ALT levels in all non-SVR patients (Figure 2*C*), and non-SVR patients according to AFP24 levels (Figure 2*D*).

Cumulative Incidence of Hepatocellular Carcinoma According to the Change in α-Fetoprotein Levels

The association between the change in serum AFP levels and the cumulative incidence of HCC was assessed in all patients, in stratified analysis according to SVR and non-SVR (Supplementary Figure 1). For those patients with a PreAFP level of 5 ng/mL or greater, the cumulative incidence of HCC was significantly lower among the patients with an AFP24 level less than 5 ng/mL than the patients with an AFP24 level of 5 ng/mL or greater in each group (all patients, $P < .001$; SVR, $P = .046$; non-SVR, $P = .003$). For those patients with an AFP24 level less than 5 ng/mL, no significant differences were found in the cumulative incidence of HCC between the patients with a PreAFP level of 5 ng/mL or greater and the patients with a PreAFP level less than 5 ng/mL in each group (all patients, $P = .074$; SVR, $P = .299$; non-SVR, $P = .139$).

Table 3. Risk Factors for HCC Incidence Among the Post-treatment Factors According to Antiviral Effect (Cox Proportional-Hazards Model) in Patients With SVR

		Univariate analysis			Multivariate analysis		
Factor	Category	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
Age, *y*	0: <55	1			1		
	1: 55–64	5.924	1.326–26.471	.020	3.007	0.638–14.181	.164
	2: ≥65	9.649	2.085–44.659	.004	5.814	1.124–30.070	.036
Sex	0: female	1	0.908–5.366	.081			
	1: male	2.207					
White blood cell count at 24 wk after EOT	0: ≥5000/mm^3	1	0.240–1.362	.207			
	1: <5000/mm^3	0.571					
Hemoglobin level at 24 wk after EOT	0: ≥14 g/dL	1	0.359–1.910	.658			
	1: <14 g/dL	0.828					
Platelet count at 24 wk after EOT	0: ≥15 × 10^4/mm^3	1	0.943–5.312	.068			
	1: <15 × 10^4/mm^3	2.238					
Total bilirubin level at 24 wk after EOT	0: <0.8 mg/dL	1	0.386–2.311	.901			
	1: ≥0.8 mg/dL	0.945					
Serum albumin level at 24 wk after EOT	0: ≥4.0 g/dL	1	0.690–8.231	.170			
	1: <4.0 g/dL	2.382					
i-ALT	0: >30 IU/L	1	0.228–1.973	.468			
	1: ≤30 IU/L	0.671					
AFP24	0: <5 ng/mL	1	2.588–22.159	<.001	1	2.738–23.942	<.001
	1: ≥5 ng/mL	7.573			8.096		

CI, confidence interval.

Table 4. Risk Factors for HCC Incidence Among the Post-treatment Factors According to Antiviral Effect (Cox Proportional-Hazards Model) in Patients Without SVR

		Univariate analysis			Multivariate analysis		
Factor	Category	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
Age, *y*	0: <55	1			1		
	1: 55–64	4.267	1.669–10.911	.002	3.546	1.310–9.596	.013
	2: ≥65	7.128	2.819–18.025	<.001	6.327	2.355–17.00	<.001
Sex	0: female	1	1.418–3.525	.001	1	1.760–5.787	<.001
	1: male	2.236			3.192		
White blood cell count at 24 wks after EOT	0: ≥5000/mm^3	1	0.632–1.617	.963			
	1: <5000/mm^3	1.011					
Hemoglobin level at 24 wks after EOT	0: ≥14 g/dL	1	0.556–1.369	.553			
	1: <14 g/dL	0.873					
Platelet count at 24 wks after EOT	0: ≥15 × 10^4/mm^3	1	1.487–3.920	<.001	1	0.591–2.063	.756
	1: <15 × 10^4/mm^3	2.414			1.104		
Total bilirubin level at 24 wks after EOT	0: <0.8 mg/dL	1	1.075–2.901	.025	1	0.466–1.489	.537
	1: ≥0.8 mg/dL	1.766			0.833		
Serum albumin level at 24 wks after EOT	0: ≥4.0 g/dL	1	1.710–4.579	<.001	1	0.961–3.140	.068
	1: <4.0 g/dL	2.799			1.737		
i-ALT	0: >60 IU/L	1			1		
	1: 31–60 IU/L	0.531	0.339–0.831	.006	0.728	0.388–1.365	.322
	2: ≤30 IU/L	0.115	0.041–0.324	<.001	0.181	0.040–0.827	.027
AFP24	0: <5 ng/mL	1			1		
	1: ≤5 to <10 ng/mL	4.340	1.949–9.663	<.001	3.347	1.371–8.171	.008
	2: ≥10 ng/mL	6.785	3.111–14.797	<.001	4.855	1.814–12.996	.002
PEG-IFN/RBV antiviral effect	0: NR	1	0.349–0.901	.017	1	0.676–2.699	.394
	1: relapse	0.561			1.351		

CI, confidence interval; RBV, ribavirin.

Fitness of Model for Hepatocellular Carcinoma Incidence

Finally, we assessed which was a more applicable model for HCC incidence among 2 models: the pretreatment factor model or the post-treatment factor model. The variables of age, sex, platelet counts, ALT levels, AFP levels, and virologic response were examined for all patients. The post-treatment model was shown to be significantly better fitted for HCC incidence than the pretreatment model ($P = .0008$) (Table 5). When the AFP levels were compared between pretreatment (PreAFP) and post-treatment (AFP24) for all patients, the AFP24 level was shown to be more applicable for HCC incidence than the PreAFP level ($P < .0001$). Furthermore, even in the stratified analysis according to the virologic response, AFP24 levels were more applicable than PreAFP levels in both groups (SVR, $P = .03$; non-SVR, $P = .001$) (Table 5).

Discussion

In the present study, the risk for HCC incidence was significantly lower in SVR patients than in non-SVR patients (at 5 years, 2.6% vs 11.7%), as previously reported.[2,12,13] However, to date, the risk factors for HCC incidence in each virologic response or the relationship between HCC incidence and the factors changed by IFN therapy had not been fully examined. Then, we examined the relationship between HCC incidence and post-treatment factors based on the antiviral effects with a large-scale cohort undergoing Peg-IFN plus ribavirin therapy.

For SVR patients, AFP24 and ALT24 levels significantly decreased compared with PreAFP and PreALT levels. HCC incidence significantly increased with higher AFP24 levels but not with higher ALT24 levels. Moreover, the multiple Cox regression showed that AFP24 levels as well as age were significant risk factors for HCC incidence. These results suggested that HCC incidence in SVR patients is accompanied by an AFP increase but not an ALT increase. Although AFP can be a comprehensive surrogate marker for HCC incidence in relation to various factors, such as liver inflammation and fibrosis, our data suggest that AFP can be a marker for HCC incidence independent of liver inflammation. In clinical practice, even if HCV was eradicated and the serum ALT level was normal, careful surveillance for HCC was needed for patients with an AFP24 of 5 ng/mL or greater.

As previously reported,[13] the cumulative incidence of HCC was significantly lower in relapsers than in NR patients in this study. However, the reason why HCC incidence was reduced among relapsers who showed transient HCV disappearance in sera has been unclear.[13] In this study, multiple Cox regression for HCC incidence among non-SVR patients using post-treatment factors, which included AFP24 levels and i-ALT levels after the EOT, showed that AFP24 and i-ALT levels were significant risk factors for HCC incidence but not factors of