included age, HBe positivity, platelets, and levels of HBsAg, HBcrAg, HBV DNA and IL-22 before treatment. Statistical analyses were carried out using SPSS software version 21.0J (IBM Japan, Tokyo, Japan).

RESULTS

Baseline clinical characteristics of patients

THE CLINICAL PROFILE of the experimental patient cohort is shown in Table 1. Among our 48 patients with chronic hepatitis, 39 (81%) achieved a VR at 24 months. A VR was attained in 11 of 20 HBeAg positive patients (55%) and in all 28 HBeAg negative patients (100%). One patient (5%) demonstrated HBeAg seroclearance through to month 24, but did not attain HBeAg seroconversion. No patient experienced a virological breakthrough.

The median age of patients achieving a VR was significantly higher than that of patients who did not (55 vs 37 years; $P = 0.031$) (Table 1). In contrast, viral responders had significantly lower median HBsAg (3.3 vs 3.9 log IU/mL; $P = 0.001$) and HBcrAg (5.0 vs 6.8 log U/mL; $P < 0.001$) levels than non-responders. We found no significant differences between patient groups with regard to sex, HBV genotype, or albumin, AST, ALT, bilirubin or platelet levels. When stratified by HBeAg positivity, HBsAg level only was significantly associated with a VR (3.2 vs 3.9 log IU/mL; $P = 0.003$). When we compared HBeAg positive and negative patients, median HBV DNA and HBcrAg levels, but not HBsAg, were significantly higher in HBeAg positive patients (Table S1).

Detection and quantification of serum markers in patients with chronic hepatitis B and controls

Serum samples obtained prior to ETV therapy were examined for the presence of six cytokines and five chemokines by multiplex assays. As shown in Table 2, the median baseline serum concentrations of IL-6 (6.5 vs 5.8 pg/mL; $P = 0.031$) and three chemokines (CCL2 [39.3 vs 31.5 pg/mL; $P = 0.022$], CXCL9 [329.2 vs 127.8 pg/mL; $P = 0.002$] and CXCL10 [217.1 vs 58.7 pg/mL; $P = 0.001$]) were significantly higher in patients with chronic hepatitis B than in healthy controls. When we subdivided patients into HBeAg positive or anti-HBe positive groups, no significant differences in the median concentrations of any cytokine or chemokine were seen, including IL-22 (Table S1).

Effect of ETV therapy on serum cytokine levels

The median levels of serum cytokines and chemokines in our cohort are shown in Table 3. Among our patients, the median baseline serum IL-22 concentration was significantly higher in virological responders than in non-responders (35.3 vs 27.8 pg/mL; $P = 0.031$) (Fig. 1a). No other cytokines or chemokines were associated with

Table 1 Demographic and clinical characteristics of 48 patients with chronic hepatitis B

Characteristics	Total, $n = 48$	VR (+), $n = 39$	VR (−), $n = 9$	P
Age, years	55 (24–81)	55 (24–81)	37 (26–67)	**0.031**
Male, *n* (%)	33 (69)	29 (74)	4 (44)	0.18
HBeAg positive, *n* (%)	20 (42)	11 (28)	9 (100)	**<0.001**
HBV genotype C, *n* (%)	45 (94)	37 (95)	8 (89)	1.00
HBV DNA (log copies/mL)	6.6 (2.7 to >9.1)	6.4 (2.7 to >9.1)	8.0 (3.9 to >9.1)	0.06
HBsAg (log IU/mL)	3.4 (−1.2 to 4.5)	3.3 (−1.2 to 4.3)	3.9 (3.3–4.5)	**0.001**
HBcrAg (log U/mL)	5.2 (3.0–6.8)	5.0 (3.0–6.8)	6.8 (5.4–6.8)	**<0.001**
Albumin (mg/dL)	4.2 (2.3–5.3)	4.2 (3.1–5.3)	4.2 (2.3–4.5)	0.80
AST (IU/L)	48 (15–1476)	51 (15–1476)	36 (28–358)	0.82
ALT (IU/L)	49 (9–2021)	63 (9–2021)	56 (29–954)	0.74
Bilirubin (mg/dL)	0.8 (0.3–3.1)	0.8 (0.3–3.1)	0.7 (0.5–1.0)	0.33
Platelet (/μL)	16.3 (8.0–28.9)	15.2 (8.0–28.9)	19.5 (11.9–27.7)	0.053

Continuous variables are expressed as median values (range).
Bolded figures indicate statistical significance.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

Table 2 Serum cytokines and chemokines in patients with chronic hepatitis B and healthy subjects

Cytokine/chemokine	Patients	Controls	*P*-value
IL-2	2.3 (0–4.9)	2.1 (1.9–2.4)	0.42
IL-6	6.5 (2.7–19.1)	5.8 (5.8–6.5)	**0.031**
IL-10	1.1 (0.0–26.8)	1.4 (1.3–1.6)	0.49
IL-12p70	12.9 (0.1–22.0)	12.9 (12.8–12.9)	0.50
IL-21	12.5 (5.0–1916.5)	11.5 (10.5–253.5)	0.68
IL-22	34.9 (27.2–75.7)	33.6 (32.3–39.0)	0.47
CCL2	39.3 (23.8–8118.8)	31.5 (26.7–39.3)	**0.022**
CCL3	4.8 (0.0–651.8)	7.0 (5.0–9.9)	0.25
CXCL9	329.2 (89.8–18 758.9)	127.8 (107.5–874.3)	**0.002**
CXCL10	217.1 (18.6–3594.3)	58.7 (24.7–859.5)	**0.001**
CXCL11	40.8 (0.7–553.8)	25.8 (12.9–90.3)	0.23

Continuous variables are expressed as median values (range) (pg/mL).
Bolded figures indicate statistical significance.
IL, interleukin.

a VR. When stratified by HBeAg positivity, serum IL-22 and IL-6 levels in the VR group were significantly higher than those in the non-VR group (35.3 vs 31.2 pg/mL [$P = 0.046$] and 6.9 vs 6.1 pg/mL [$P = 0.031$], respectively).

Several clinical findings (HBV DNA, HBsAg, HBcrAg, albumin, AST, ALT, bilirubin and platelet) at baseline were examined for their correlation with serum cytokines or chemokines in patients with chronic hepatitis B. Serum IL-6, CXCL9, CXCL10 and CXCL11 were all positively correlated with values for AST, ALT and bilirubin, but were negatively correlated with serum HBsAg (Table 4). CXCL9, CXCL10 and CXCL11 were also significantly correlated with each other (data not shown). There was a negative correlation between HBsAg and AST, ALT and bilirubin (data not shown).

Prediction of VR in patients with chronic hepatitis B

We performed ROC analysis to determine the optimal cut-off values for serum IL-22, HBsAg and HBcrAg in predicting a VR for chronic HBV infection with the values obtained from the 39 patients who achieved a VR and the nine who did not. The selection of optimal cut-off point values was based on the IL-22, HBsAg and HBcrAg levels at which accuracy was maximal. Optimal cut-off value, sensitivity, specificity, positive predictive value, negative predictive value and calculated area

Table 3 Serum cytokines and chemokines in treatment outcome to antiviral therapy

Cytokine/chemokine	VR	Non-VR	*P*-value
IL-2	2.3 (0.0–4.9)	3.1 (0.0–3.3)	0.60
IL-6	6.8 (2.7–19.1)	6.1 (4.3–12.5)	0.22
IL-10	0.6 (0.0–26.8)	1.5 (0.0–5.0)	0.86
IL-12p70	12.9 (0.1–22.0)	12.9 (1.2–18.0)	0.74
IL-21	12.2 (5.0–1916.5)	19.9 (5.9–27.8)	0.70
IL-22	35.3 (27.2–75.7)	27.8 (27.3–46.7)	**0.031**
CCL2	40.8 (24.4–118.8)	34.8 (23.8–60.3)	0.13
CCL3	4.5 (0.0–651.8)	6.5 (2.7–22.9)	0.57
CXCL9	322.5 (115.4–18 758.9)	353.6 (89.8–1545.1)	0.60
CXCL10	206.3 (29.1–3594.3)	294.2 (18.6–2240.7)	0.94
CXCL11	39.9 (0.7–553.8)	48.8 (12.6–428.2)	0.80

Continuous variables are expressed as median values (range) (pg/mL).
Bolded figure indicates statistical significance.
IL, interleukin; VR, virological response.

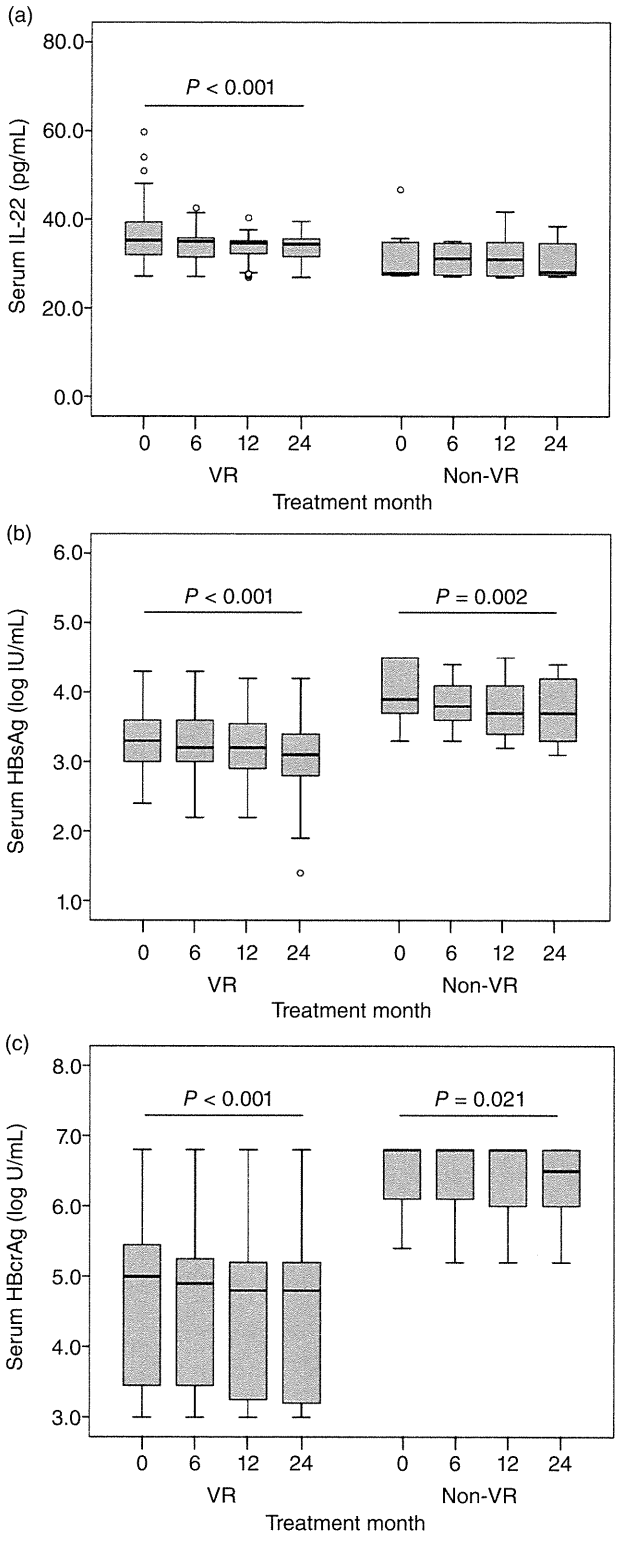

Figure 1 Comparison of serum (a) IL-22, (b) HBsAg and (c) HBcrAg levels during entecavir therapy in the VR ($n = 39$) and non-VR ($n = 9$) groups. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The harsh marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. IL, interleukin; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis core-related antigen; VR, virological response.

under the curve (AUC) values for each parameter are listed in Table 5. The AUC values were consistently high and ranged between 0.731 (IL-22) and 0.858 (HBcrAg).

Several factors found in association with a VR to ETV therapy were evaluated for their independence by multivariate analysis. We determined that IL-22 of 27.8 pg/mL or more (hazard ratio [HR] = 13.67 [95% confidence interval [CI] = 1.05–178.11], $P = 0.046$) and HBcrAg of 5.7 log U/mL or less (HR = 10.88 [95% CI = 1.02–115.44], $P = 0.048$) were independent factors related to a VR. HBsAg did not have a significant independent association in this study ($P = 0.071$).

Serum cytokine and chemokine changes during treatment

Longitudinal analysis of IL-22, HBsAg and HBcrAg levels was carried out at 6, 12 and 24 months after the initiation of therapy and showed significant gradual reductions in IL-22 ($P < 0.001$, Friedman test), HBsAg ($P < 0.001$) and HBcrAg ($P < 0.001$) in samples collected from patients who achieved a VR (Fig. 1). We noted a higher median serum IL-22 concentration at month 6 in the VR group than in the non-VR group ($P = 0.012$), and there were significant differences at each time point for HBsAg (6 months, $P = 0.002$; 12 months, $P = 0.006$; and 24 months, $P = 0.004$) and HBcrAg (6 months, $P < 0.001$; 12 months, $P < 0.001$; and 24 months, $P < 0.001$) between responders and non-responders.

DISCUSSION

IN THE PRESENT study, we measured the levels of six cytokines and five chemokines in patients with chronic hepatitis B and analyzed their association with ETV therapy outcome using a bead-array multiplex immunoassay system. Four of our observations are noteworthy and require further comment. First, serum IL-6, CCL2, CXCL9 and CXCL10 concentrations were

Table 4 Correlation between cytokines, chemokines and clinical parameters

		IL-2	IL-6	IL-10	IL-12	IL-21	IL-22	CCL2	CCL3	CXCL9	CXCL10	CXCL11
HBV DNA	*r*	0.08	0.01	0.10	0.06	0.08	0.17	−0.13	0.01	−0.13	−0.10	0.20
	P	0.61	0.97	0.51	0.69	0.61	0.25	0.39	0.95	0.39	0.50	0.18
HBsAg	*r*	−0.99	−0.35	−0.14	0.22	−0.08	−0.05	−2.5	0.02	−0.78	−0.61	−0.32
	P	0.51	**0.015**	0.35	0.14	0.61	0.74	0.09	0.89	**<0.001**	**<0.001**	**0.025**
HBcrAg	*r*	0.04	0.05	−0.16	0.24	0.18	0.14	−0.13	0.14	−0.14	−0.15	0.11
	P	0.79	0.76	0.29	0.11	0.21	0.35	0.40	0.33	0.36	0.31	0.45
Albumin	*r*	0.17	0.02	0.17	−0.02	0.05	−0.02	0.12	0.08	0.13	−0.09	0.02
	P	0.25	0.91	0.24	0.89	0.75	0.88	0.40	0.60	0.39	0.53	0.91
AST	*r*	0.05	0.40	0.11	−0.11	−0.03	0.14	0.13	−0.07	0.78	0.75	0.36
	P	0.72	**0.004**	0.45	0.47	0.83	0.33	0.39	0.66	**<0.001**	**<0.001**	**0.013**
ALT	*r*	0.02	0.42	0.12	−0.11	−0.06	0.16	0.10	−0.08	0.69	0.71	0.46
	P	0.91	**0.003**	0.40	0.44	0.70	0.28	0.52	0.57	**<0.001**	**<0.001**	**0.001**
Bilirubin	*r*	−0.03	0.36	0.07	0.08	−0.03	0.13	0.27	−0.12	0.33	0.65	0.35
	P	0.83	**0.012**	0.64	0.58	0.84	0.38	0.07	0.42	**0.023**	**<0.001**	**0.015**
Platelet	*r*	0.08	0.12	0.15	−0.09	0.13	0.25	−0.05	0.19	0.31	0.04	0.13
	P	0.57	0.42	0.33	0.55	0.38	0.09	0.74	0.20	**0.033**	0.82	0.39

Bolded figures indicate statistical significance.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IL, interleukin; *r*, Spearman's rank correlation.

higher in patients with chronic hepatitis B than in healthy subjects. Second, serum IL-22 concentration before treatment was significantly higher in patients achieving a VR to ETV therapy. In contrast, responders had lower serum levels of HBsAg and HBcrAg at baseline. Third, IL-22, HBsAg and HBcrAg decreased during treatment and remained low in patients with a VR. Fourth, serum IL-6, CXCL9, CXCL10 and CXCL11 were positively correlated with serum values of AST, ALT and bilirubin, but were negatively correlated with HBsAg.

Interleukin-6 is a well-recognized multifunctional cytokine that may reflect more active hepatic necroinflammation and be associated with chronic HBV infection severity. As in previous studies,[18,21] serum IL-6 was significantly higher in the HBV-infected group than in healthy controls and was positively correlated with such clinical parameters as transaminases and bilirubin. Hence, our data support that IL-6 is strongly associated with the severity of liver diseases.

CXCL9, CXCL10 and CXCL11 appear to be particularly important in chronic HCV infection by promoting the development of intrahepatic inflammation that leads to fibrogenesis.[22,23] These chemokines are also significantly elevated in patients with necroinflammatory activity of acute and chronic hepatitis C.[24,25] In our study, serum CXCL9 and CXCL10 were higher in patients with chronic HBV infection than in healthy individuals, which was in agreement with a previous

Table 5 Optimal cut-off value, sensitivity, specificity, AUC, and predictive value of serum IL-22, HBsAg and HBcrAg at baseline of treatment in 48 patients with chronic hepatitis B

	Cut-off value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
IL-22	27.8 pg/mL	56 (21–86)	90 (76–97)	0.731 (0.533–0.929)	90	56
HBsAg	3.6 log IU/mL	78 (40–97)	77 (61–89)	0.838 (0.704–0.971)	44	94
HBcrAg	5.7 log U/mL	89 (52–100)	82 (67–93)	0.858 (0.754–0.962)	53	97

All AUC values were significantly higher than a 0.50 non-predictive value ($P < 0.01$ for all comparisons). Cut-off values were determined by constructing receiver–operator curves.
AUC, area under the curve; CI, confidence interval; HBcrAg, hepatitis core-related antigen; HBsAg, hepatitis B surface antigen; IL, interleukin; NPV, negative predictive value; PPV, positive predictive value.

report.[12] Moreover, the serum CXCR3-associated chemokines CXCL9, CXCL10 and CXCL11 were all well correlated with serum values of AST, ALT and bilirubin. Because we observed a significant correlation between these chemokines and IL-6, our findings suggest that CXCR3-associated chemokines may too contribute to necroinflammatory activity in chronic HBV infection. However, there were insufficient histological data in our study to assess whether IL-6 and CXCR3-associated chemokines were correlated with degree of fibrosis, in addition to a lack of biochemical evidence of inflammation. We furthermore showed a striking negative association between HBsAg concentration and levels of IL-6 and CXCR3-associated chemokines. As HBsAg was also negatively correlated with transaminases and bilirubin, this HBsAg decline may be linked to increased immunological activity.

Interestingly, this study demonstrated a beneficial role of IL-22 in achieving a VR during ETV therapy. IL-22 is an IL-10 family cytokine that is important for the modulation of tissue responses during inflammation and is expressed by many types of lymphocytes of both the innate and adaptive immune systems, most notably T-helper 17 cells, γδ T cells, natural killer cells and lymphoid tissue inducer-like cells. The IL-22 receptor is highly expressed on hepatocytes.[26,27] At present, several studies support a protective role of IL-22 in the prevention of hepatocellular damage, although there is evidence indicating dual protective and pathogenic roles for this cytokine in the liver.[17,28–30] Some groups have examined the association between IL-22 and liver fibrosis in humans and mice.[31,32] In one report, tumor-infiltrating lymphocytes in HCC exhibited elevated IL-22 expression, and these IL-22$^+$ lymphocytes promoted tumor growth and metastasis in mice.[33] Although human patients with chronic hepatitis B show increased percentages of T-helper 17 cells in the peripheral blood and liver and an increased concentration of IL-22 in the serum,[14,34] there have been no reports on treatment outcome in patients with chronic HBV infection during ETV therapy. In our study, IL-22 levels decreased over time in both the VR and non-VR groups, but they were consistently higher in the VR group. This difference in IL-22 levels between the two groups further supports the possibility that IL-22 may be important for the activation of immune cells that contribute to viral control. When stratified by HBe positivity, although IL-22 was still significantly associated with a VR, the number of patients was only 20 in this study. Further research is needed to clarify the association between IL-22 and treatment response.

Lastly, we uncovered that lower baseline serum HBsAg and HBcrAg levels were associated with a VR. HBcrAg assays measure serum levels of HB core, e and 22-kDa precore antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these three denatured antigens.[35] Because this assay measures all antigens transcribed from the precore/core gene, it is regarded as core related.[36] The AUC values for baseline HBsAg and HBcrAg levels were high at 0.838 and 0.858, respectively. Several studies have shown that HBsAg is useful for the management of ETV therapy,[37,38] whereby an HBsAg decline is most profound in patients losing HBeAg detectability during treatment.[39] HBeAg positivity was also significantly associated with treatment outcome in the present study. However, because HBcrAg, but not HBsAg or HBeAg, was an independent factor related to a VR in multivariate analysis, our results indicated that serum HBcrAg quantitation may offer clinicians a new tool in predicting treatment outcome in HBV infection. Further investigation of large cohorts must be done to validate the significance of our findings.

With a VR at 12 months established as a parameter, 38 patients (79%) achieved this event. Serum IL-22, HBsAg and HBcrAg levels were all still significantly associated with a VR at 12 months. AUC values were as high as between 0.737 (IL-22) and 0.878 (HBcrAg). Furthermore, ALT normalization was achieved in 40 (83%) and 42 (88%) patients at 12 and 24 months, respectively. Although lower median pretreatment levels of HBsAg and HBcrAg were significantly associated with ALT normalization, there was no such statistically significant relation for IL-22 (data not shown).

In summary, a cytokine (IL-6) and several chemokines (CCL2, CXCL9 and CXCL10) were seen to be elevated in patients with chronic hepatitis B. Our results indicate that serum IL-6 and CXCR3-associated chemokines are correlated with liver injury, serum IL-22 is a useful biomarker for predicting a VR to ETV therapy, and a lower level of serum HBcrAg is related to a favorable response to antiviral therapy.

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SUPPORTING INFORMATION

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

Table S1 Demographic, clinical characteristics, and serum cytokines and chemokines in patients with hepatitis B e-antigen (HBeAg) positive and hepatitis B e-antigen (HBeAg) negative patients.

Hepatology Research

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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-

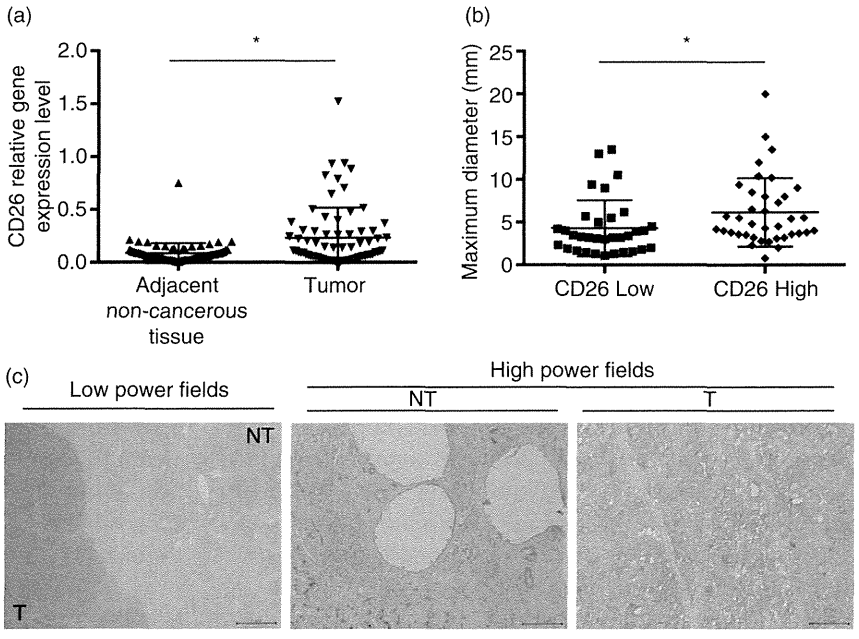

Figure 1 CD26 expression is frequently upregulated in hepatocellular carcinomas (HCC) and positively correlated with tumor size. (a) CD26 mRNA levels were examined in HCC and adjacent non-cancerous liver tissues. Relative CD26 mRNA levels normalized to β-actin mRNA expression (*$P < 0.05$ by Student's 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

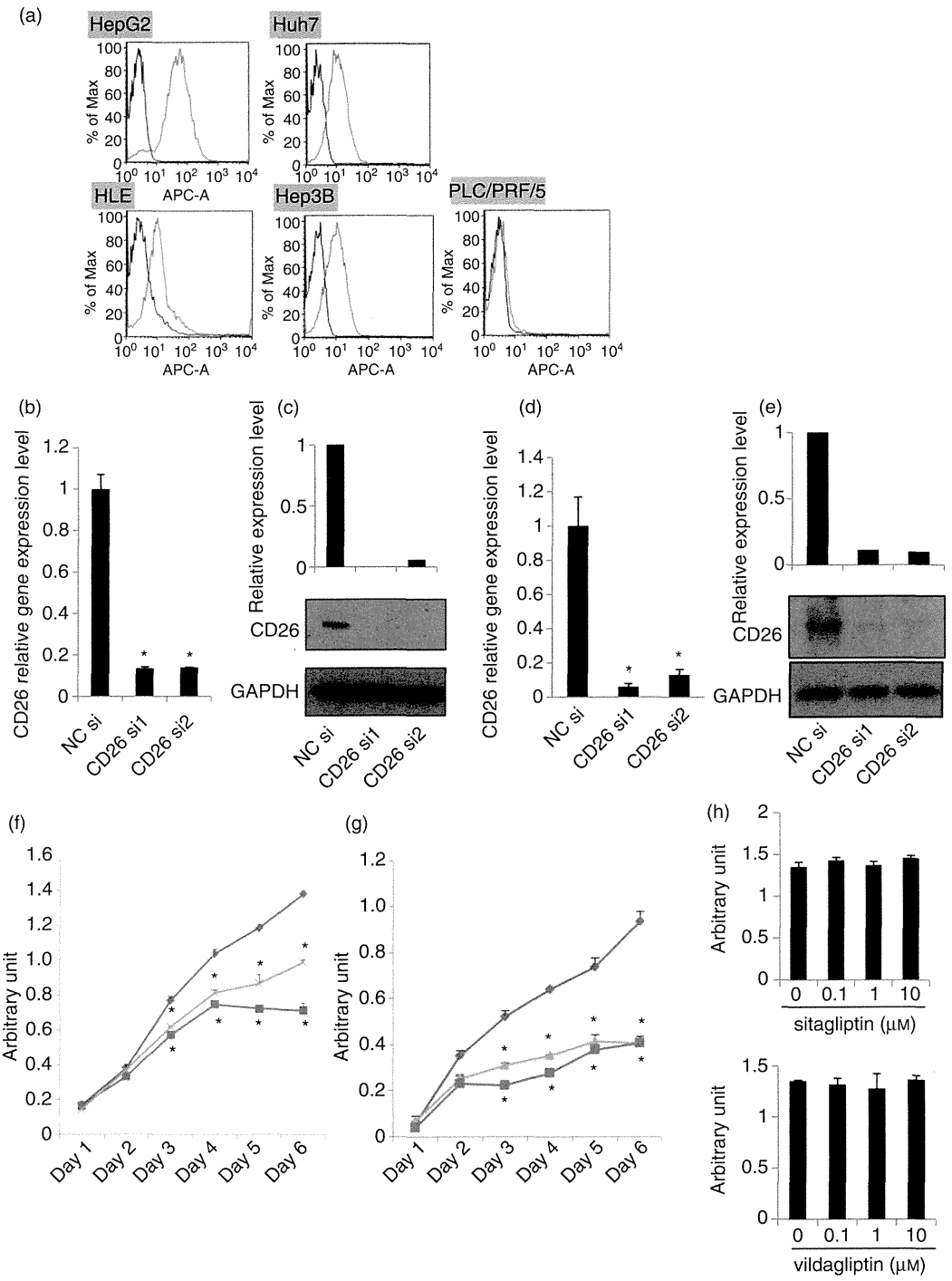

Figure 2 CD26 knockdown inhibits hepatoma cell growth. (a) CD26 expression levels were examined by flow cytometry in various hepatoma cell lines. (b–g) Two different siRNA oligos targeting CD26 or a negative control siRNA oligo were individually transfected into (b,c,f) HepG2 or (d,e,g) Huh7 cells. (b,d) 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.

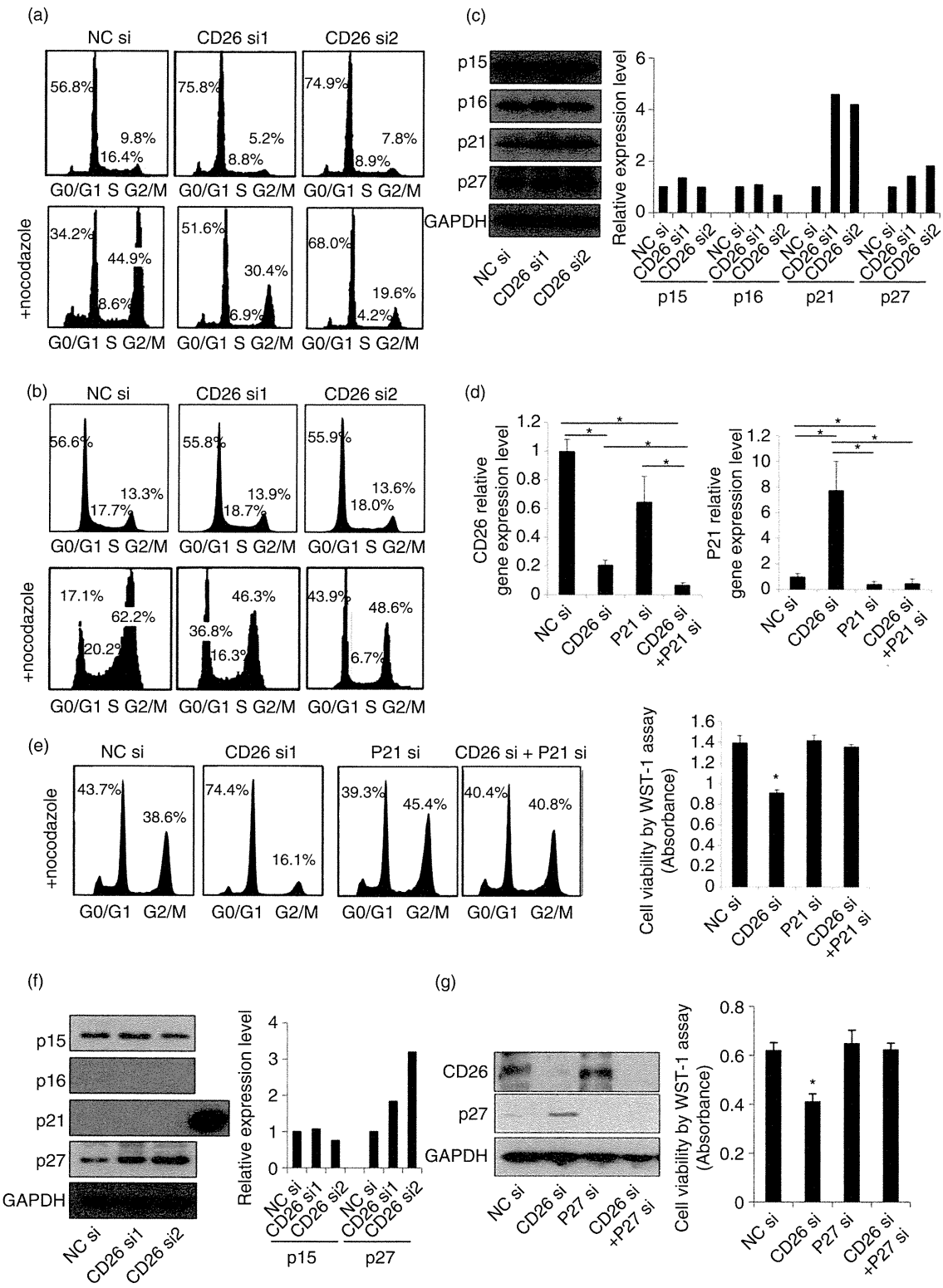

Figure 3 CD26 knockdown induces cell cycle arrest at the G0/G1 phase via upregulation of Cip/Kip family proteins. (a–c) Two different siRNA oligos targeting CD26 or a negative control siRNA oligo were individually transfected into (a,c) HepG2 or (b) Huh7 cells. The cell cycle was analyzed by flow cytometry 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

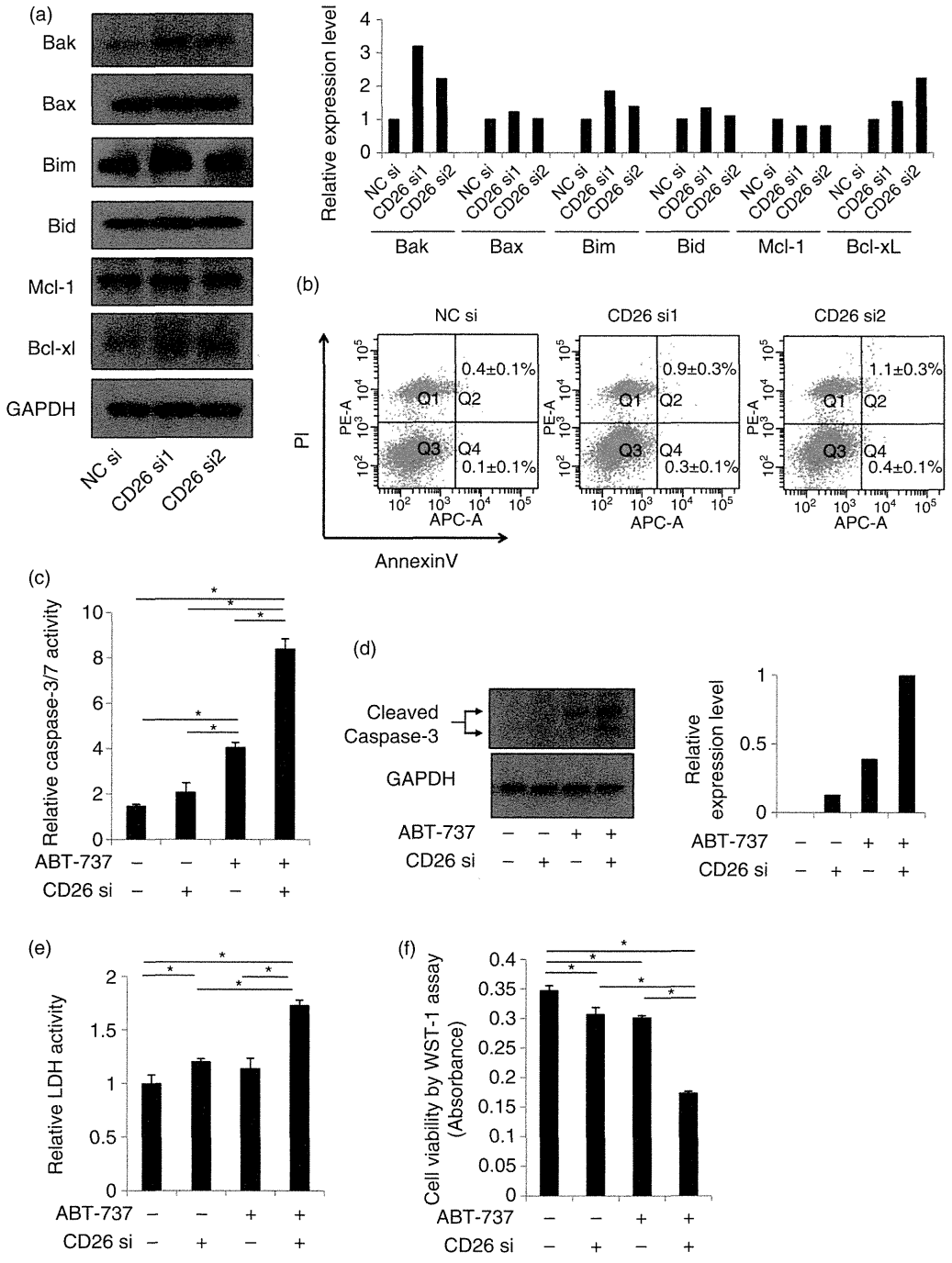

Figure 4 Synthetic lethal interaction of combined CD26 and Bcl-xL inhibition induces substantial apoptosis in hepatoma cells. Two different siRNA oligos targeting CD26 or a negative control siRNA oligo were individually transfected into Huh7 cells. (a) Western blotting of several Bcl-2 family proteins (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.

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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.