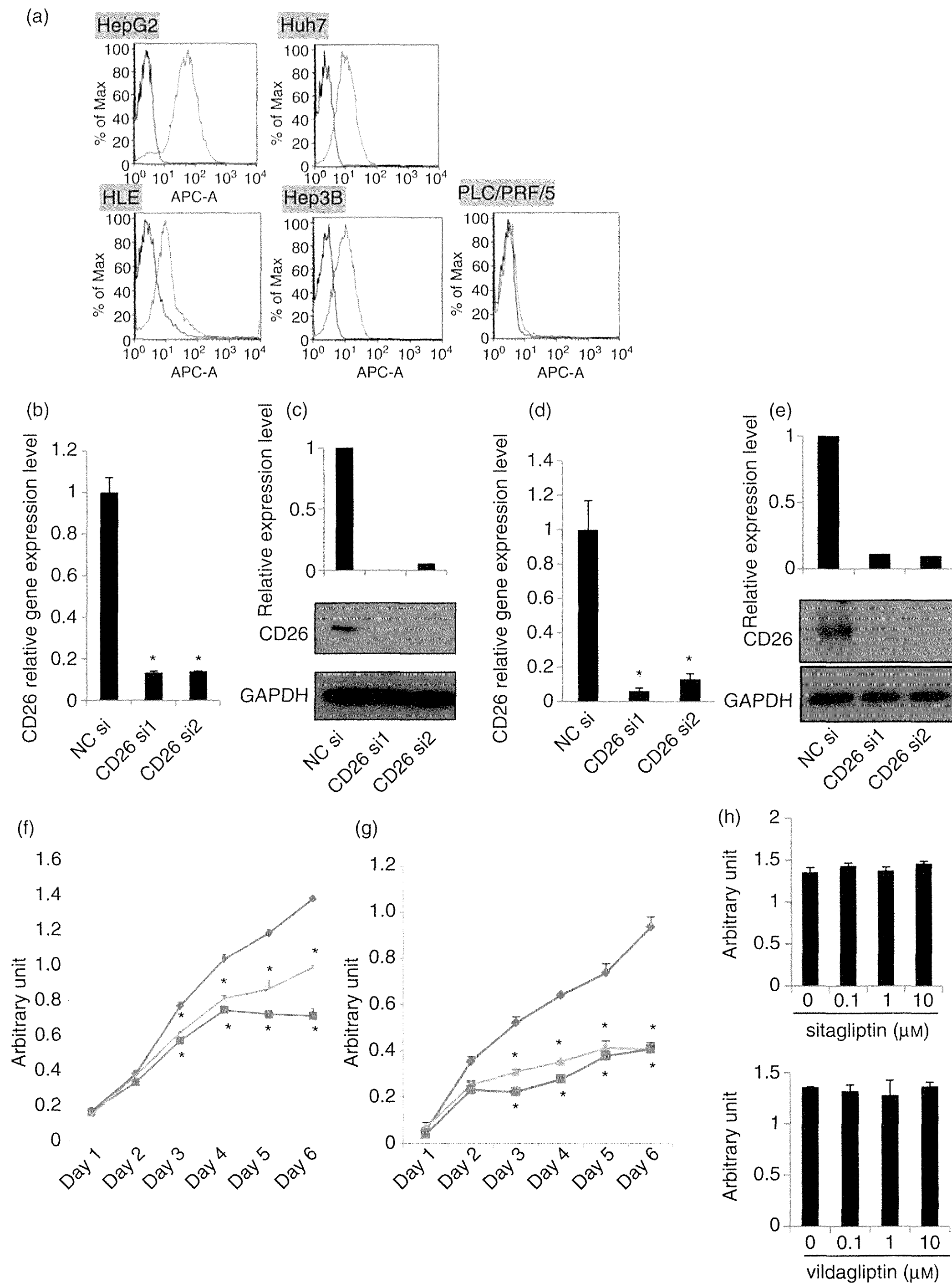

Figure 2 CD26 knockdown inhibits hepatoma cell growth. (a) CD26 expression levels were examined by flow cytometry in various hepatoma cell lines. (b–g) Two different siRNA oligos targeting CD26 or a negative control siRNA oligo were individually transfected into (b,c,f) HepG2 or (d,e,g) Huh7 cells. (b,d)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

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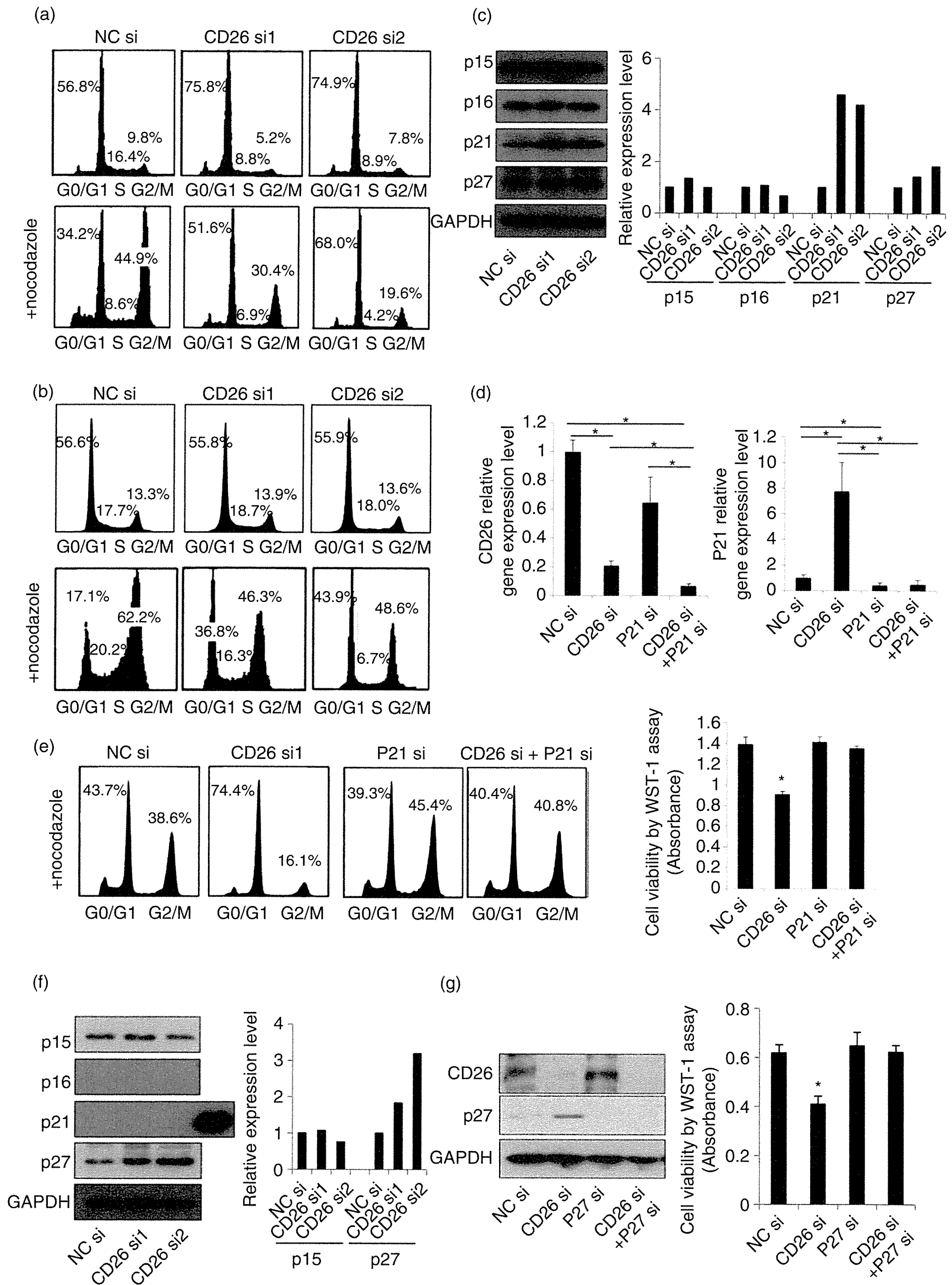

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Figure 3 CD26 knockdown induces cell cycle arrest at the G0/G1 phase via upregulation of Cip/Kip family proteins. (a–c) Two different siRNA oligos targeting CD26 or a negative control siRNA oligo were individually transfected into (a,c) HepG2 or (b)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

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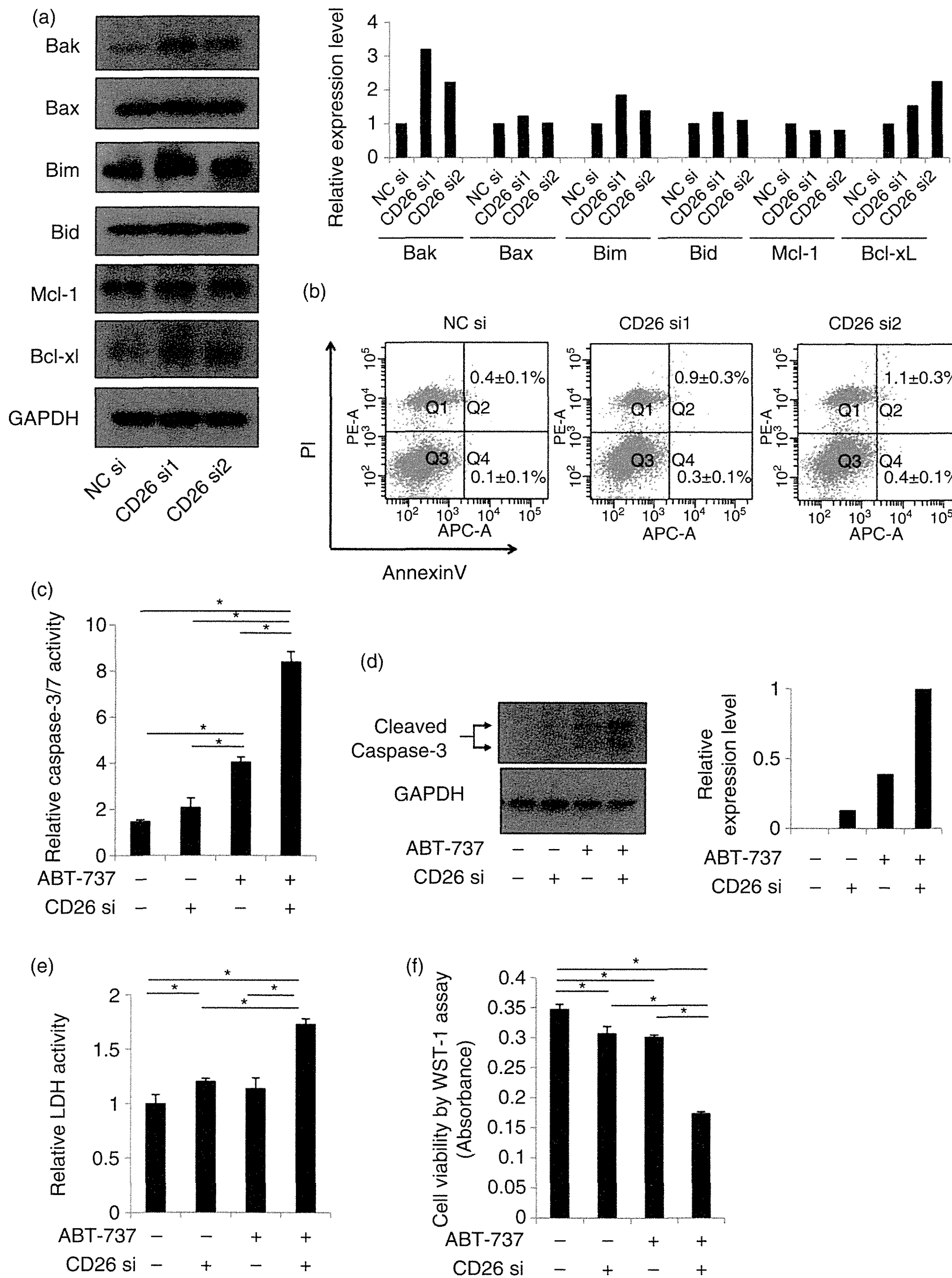

Figure 4 Synthetic lethal interaction of combined CD26 and Bcl-xL inhibition induces substantial apoptosis in hepatoma cells. Two different siRNA oligos targeting CD26 or a negative control siRNA oligo were individually transfected into Huh7 cells. (a) Western blotting of several Bcl-2 family proteins (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 (*$P < 0.05$).

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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.

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