Effects of fucoidan on proliferation, AMP-activated protein kinase, and downstream metabolism- and cell cycle-associated molecules in poorly differentiated human hepatoma HLF cells

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Abstract. Survival rates are low in patients with poorly differentiated hepatocellular carcinoma (HCC). Fucoidan, a sulfated polysaccharide derived from brown seaweed, has anticancer activity; however, the effects of fucoidan on poorly differentiated HCC remain unclear. In this study, we investigated the effects of fucoidan on AMP-activated protein kinase (AMPK), a proliferation regulator, and its downstream metabolism- and cell cycle-related molecules in a poorly differentiated human hepatoma HLF cell line. HLF cells were treated with fucoidan (10, 50, or 100 μg/ml; n=4) or phosphate buffered saline (control; n=4) for 96 h. Proliferation was evaluated by counting cells every 24 h. AMPK, TSC2, mTOR, GSK3β, acetyl-CoA carboxylase (ACC), ATP-citrate lyase, p53, cyclin D1, cyclin-dependent kinase (CDK) 4, and CDK6 expression and/or phosphorylation were examined by immunoblotting 24 h after treatment with 100 μg/ml fucoidan. Cell cycle progression was analyzed by fluorescence-activated cell sorter 48 h after treatment. Treatment with 50 or 100 μg/ml fucoidan significantly and dose- and time-dependently suppressed HLF cell proliferation (P<0.0001). Fucoidan induced AMPK phosphorylation on Ser172 24 h after treatment. Although no differences were seen in expression and phosphorylation levels of TSC2, mTOR, GSK3β, ATP-citrate lyase, and p53 between the control and fucoidan-treated HLF cells, fucoidan induced ACC phosphorylation on Ser79. Moreover, fucoidan decreased cyclin D1, CDK4 and CDK6 expression 24 h after treatment. Furthermore, HLF cells were arrested in the G1/S phase 48 h after fucoidan treatment. We demonstrated that fucoidan suppressed HLF cell proliferation with AMPK phosphorylation. We showed that fucoidan phosphorylated ACC and downregulated cyclin D1, CDK4 and CDK6 expression. Our findings suggest that fucoidan inhibits proliferation through AMPK-associated suppression of fatty acid synthesis and G1/S transition in HLF cells.

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Abbreviations: HCC, hepatocellular carcinoma; AMPK, adenosine monophosphate-activated protein kinase; p, phosphorylated; TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; eEF, eukaryotic elongation factor; eEF2K, eukaryotic elongation factor 2 kinase; GSK, glycogen synthase kinase; ATP, adenosine triphosphate; ACL, ATP-citrate lyase; ACC, acetyl-CoA carboxylase; Rb, retinoblastoma; CDK, cyclin-dependent kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate buffered saline

Key words: fucoidan, hepatocellular carcinoma, AMP-activated protein kinase, cyclin D1, acetyl-CoA carboxylase

Introduction

Hepatocellular carcinoma (HCC) is a common cancer with high morbidity and mortality rates (1). Although prognosis of patients with HCC has improved due to the development of techniques for early detection and treatment, the overall survival rates in patients with poorly differentiated HCC remain low (2).

Fucoidan is a water-soluble dietary fiber derived from brown seaweed. It is composed of polysaccharides containing L-fucose and sulfate ester groups (3). Fucoidan has various biological activities including anti-inflammatory, anti-viral and antihypertensive actions (4,5). Furthermore, fucoidan exerts anticancer activity and is reported to inhibit the proliferation of colon, breast and prostate cancer cell lines (6-8). Although fucoidan suppresses HCC proliferation, most of the cell lines used in previous studies, including HepG2 and Huh-7 cells, were well-differentiated (9-14). Thus, the anticancer activity of fucoidan against poorly differentiated HCC cells, such as the HLF cell line, remains unclear.

The mechanisms of fucoidan-mediated hepatoma inhibition have been investigated. However, Zhu *et al* reported that fucoidan does not inhibit vascular endothelial growth factor, basic fibroblast growth factor, interleukin-8, or heparanase expression in HCC cells and/or tumor tissues, and that it had no effect on angiogenesis and apoptosis *in vivo* (12). Thus, the mechanisms of fucoidan-mediated hepatoma inhibition remain unclear. Adenosine monophosphate-activated protein

kinase (AMPK) is a sensor of cellular energy and nutrient status and can crosstalk with signaling pathways that promote proliferation (15). Activated AMPK regulates molecules associated with protein, glucose and lipid metabolism and the cell cycle, thereby suppressing cancer proliferation (16,17). However, the effects of fucoidan on AMPK and its downstream molecules are unknown.

The aim of this study was to investigate the effects of fucoidan on the proliferation of the poorly differentiated HCC cell line HLF. Additionally, we examined the effects of fucoidan on AMPK and its downstream metabolism and cell cycle-associated molecules in HLF cells.

Materials and methods

Reagents and antibodies. Fucoidan from *Fucus vesiculosus* was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). All other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise indicated. Antibodies against AMPK, phosphorylated (p)-AMPK (Thr172), tuberous sclerosis complex (TSC) 2, p-TSC2 (Thr1462), mammalian target of rapamycin (mTOR), p-mTOR (Ser2448), p-eukaryotic elongation factor (eEF) 2 (Thr56), eukaryotic elongation factor 2 kinase (eEF2K), p-eEF2K (Ser366), glycogen synthase kinase (GSK) 3β, p-GSK3α (Ser21), p-GSK3β (Ser9), adenosine triphosphate (ATP)-citrate lyase (ACL), p-ACL (Ser454), acetyl-CoA carboxylase (ACC), p-ACC (Ser79), retinoblastoma (Rb), p16, p53, p-p53 (Ser9), p-p53 (Ser15), cyclin D1, cyclin-dependent kinase (CDK) 4, CDK6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against p21 were purchased from BD Biosciences (San Jose, CA, USA).

Cell lines. HLF cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies; Japan, Tokyo, Japan), penicillin (100 U/ml) and streptomycin (100 U/ml) at 37˚C in a humidified atmosphere containing 5% CO_2.

Fucoidan treatment. HLF cells were seeded ($1x10^4$ cells) in 10-cm dishes. Fucoidan was dissolved in phosphate buffered saline (PBS; 130 mM NaCl, 2 mM NaH_2PO_4 and 7 mM Na_2HPO_4, pH 7.4), and 10, 50 or 100 μg/ml of fucoidan was added 2 h after seeding. The culture medium containing fucoidan was replaced every 24 h, until 96 h.

Cell proliferation analysis. Cell proliferation was evaluated by counting cells. Cells were counted 0, 24, 48, 72 and 96 h after treatment with fucoidan (n=4 per condition) or PBS (control; n=4). Cells were trypsinized after washing with PBS. Then, cell numbers were determined using an automated cell counter (CDA-500; Sysmex Corp., Kobe, Japan) as previously described (18).

Immunoblotting analysis. After washes with PBS, cells were lysed in lysis buffer (50 mM HEPES, 250 mM NaCl, 20 mM EDTA and 0.1% Nonidet P-40, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor cocktail (Sigma-Aldrich Co. LLC.), 10 nM NaF and 1 mM Na_3VO_4. Cell lysates were centrifuged at 12,000 x g for 20 min at 4˚C, and the supernatant was collected. The protein concentration was determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The samples were then mixed with an equal volume of 2X sample loading buffer containing 2% sodium dodecyl sulfate (SDS) and 2-mercaptoethanol, and were incubated at 95˚C for 5 min. Samples were loaded on SDS-polyacrylamide gels and transferred onto equilibrated polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked for 1 h at room temperature with 5% skim milk, and then incubated with primary antibodies overnight at 4˚C. The membranes were washed and incubated with horseradish peroxidase-labeled secondary antibodies (GE Healthcare UK Ltd., Buckinghamshire, UK) for 1 h at room temperature. After several washes, the membranes were incubated with chemiluminescent reagents (ECL Advanced Western Blotting Detection kit; GE Healthcare UK Ltd.), and specific bands were visualized by an image analyzer LAS-1000 plus (Fuji Film, Tokyo, Japan) as previously described (19).

Cell cycle analysis. After treatment with PBS or 100 μg/ml fucoidan for 24 or 48 h, the cells were trypsinized, washed with PBS, and incubated in 0.2% Triton X-100 for 20 min at 37˚C. Then, DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACS-Calibur (Becton Dickinson, Franklin Lakes, NJ, USA). The flow cytometry data were collected, and cell cycle distributions were analyzed with Cell Quest software (Becton Dickinson) as previously described (20).

Statistical analysis. All data are expressed as the mean ± SD. Comparisons among multiple groups were made using one-way analyses of variance, followed by Fisher's protected least-significant-difference post-hoc test as previously described (21). A P-value <0.05 was considered statistically significant.

Results

Effects of fucoidan on HLF cell proliferation. The cell number did not differ between the control cells and those treated with 10 μg/ml fucoidan. However, treatment with 50 and 100 μg/ml fucoidan significantly and dose- and time-dependently suppressed cell proliferation (Fig. 1A and B, P<0.0001). Cell number was ~50% lower 96 h after treatment with 100 μg/ml fucoidan, as compared to the controls ($22.4\pm1.8x10^5$ vs. $0.9\pm0.2x10^5$ cells/dish; Fig. 1A and B). Cell viability was evaluated by trypan blue staining. Cell death was rarely observed beyond 96-h fucoidan treatment at any dose (data not shown).

Effects of fucoidan on AMPK expression and phosphorylation in HLF cells. After 24 h of treatment with PBS or 100 μg/ml fucoidan, AMPK expression and Ser172 phosphorylation were evaluated by immunoblotting. Although AMPK protein expression level did not differ between the controls and fucoidan-treated HLF cells, treatment with fucoidan enhanced Ser172 phosphorylation, as compared to control cells (Fig. 2).

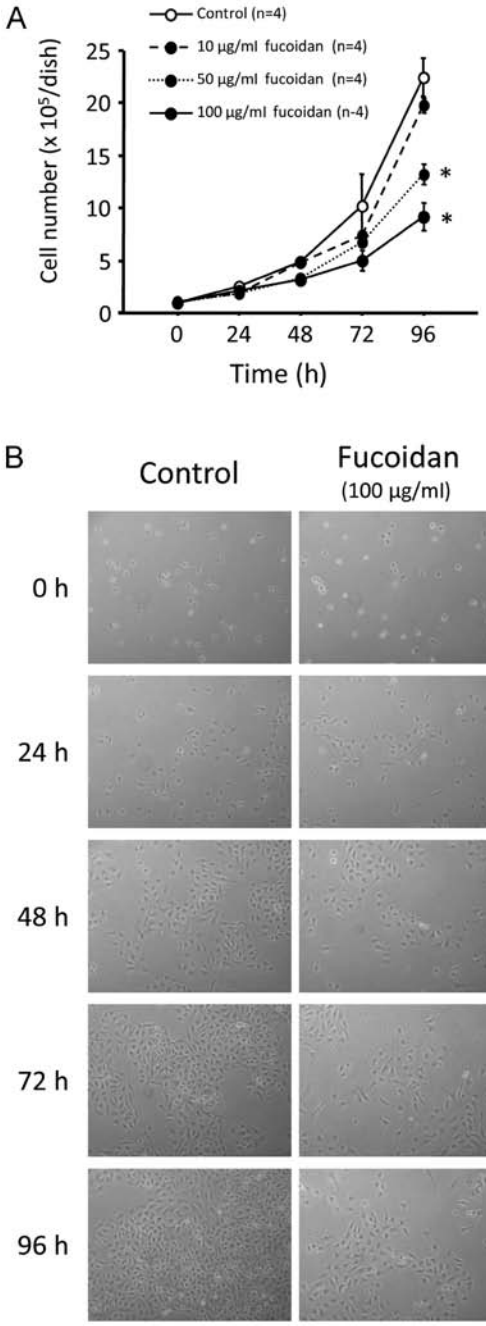

Figure 1. Effects of fucoidan on HLF cell proliferation. (A) HLF cells were treated with PBS (control) or fucoidan (10, 50, or 100 μg/ml). Cells were counted every 24 h, up to 96 h after initial treatment. *P<0.0001 as compared to the control. (B) Representative phase-contrast microscopic images of control and fucoidan (100 μg/ml)-treated HLF cells 0, 24, 48, 72 and 96 h after treatment.

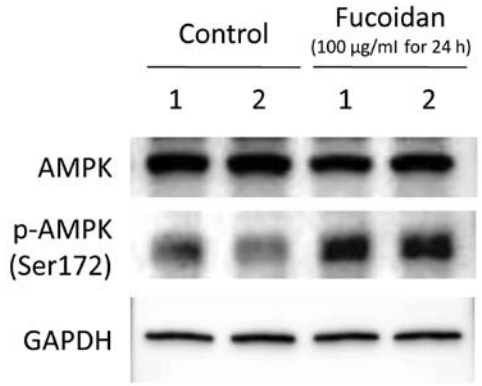

Figure 2. Effects of fucoidan on AMPK expression and phosphorylation. After 24 h of treatment with PBS (control) or fucoidan (100 μg/ml), AMPK and p-AMPK (Ser172) expression in HLF cells was examined by immunoblotting. GAPDH was used as a loading control.

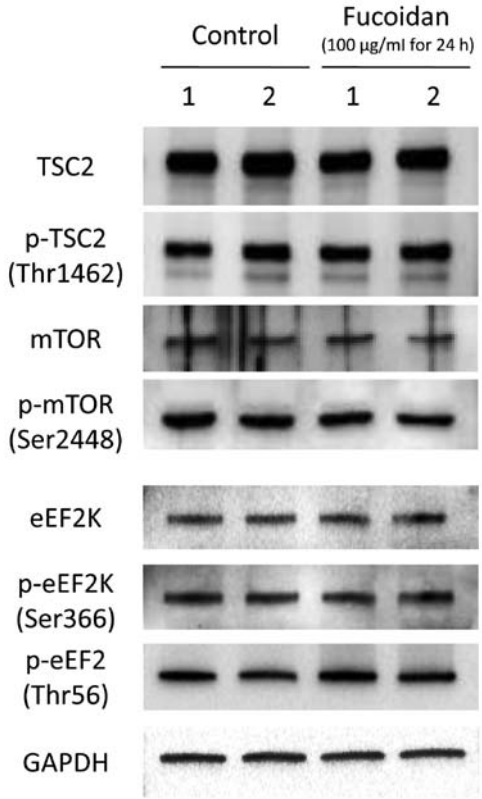

Figure 3. Effects of fucoidan on metabolism-associated molecule expression. After 24 h of treatment with PBS (control) or fucoidan (100 μg/ml), TSC2/mTOR and eEF2/eEF2K expression and phosphorylation in HLF cells were examined by immunoblotting. GAPDH was used as a loading control.

Effects of fucoidan on metabolism-associated molecule expression and phosphorylation in HLF cells. The effects of fucoidan on metabolism-associated molecules, such as TSC2/mTOR and eEF2/eEF2K were examined by immunoblotting. TSC2 and mTOR expression was not altered by fucoidan treatment (Fig. 3). Furthermore, fucoidan treatment did not affect TSC2 Thr1462 or mTOR Ser2448 phosphorylation in HLF cells (Fig. 3). Similarly, eEF2K expression and EF2K Ser366 and eEF2 Thr56 phosphorylation were not altered by fucoidan treatment (Fig. 3).

Effects of fucoidan on glucose and lipid metabolism-associated molecule expression in HLF cells. We next assessed the effects of fucoidan on the expression and phosphorylation of glucose and lipid metabolism-associated molecules, including GSK3β, ACL and ACC, by immunoblotting. The expression of GSK3β, and the phosphorylation of GSK3β at Ser9 and GSK3α at Ser21 were not altered by fucoidan treatment (Fig. 4). Additionally, ACL expression and phosphorylation at Ser366 did not differ between the control and fucoidan-treated HLF cells (Fig. 4). Although there was no difference in ACC expression after

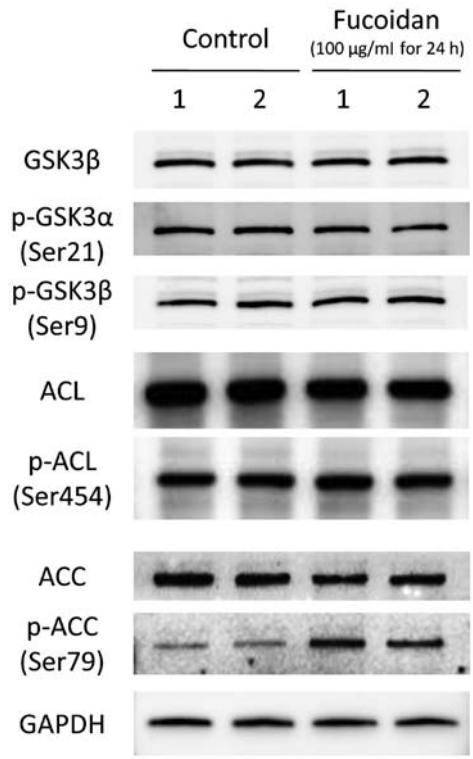

Figure 4. Effects of fucoidan on glucose and lipid metabolism-associated molecule expression and phosphorylation. After treatment with PBS (control) or fucoidan (100 μg/ml) for 24 h, GSK3β, GSK3α, ACL, and ACC expression and phosphorylation in HLF cells were examined by immunoblotting. GAPDH was used as a loading control.

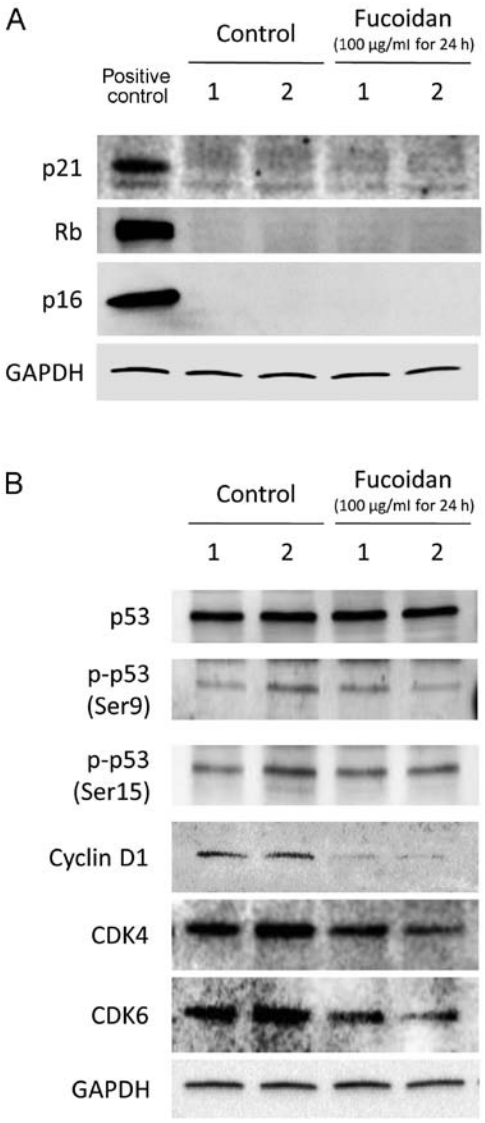

Figure 5. Effects of fucoidan on cell cycle-associated molecule expression and phosphorylation. The effects of fucoidan on (A) expression of Rb, p21 and p16 and (B) expression of p53, cyclin D1, CDK4 and CDK6, and phosphorylation of p53 at Ser9 and Ser15 in HLF cells were examined by immunoblotting. GAPDH was used as a loading control.

fucoidan treatment, fucoidan enhanced ACC phosphorylation at Ser79 (Fig. 4).

Effects of fucoidan on cell cycle-associated molecule expression and phosphorylation in HLF cells. The effects of fucoidan on cell cycle-associated molecule expression and phosphorylation were examined by immunoblotting. Fucoidan did not alter p21, Rb and p16 expression in HLF cells (Fig. 5A). Furthermore, p53 expression and phosphorylation at Ser9 and Ser15 were similar in the control and fucoidan-treated HLF cells (Fig. 5B). In contrast, fucoidan decreased the expression of cyclin D1, CDK4 and CDK6 (Fig. 5B).

Effects of fucoidan on HLF cell cycle. At 24 h after initial treatment, there was no significant difference in cell cycle between the control and fucoidan-treated HLF cells (Fig. 6). However, 48 h after initial treatment, the percentage of cells in the G0/G1 phase was 57.4 and 29.3%, that in the S phase was 18.9 and 57.0%, and that in the G2/M phase was 23.7 and 13.7% in the control and fucoidan-treated HLF cells, respectively (Fig. 6). Thus, 100 μg/ml fucoidan treatment arrested HLF cells in the G1/S phase. Apoptosis was not observed in the control or fucoidan-treated HLF cells (Fig. 6).

Discussion

We demonstrated that fucoidan suppressed the proliferation of HLF cells, a poorly differentiated HCC. We also showed that fucoidan induces AMPK accompanied by ACC phosphorylation and downregulation of cyclin D1, CDK4 and CDK6 expression. These findings suggest that fucoidan inhibits proliferation via AMPK-associated suppression of fatty acid synthesis and G1/S transition in HLF cells.

In this study, we showed that fucoidan suppressed proliferation, but not apoptosis, in HLF cells. In contrast, previous studies reported that fucoidan induces apoptosis in several human HCC cell lines, including HepG2, Huh7 and SMMC-7721 cells (9,11,13,14). It is unclear why fucoidan did not induce apoptosis in HLF cells; however, one possibility is that there are different phenotypes between HCC cell lines. HLF cells were established from poorly differentiated HCC (22) and are resistant to apoptosis due to low tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 expression (23). Thus, the mechanisms mediating fucoidan-induced proliferation suppression may differ depending on HCC phenotype.

AMPK is a central regulator of proliferation and exerts its effects via the regulation of metabolism and the cell

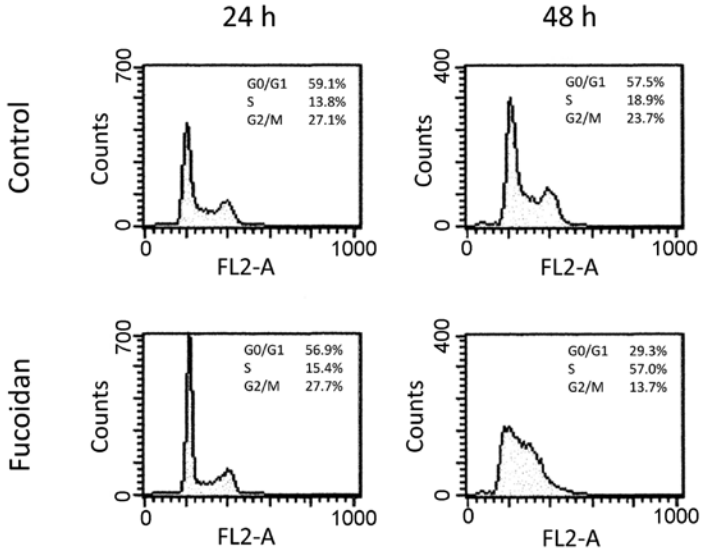

Figure 6. Effects of fucoidan on HLF cell cycle. After treatment with PBS (control) or fucoidan (100 μg/ml) for 24 or 48 h, cell cycle progression was assessed based on DNA content by using flow cytometry.

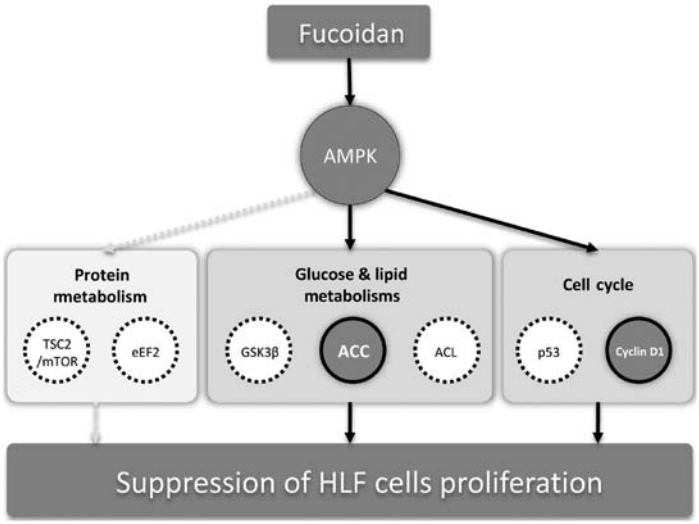

Figure 7. Proposed molecular mechanisms for fucoidan-induced suppression of HLF cell proliferation.

cycle. Thus, AMPK is a potential therapeutic target for cancer (16,17). However, the effects of fucoidan on AMPK have not been reported. We first demonstrated that fucoidan phosphorylated AMPK in HLF cells. Although the mechanisms regulating fucoidan-induced AMPK activation remain unclear, AMPK is a member of the metabolite-sensing protein kinase family and is activated by depletion of intracellular ATP levels (16,17). Fucoidan causes mitochondrial dysfunction in cancer cells (24). Since mitochondria produce ATP, fucoidan may activate AMPK through depletion of ATP via mitochondrial injury.

Phosphorylated AMPK inhibits protein synthesis, thereby suppressing cell proliferation through downregulation of the TCS2/mTOR (17) and eEF2/eEF2K pathways (25). Additionally, Lee *et al* reported that fucoidan inhibits human lung cancer cell migration and invasion via mTOR regulation (26). However, fucoidan did not affect the TCS2/mTOR or eEF2/eEF2K pathways in this study, suggesting that impairment of protein synthesis is not a major mechanism for fucoidan-induced suppression of HLF cell proliferation.

Phosphorylated AMPK also inhibits gluconeogenesis and lipogenesis, thereby suppressing proliferation through GSK3β, ACL and ACC (10,17,27). In this study, fucoidan did not affect the expression or phosphorylation of GSK3β and ACL; however, fucoidan induced ACC phosphorylation at Ser79. Phosphorylation at Ser79 inhibits malonyl-CoA production, an initial step of fatty acid synthesis (28). Various cancer cells exhibit a markedly increased rate of *de novo* fatty acid synthesis (17). Cancer cells activate fatty acid biosynthesis to sustain an increasing demand for phospholipids for membrane biogenesis (29). Moreover, high levels of arachidonic acid can promote breast cancer cell proliferation (30). Taken together, the suppression of fatty acid synthesis through AMPK-induced ACC phosphorylation may mediate fucoidan-induced proliferation suppression (Fig. 7). AMPK activation phosphorylates ACC, resulting in the suppression of

prostate, breast and ovarian cancer cell proliferation (31-33), supporting our hypothesis.

AMPK is reported to regulate proliferation via the cell cycle machinery (17). Phosphorylated AMPK suppresses proliferation through p21, Rb, p16 and p53 (17). However, these molecules were not expressed in HLF cells, and fucoidan did not alter the expression or phosphorylation of p53. In contrast, fucoidan downregulated cyclin D1, CDK4 and CDK6 expression in HLF cells. Cyclin D1 is required for cell cycle G1/S transition (34) and G1/S arrest was seen in this study. Sikka *et al* reported that AMPK activation downregulates cyclin D1, CDK4 and CDK6, thereby inhibiting squamous cancer cell proliferation (35). Similar findings were also reported in myeloma cells (36). Moreover, Banafa *et al* demonstrated that fucoidan decreases cyclin D1 and CDK4 gene expression in human breast cancer MCF-7 cells (37). Thus, our findings, along with previous studies, suggest that AMPK-induces G1/S phase arrest via downregulation of cyclin D1, CDK4 and CDK6, thereby mediating fucoidan-induced suppression of HLF cell proliferation (Fig. 7).

In conclusion, we showed that fucoidan inhibited the proliferation of HLF cells, a poorly differentiated HCC. In addition, we demonstrated that fucoidan phosphorylated AMPK accompanied by phosphorylation of ACC and downregulates cyclin D1, CDK4 and CDK6 expression. These findings suggest that fucoidan suppresses proliferation via AMPK-mediated inhibition of fatty acid synthesis and cell cycle G1/S transition in HLF cells.

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