The anti-tumor activity of brown seaweed oligo-fucoidan via lncRNA expression modulation in HepG2 cells

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Abstract Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death in Asia. HCC is less sensitive to chemotherapy and is known to express multidrug resistant genes to acquire resistance to chemotherapeutic agents, therefore the development of a potent HCC suppressor is essential in treating HCC. Our previous reports demonstrated that oligo-fucoidan from the brown seaweed *Sargassum hemiphyllum* elevates microRNA-29b to inhibit epithelial-mesenchymal transition in hepatoma cells. In this study, we aimed to examine in vitro effect of oligo-fucoidan in hepatocellular carcinoma through apoptosis and long noncoding RNA (lncRNA) pathway. Oligo-fucoidan was studied for its anti-hepatoma cells by MTT and DNA ladder analysis. And the mechanism was studied by flow cytometry, qPCR and western blot analysis. In this study, oligo-fucoidan

induced sub-G1 phase cell cycle arrest and activation of caspases, indicating that the intrinsic and extrinsic apoptotic pathways were involved in the mechanism of oligo-fucoidan-induced cell death. Moreover, oligo-fucoidan significantly increased the expression of p53, p21, and p27, while cyclin-B1 and -D1 were decreased at the mRNA and protein levels. Finally, we showed that targeting apoptosis and cell cycle pathways could also contribute to the induction of the lncRNA-Saf and lncRNA-p21. Through human lncRNA profiler array analysis, the differential expression of lncRNAs in HCC cells following oligo-fucoidan exposure was further examined. These findings indicated that lncRNAs switched oligo-fucoidan-induced apoptosis, which might be potentially valuable in HCC adjuvant therapy.

Keywords Fucoidan · Hepatocellular carcinoma · Apoptosis · lncRNA-Saf · lncRNA-p21

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third most common cause of cancer-related death in Asia. With approximately 1% of all deaths related to HCC, it has one of the highest mortality rates worldwide (Kew 2010). Surgery is considered to be the first choice for HCC treatment, but some patients cannot undergo

surgery due to poor liver function, and only 10%–25% resectability rate for HCC patients (Nagasue et al. 1993). Furthermore, HCC is less sensitive to chemotherapy and is known to express multidrug resistant genes to acquire resistance to chemotherapeutic agents, which is one of the important obstacles in successful chemotherapy treatment against HCC (Kato et al. 2001). The development of a potent HCC suppressor is therefore essential in treating HCC.

Fucoidan is the general term for a class of sulfated and fucosylated polysaccharides found in brown seaweed. The intensity of the biological activities of fucoidan varies depending on species, molecular weight, composition, structure, and method of extraction (Fitton 2011). Our previous reports demonstrated that oligo-fucoidan from *Sargassum hemiphyllum* acts against breast and lung cancer through degradation of transforming growth factor- β receptor and inhibition of epithelial-mesenchymal transition (EMT) (Hsu et al. 2013, 2014). It also ameliorates tumor- and chemotherapy-induced skeletal muscle atrophy in bladder cancer-bearing mice (Chen et al. 2016). Moreover, Chen et al. (2014) and Park et al. (2014) have reported that fucoidan induces apoptosis to eliminate breast cancer, colon cancer, and urinary bladder cancer cells. However, the precise regulatory mechanisms by which oligo-fucoidan induces apoptosis are not completely clear. Furthermore, oligo-fucoidan displays no toxicological indications over 28 days of repeated oral administration to rats and exhibits no mutagenicity (Hwang et al. 2016). Due to its low toxicity and antitumor activity, oligo-fucoidan represents a potentially valuable candidate for HCC chemotherapy.

The nonprotein-coding RNAs (ncRNAs) have become important in understanding the basic biology and major pathologies of cancer (Naeini and Ardekani 2009). The ncRNAs can be divided into two groups, namely small noncoding RNAs, such as small interfering RNAs (siRNAs) and microRNAs (miRNAs; < 200 bp) and long noncoding RNAs (lncRNAs; > 200 bp) (Gomes et al. 2013). Many studies report that miRNAs exert various effects in preventing cancer progression by apoptosis induction (Xiong et al. 2010), and that lncRNAs are associated with HCC (Yu et al. 2015). Some dietary natural agents such as curcumin, resveratrol, and genistein have been shown to exert their apoptotic effects in cancer cells through the regulation of one or more miRNAs (Phuah and

Nagoor 2014). Moreover, we recently found that oligo-fucoidan increases miRNA-29b levels to regulate the DNMT3B-MTSS1 axis and inhibit EMT in HepG2 human HCC cells (Yan et al. 2015). Compared with siRNAs and miRNAs, the role of lncRNA in dietary natural agents for cancer treatment remains limited.

In this study, the effects and mechanism of oligo-fucoidan in HCC cells were investigated, and we further examined the differential expression of lncRNAs in HCC cells following oligo-fucoidan exposure. Our results indicated that lncRNAs might be used as novel biomarkers and that oligo-fucoidan may represent a natural dietary supplement for patients with HCC.

Materials and methods

Oligo-fucoidan preparation

Oligo-fucoidan was a gift from Hi-Q Marine Biotech International Ltd (Xinbei, Taiwan). Fresh *S. hemiphyllum* was collected from the coast of Penghu County, Taiwan during a period from February to April, 2015. The specie was identified by Dr. Huei-Meei Su (Fisheries Research Institute, Council of Agriculture, Keelung, Taiwan) with morphology, and collection code was FA37640 in Taiwan Biodiversity Information Facility (<http://taibif.tw/zh/namecode/123312>). According to the information provided by the manufacturer: To prepare the oligo-fucoidan, fresh dried *S. hemiphyllum* (100 g) was added in 5 l distilled water and boiled at 100 °C for 30 min. Then, the hot water extract was centrifuged at 5000×g for 15 min and lyophilized under the reduced pressure followed by addition of 4 volumes of 95% ethanol for overnight at 4 °C, and isolated the sulfate rich portions by ion exchange gel as fucoidan. For hydrolysis of fucoidan, 5 g of fucoidan (about 200–260 kDa) was suspended in 125 ml distilled water at 55 °C with at 20×g stirring speed followed by addition of glycolytic enzyme at a concentration of 1 mg/g fucoidan for 6 h. After centrifugation at 10,000×g for 20 min at 4 °C, the supernatants were passed through a 30 kDa molecular weight cut-off membrane (ProStream™ PP, TangenX Technology Co., Boston, MA, USA) and the filtrate was further passed through a 1 kDa molecular weight cut-off membrane. The characteristics of oligo-

fucoidan were average molecular weight of 0.8 kDa (92.1%) (Hwang et al. 2017), fucose content $210.9 \pm 3.3 \mu\text{mol/g}$ (Hwang et al. 2011), and sulfate content $38.9 \pm 0.4\%$ (w/w) (Hung et al. 2010), and we named it as sulfate rich oligosaccharide. It was dissolved in phosphate-buffered saline (PBS) and completely dissolved by stirring at room temperature for 30 min.

Cell culture

HepG2 cell, the human liver cancer cell line was obtained from the American Type Culture Collection. L02 cell, the normal human liver cell line was obtained from Dr. Ya-Wen Lin's laboratory (National Defense Medical Center, Taipei, Taiwan). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin at 37 °C in an atmosphere of 5% CO₂. The medium was renewed every 2 days. Cells grown in a medium containing PBS without Oligo-fucoidan served as a control (0 $\mu\text{g/ml}$ oligo-fucoidan).

Cell viability

Cell viability was analyzed using the MTT assay. Cells were seeded 2×10^3 cells per well of 96-well plate and incubated for 48 h with or without different concentrations of oligo-fucoidan. At the required time point, cells were reacted with MTT (1 mg/ml) for 4 h, and absorbance were recorded at 570 nm. The cell viability (%) was determined as $(A1/A0) \times 100\%$, where A0 and A1 were the absorbance of control group (0 $\mu\text{g/ml}$ oligo-fucoidan) and oligo-fucoidan group (50, 100 and 200 $\mu\text{g/ml}$), respectively.

DNA ladder analysis

HepG2 cells (2×10^3 cells per well) were treated with or without oligo-fucoidan for 48 h. The fragmented DNA was selectively extracted using a K170-50 Apoptotic DNA Ladder Isolation Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. The extracted DNA samples were separated on a 1.5% agarose gel containing 0.1 $\mu\text{g/ml}$ of ethidium bromide (EtBr) in Tris–acetic acid–ethylene-diaminetetraacetic acid (EDTA) buffer at 100 V, and electrophoresis was carried out.

Cell cycle analysis

Evaluation of the cell cycle distribution and sub-G1 peaks by flow cytometric analysis. HepG2 cells (2×10^3 cells per well) were treated with or without oligo-fucoidan for 48 h. After 48 h, cells were collected by trypsin, washed in cold PBS, and fixed with 70% ethanol on ice for 1 h. After removing the ethanol by washing with PBS, the cells were incubated in 10 mg/ml RNase A for 30 min at room temperature and then in 20 mg/ml propidium iodide (PI) for 10 min in the dark. Cells were then analyzed by flow cytometry (Becton, Dickinson and Company).

Caspase-3, -8 and -9 activities analysis

Activities of caspase-3, -8 and -9 were tested with Caspase Colorimetric Protease Assay Sampler Kit according to the manufacturer's instructions (Invitrogen, Frederick, MD, USA). HepG2 cells (2×10^3 cells per well) were treated with or without oligo-fucoidan for 48 h. After 48 h, cells were washed twice in ice-cold PBS and lysis. The cell lysates were incubated with DEVD-pNA (for caspase-3), IETD-pNA (for caspase-8) and LEHD-pNA (for caspase-9) and the release of pNA was measured at 405 nm.

Western blot analysis for apoptosis related proteins

HepG2 cells (2×10^3 cells per well) were treated with or without oligo-fucoidan for 48 h, and cellular proteins were extracted using a lysis buffer [10 mM of Tris–HCl pH 7.8, 5 mM MgCl₂, 0.3 mM of ethylene glycol tetraacetic acid (EGTA), 10 mM KCl, 1 mM of dithiothreitol, 0.1 mM of phenyl-methylsulfonylfluoride (PMSF), and protease inhibitors]. The protein contents was determined by the Bio-Rad detergent compatible protein assay (Bio-Rad, CA, USA). Fifty micrograms of cellular protein were separated on an SDS-15% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, CA, USA). The immunoblot was incubated overnight with blocking solution (5% skim milk in pH 7.5, 20 mM Tris-150 mM NaCl-0.2% Tween 20) and followed by incubation at 4 °C overnight with primary antibody. The secondary antibody was incubated with the membrane at room temperature for 1 h. Expression of protein was detected by staining with NBT/BCIP (Pierce, USA).

and quantitated by densitometric analysis (Pharmacia, ImageMaster VDS, Sweden). Primary antibodies, anti-Bcl-2, anti-Bid, anti-Bax, anti-Bad, anti-cleaved PARP (89 kDa), anti-p53, anti-p21, anti-p27, anti-cyclin (Cyc)-B1, anti-Cyc-D1 and anti-GAPDH (BioVision, USA), were applied at 1:1000 dilution. Secondary antibody, anti-mouse IgG-peroxidase from goat (Sigma, USA), was applied at 1:5000 dilutions. Images of resulting gels were captured and analyzed by ImageMaster VDS and ImageMaster 1D Elite software (Amersham Pharmacia Biotech, Sweden).

RNA isolation and quantitative real time PCR (qPCR) assay for apoptosis related genes

HepG2 cells (2×10^3 cells per well) were treated with or without oligo-fucoidan for 48 h. Total RNA was isolated by RNeasy B (Amersham Pharmacia Biotech, Sweden), and the concentration of total RNA was detected by spectrophotometer (Hitachi, Japan). The synthesis of complementary DNA (cDNA) was using Improm-II TM Reverse Transcriptase (Promega, WI, USA) according to the manufacturer's instructions. Primer sequences used to amplify the desired cDNA were as Table 1. Above primers were purchased from Mission Biotech Co., Ltd. (Taipei, Taiwan). The cycling conditions were 40 cycles consisting of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s and extension at 72 °C for 18 s. Gene expression fold was determined by qPCR (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany) using SYBR Green as the fluorophore (Molecular Probes, Eugene, OR, USA). The level

mRNA expression was calculated as the relative intensity of the qPCR product compared with that from GAPDH gene using the $2^{-\Delta\Delta C_t}$ method. All qPCR experiments were performed in triplicate.

LncRNA array

Based on the protocol of the Human lncRNA Profiler™ qPCR Array Kit (SBI, Mountain View, CA, USA), which sets to quantitate 90 different lncRNAs, 5 positive housekeeping reference controls and one negative control. One μg RNA was mixed with PolyA buffer, MnCl_2 , ATP, and PolyA polymerase and incubated for 30 min at 37 °C, and then added Oligo dT adapter heating for 5 min at 60 °C. Following, RT buffer, dNTP, DTT, random primer mix, and reverse transcriptase were added into the mixture and incubated for 1 h at 42 °C for synthesis lncRNA cDNA. Finally, the lncRNA cDNA was heated for 10 min at 95 °C and stored at -80 °C for further study. Mastermix reagent (contained SYBR Green qPCR Mastermix buffer, lncRNA cDNA and RNase-free water) and lncRNA primer were mixed for qPCR assay, and cycling conditions was mentioned above. The qPCR array plate contains assays for 90 lncRNAs and also includes 5 endogenous reference RNAs as normalization signals. The relative lncRNA level was calculated according to the $2^{-\Delta\Delta C_t}$ method as compared to endogenous reference RNAs.

Table 1 Primer sequences and reaction conditions for quantitative real time PCR assay of apoptosis related genes

Gene name	Oligonucleotide sequences	Product size (bp)
p53	F ¹ : CAGCACATGACGGAGGTTGT R ² : TCATCCAAATACTCCACACGC	125
p21	F: TGTCCTGTCAGAACCCATGC R: AAAGTCGAAGTTCATCGCTC	139
p27	F: AACGTGCGAGTGTCTAACGG R: CCCTCTAGGGGTTTGTGATTCT	209
Cyc-B1	F: AATAAGGCGAAGATCAACATGGC R: TTTGTTACCAATGTCCCCAAGAG	111
Cyc-D1	F: GCTGCGAAGTGGAACCATC R: CCTCCTTCTGCACACATTTGAA	135
GAPDH	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG	197

¹Forward primer for sequence

²Reverse primer for sequence

Statistical analysis

Numerical data are presented as means $\pm$ standard deviation. The data was analyzed by a one-way analysis of variance (ANOVA) and followed by the Least Significant Difference test using SPSS (Chicago, IL, USA) version 10 software.

Results and discussion

Oligo-fucoidan suppressed cell viability in HepG2 cells

Since apoptosis is characteristic of antitumor substance-induced cell death, we first examined the cell viability of HepG2 human liver cancer cells and L02 normal human liver cells when treated with oligo-fucoidan. Through MTT assays, 50, 100, and 200 $\mu\text{g/ml}$ oligo-fucoidan were observed to significantly inhibit HepG2 cell growth by $81.34\% \pm 4.84\%$, $65.03\% \pm 1.64\%$, and $56.36\% \pm 2.64\%$, respectively. Thus, cell viability was gradually reduced by Oligo-fucoidan treatment in a dose-dependent manner in HepG2 cells. In contrast, oligo-fucoidan did not inhibit the growth of L02 normal human liver cells at the same doses (Fig. 1a). It has been suggested that the suppression of cell growth by oligo-fucoidan was selective for cancer cells. Changes in HepG2 cell morphology were observed by inverted microscopy under $200\times$ magnification. The average number of HepG2 cells was significantly less than that in control cells following oligo-fucoidan treatment. HepG2 cells treated with oligo-fucoidan also exhibited the typical characteristics of apoptosis, including smaller size, single cells, or small clusters of cells (Fig. 1b). Oligo-fucoidan clearly demonstrated cytotoxicity to HepG2 cells.

Oligo-fucoidan induced apoptosis cell death in HepG2 cells

Apoptosis is one of the factors contributing to cell proliferation inhibition, with internucleosomal DNA fragmentation being a hallmark of apoptosis in mammalian cells. Therefore, we examined internucleosomal DNA fragmentation by DNA ladder analysis. After treatment with oligo-fucoidan for 48 h, oligo-fucoidan induced internucleosomal DNA

fragmentation (Fig. 2a). We further investigated whether induction of apoptosis by oligo-fucoidan was associated with cell cycle arrest. The cell cycle distribution was analyzed using flow cytometry. As shown in Fig. 2b, the percentages of sub-G1 phase apoptotic HepG2 cells significantly increased following treatment with 50, 100, and 200 $\mu\text{g/ml}$ oligo-fucoidan to $39.5\% \pm 3.4\%$, $56.8\% \pm 6.1\%$, and $64.2\% \pm 5.4\%$, respectively, compared with $4.8\% \pm 1.3\%$ in the control (0 $\mu\text{g/ml}$ oligo-fucoidan). This data suggested that oligo-fucoidan induces sub-G1 phase cell cycle arrest at concentrations as low as 50 $\mu\text{g/ml}$.

Oligo-fucoidan induced apoptosis in HepG2 cells through caspase activation

The enzyme responsible for apoptotic DNA fragmentation is the caspase-activated DNase (CAD). Under normal conditions, CAD is inhibited by inhibitor of caspase-activated DNase (ICAD). During apoptosis, initiator caspase-8 and -9 activate effector caspase-3 to cleave ICAD and releases DNase activity, causing DNA fragmentation in the nuclei (Enari et al. 1998). To assess whether the oligo-fucoidan-induced apoptosis in HepG2 cells was caspase dependent, the enzyme activities of caspase-8, -9 and -3 were analyzed using commercial colorimetric assays. As shown in Fig. 3, 100 and 200 $\mu\text{g/ml}$ oligo-fucoidan significantly elevated activity of caspase-8 and -9 and then downregulated the activity of caspase-3. Our results suggested that oligo-fucoidan induced activation of caspase-3 through upregulation of caspase-8 and -9, indicating that the caspase apoptotic pathway is involved in the mechanism of oligo-fucoidan-induced cell death.

Effects of oligo-fucoidan on apoptosis related protein in HepG2 cells

Bid is a pro-apoptotic member of the Bcl-2 family that, as an initiator, interacts with pro-apoptotic Bax, Bad, and anti-apoptotic Bcl-2. Bax is required for mitochondrial outer membrane permeabilization to induce the no return form of apoptotic cell death (Billen et al. 2008). Since the permeability of mitochondrial membrane makes cell apoptosis, we subsequently attempted to determine whether or not oligo-fucoidan treatment induces changes in the levels of Bcl-2 family

Fig. 1 The effects of oligo-fucoidan on cell viability. **a** HepG2 cells and immortalized normal hepatocytes L02 cells were incubated for 48 h with various concentrations of oligo-fucoidan (0, 50, 100, and 200 µg/ml), and the proliferation of cells was measured using an MTT assay. Values are expressed as the mean $\pm$ standard error of three independent experiments.

proteins, including Bcl-2, Bid, Bax, and Bad. Oligo-fucoidan was found to activate Bid then suppressed Bcl-2 expression and increased pro-apoptotic Bax and Bad expression (Fig. 4). Full-length poly (ADP-ribose) polymerase (PARP) is a 116 kDa protein involved in the repair of DNA. During apoptosis, PARP is cleaved by caspase-3 into an 89-kDa fragment, thereby losing its function (Lazebnik et al. 1994). Thus, cleaved PARP is a useful marker for apoptosis. As shown in Fig. 4, oligo-fucoidan treatment resulted in increased levels of cleaved PARP in HepG2 cells.

Effects of oligo-fucoidan on cell cycle-related genes and proteins in HepG2 cells

The intrinsic apoptotic pathway is regulated by p53, which can activate p21 and p27 (Macleod et al. 1995),

and then inhibit the expression of Cyc-B1 (Nigam et al. 2009) and Cyc-D1 (Han et al. 1999) to arrest tumor cell growth. To further study how oligo-fucoidan induced apoptosis and blocked the cell cycle in HepG2 cells, we tested the cell cycle-related factors p53, p21, p27, Cyc-B1, and Cyc-D1. Compared with the control (0 µg/ml oligo-fucoidan), 100 and 200 µg/ml oligo-fucoidan significant increased p53, p21, and p27 promoter activity in HepG2 cells, while levels of Cyc-B1 and Cyc-D1 were decreased at the mRNA (Fig. 5a) and protein expression (Fig. 5b). These results were in agreement with the data presented in Fig. 2 that oligo-fucoidan induced cell cycle arrest in HepG2 cells, thereby inhibiting tumor cell proliferation.

Fig. 2 Induction of apoptosis by oligo-fucoidan in HepG2 cells. **a** The cells were collected, and the extracted fragmented DNA was separated on 1.5% agarose gel and UV light by EtBr

staining. M means marker. **b** The cells were stained with PI for DNA flow cytometry analysis. Percentages of apoptotic cells were determined by counting the sub-G1 phase cell population

Fig. 3 Effects of oligo-fucoidan on the caspase activity assay of caspase-3, -8 and -9 in HepG2 cells. HepG2 cells were pretreated with oligo-fucoidan for 48 h, quantitative analysis of caspases-3, -8 and -9 activity in vitro. Cell lysates were prepared and assayed for in vitro caspase-3, -8 and -9 activity using DEVD-pNA, IETD-pNA and LEHD-pNA, respectively, as substrates. Values are expressed as the mean $\pm$ standard error of three independent experiments. Significant differences are shown (* $p < 0.05$, compared with the control group)

Fig. 4 Effects of oligo-fucoidan on the protein expression of apoptosis related proteins in HepG2 cells. After treatment with oligo-fucoidan, the HepG2 cell lysates were prepared and immunoprecipitated. The immunocomplexes were separated on 15% polyacrylamide gels, transferred onto membranes, probed with anti-Bcl-2, anti-BAD, anti-BAX, anti-BID and anti-cleaved PARP antibodies. Values are expressed as the mean $\pm$ standard error of three independent experiments. Significant differences are shown (* $p < 0.05$, compared with the control group)

Oligo-fucoidan inhibited lncRNA-Saf and increased lncRNA-p21 expression, accompanied by HepG2 apoptosis

lncRNAs interact with other RNAs to regulate gene expression through transcriptional mechanisms (Kung

et al. 2013). After determining the expression of cell cycle-related genes (Fig. 5a), we investigated lncRNA expression in HepG2 cells to identify specific lncRNAs that modulate HepG2 apoptosis following oligo-fucoidan treatment and to establish the interaction between lncRNA and coding genes. Among the lncRNAs whose expression levels altered following 48 h treatment with 200 $\mu\text{g/ml}$ oligo-fucoidan, the following were found to decrease: lncRNA-Saf (6.4 fold), HULC (5.3 fold), NEAT1 (3.6 fold), MEG3 (2.9 fold), HOTAIR (1.8 fold), H19 (1.6 fold), and UCA1 (1.3 fold), while expression of lncRNA-p21 (3.1 fold), SNHG1 (2.2 fold), Malat1 (1.9 fold), Tsix (1.7 fold), ANRIL (1.3 fold), and IPW (1.2 fold) were observed to increase (Table 2).

Fucoidans are potent inducers of apoptosis in various tumors both in vitro (Hsu et al. 2014; Chen et al. 2014; Park et al. 2014) and in vivo (Hsu et al. 2014; Chen et al. 2016). A number of studies have demonstrated that fucoidan activates the intrinsic (Yang et al. 2013; Min et al. 2014; Park et al. 2017) or extrinsic (Cho et al. 2015) apoptosis pathways in HCC by altering the expression of apoptosis-associated factors. Although these fucoidans have been shown to induce apoptosis in HCC cells, the cellular mechanisms underlying the apoptotic effects of oligo-fucoidan have not yet been determined. This study is the first to demonstrate the regulation of lncRNA

Fig. 5 The effect of oligo-fucoidan on the expression of the cell cycle associated genes and proteins in HepG2 cells. **a** Total RNA was isolated and reverse transcribed. The resulting cDNAs were then subjected to qPCR with the indicated. **b** Total cell lysates were subjected to SDS-PAGE, transferred, and probed

with the indicated antibodies. GAPDH was used as internal controls Western blot assays. The 15% polyacrylamide gels, transferred onto membranes, probed with anti-p53, anti-p21, anti-p27, anti-Cyc-B1, and anti-Cyc-D1 antibodies

Table 2 Effects of oligo-fucoidan on the lncRNA profile of HepG2 cells

lncRNA name	Decreased fold-changes	lncRNA name	Increased fold-changes
Saf	6.4	p21	3.1
HULC	5.3	SNHG1	2.2
NEAT1	3.6	Malat1	1.9
MEG3	2.9	Tsix	1.7
HOTAIR	1.8	ANRIL	1.3
H19	1.6	IPW	1.2
UCA1	1.3		

Total RNA was isolated from HepG2 cells treated with vehicle or oligo-fucoidan at a dose of 200 µg/ml for 48 h. Samples were then analyzed using an Human lncRNA Profiler™ qPCR Array Kit was used for the detection of lncRNAs under the treatment of oligo-fucoidan. Values shown were the increased or decreased multiples of change in expression

expression by oligo-fucoidan to activate tumor apoptosis in HCC cells.

To investigate the effects of oligo-fucoidan (92.1% approximately 0.8 kDa) in human HCC cells, HepG2 cells were exposed to various concentrations of the oligo-fucoidan. Oligo-fucoidan was shown to inhibit HepG2 cell viability in a dose-dependent manner and induced sub-G1 phase cell cycle arrest at concentrations as low as 50 µg/ml. However, high-molecular weight fucoidan (20–200 kDa) enhances apoptosis of HepG2 cells at 500 µg/ml (Min et al. 2014), while *Undaria pinnatifida* fucoidan, with a molecular mass of approximately 100 kDa, enhances apoptosis of Huh-7 HCC cells at 1000 µg/ml (Cho et al. 2015). These findings indicated that oligo-fucoidan showed stronger antitumor activity than high-molecular weight fucoidan, a finding supported by Cho et al. (Cho et al. 2010), who shows that fucoidan with molecular weight in the range of 5–30 kDa inhibits human gastric carcinoma (AGS cell) growth by 37.3%–68.0% and that fucoidan of molecular weight > 30 kDa inhibited growth by 20.6%–35.8%. The two best-understood activation mechanisms are the intrinsic pathway (or mitochondrial pathway) and the extrinsic pathway. In the present study, oligo-fucoidan activated the initiator pro-apoptotic protein Bid, suppressed Bcl-2 expression and evaluated Bax and Bad expression. It also activated p53, p21, and p27 expression to inhibit the expression of Cyc-B1 and Cyc-D1 via the intrinsic pathway. Furthermore, oligo-fucoidan activated caspase-3, -8, and -9 in concentration-dependent manner. Caspase-8 plays an important role in extrinsic apoptosis, combining with fas-

associated death domain protein (FADD) to form the death-inducing signaling complex, undergoing self-cleavage to convert to the active form (Kischkel et al. 1995). Activated caspase-8 is released into the cytosol where it cleaves downstream effector caspases. Therefore, it acts between the intrinsic and extrinsic apoptosis pathways (Kantari and Walczak 2011). It has been suggested that oligo-fucoidan-induced apoptosis involved co-activation of these same pathways. Following exposure of HepG2 cells to different concentrations of oligo-fucoidan, we found that oligo-fucoidan induced concentration-dependent apoptosis. Western blotting analysis showed that caspase-3, -8, and -9 were activated, a finding which was further verified by analyzing expression of Bid and Bax and PARP cleavage, the results of which indicated that the intrinsic apoptotic pathway was involved in oligo-fucoidan-induced apoptosis. We therefore concluded that oligo-fucoidan-induced apoptosis involved the extrinsic apoptotic pathway through lncRNA-Saf.

Recently, increasing evidence has revealed that lncRNAs play critical roles in biological processes. According to the lncRNADisease database (<http://www.cuilab.cn/lncrnadisease>), more than 200 diseases have been found to be associated with various lncRNAs and more than 300 lncRNAs have been reported to be involved in various human complex diseases (Chen et al. 2013). More and more evidences show the involvement of lncRNAs in HCC. lncRNA-HULC enhances EMT to promote tumorigenesis and metastasis of HCC (Li et al. 2016; Wang et al. 2017). Furthermore, lncRNA-NEAT1 also promotes HCC

cell proliferation through the regulation of miRNA-129 (Fang et al. 2017). Moreover, lncRNA-p21 is shown to inhibit invasion and metastasis of HCC cells (Ding et al. 2017). lncRNA-p21, which locates 15-kb upstream of the cell cycle regulator gene p21, is a novel regulator of cell proliferation, apoptosis, and DNA damage response and plays an important role in the development and progression of human diseases. lncRNA-p21 recruits hnRNP-K to the promoter of p21, activating its expression (Tang et al. 2015). Oligo-fucoidan was observed to upregulate lncRNA-p21 while downregulating lncRNA-HULC and lncRNA-NEAT1. These observations suggested that lncRNA-HULC, lncRNA-NEAT1, and lncRNA-p21 were involved in the cell death of HepG2 cells following oligo-fucoidan treatment. However, it is known from the following references, variation of the expression of these lncRNAs correlate with the aforementioned apoptotic parameters. lncRNA-Saf influences the alternative slicing of Fas pre-mRNA at regions that flank exon 6 and increases the resistance to apoptosis. The regulation of lncRNA-p21 may involve to p53 pathway converge to induce apoptosis was reference Tran et al. (2015). Fas activation triggers apoptosis in many cell types; Yan et al. (2005) demonstrates that lncRNA-Saf influences the alternative slicing of Fas pre-mRNA at regions that flank exon 6 and increases the resistance to apoptosis. lncRNA array analysis showed that lncRNA-Saf was downregulated 6.4 fold, an effect that could cause abnormal Fas signaling and pathology in the extrinsic apoptosis pathway. Although the dysregulation of several other lncRNAs were also observed, further studies are needed to establish in detail the molecular mechanisms involved in the cooperation of these lncRNAs to regulate carcinogenesis in HCC.

Finally, we chose the greatest reduction and induction one, lncRNA-Saf and lncRNA-p21, in response to dandelion as the study object. lncRNA-Saf, located in the first intron 1 of Fas, was found to be abnormally expressed in HCC and has been reported to be dysregulated in many other cancers (Villamizar et al. 2016; Sehgal et al. 2014). However, the mechanism by which lncRNA-Saf exhibits its oncogenic functions in oligo-fucoidan-induced apoptosis of HCC remains to be investigated. We have previously shown that fucoidan reduces prostate cancer growth and metastasis by inhibiting the miR-29b-DNMT3B-MTSS1 axis (Yan et al. 2015). In this

study, we showed that targeting apoptosis and cell cycle pathways could also contribute to the induction of lncRNA-Saf and lncRNA-p21 by oligo-fucoidan.

Conclusions

The overall impact of this study was significant as it provided evidence for the suppression of lncRNA-Saf expression by the oligo-fucoidan signaling pathway. Furthermore, it provided evidence that a clinically natural compound, oligo-fucoidan, induced cancer cells apoptotic signaling to reduce the expression of lncRNA-Saf and facilitated its antitumor action. We also hypothesized the mechanisms by which oligo-fucoidan regulated lncRNAs, resulting in enhanced apoptotic signaling and eventual inhibition of cancer cellular proliferation. Together, these findings indicated that lncRNAs switched oligo-fucoidan-induced apoptosis, which might be potentially valuable in HCC therapy.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest in this manuscript.

References

- Billen LP, Shamas-Din A, Andrews DW (2008) Bid: a Bax-like BH3 protein. *Oncogene* 27:S93–S104
- Chen G, Wang Z, Wang D, Qiu C, Liu M, Chen X, Zhang Q, Yan G, Cui Q (2013) lncRNADisease: a database for long-non-coding RNA-associated diseases. *Nucleic Acids Res* 41:D983–D986
- Chen S, Zhao Y, Zhang Y, Zhang D (2014) Fucoidan induces cancer cell apoptosis by modulating the endoplasmic reticulum stress cascades. *PLoS ONE* 9:e108157
- Chen MC, Hsu WL, Hwang PA, Chen YL, Chou TC (2016) Combined administration of fucoidan ameliorates tumor and chemotherapy-induced skeletal muscle atrophy in bladder cancer-bearing mice. *Oncotarget* 7:51608–51618
- Cho ML, Lee BY, You SG (2010) Relationship between over-sulfation and conformation of low and high molecular weight fucoidans and evaluation of their in vitro anticancer activity. *Molecules* 16:291–297
- Cho Y, Yoon JH, Yoo JJ, Lee M, Lee DH, Cho EJ, Lee JH, Yu SJ, Kim YJ, Kim CY (2015) Fucoidan protects hepatocytes from apoptosis and inhibits invasion of hepatocellular

- carcinoma by up-regulating p42/44 MAPK-dependent NDRG-1/CAP43. *Acta Pharm Sin B* 5:544–553
- Ding G, Peng Z, Shang J, Kang Y, Ning H, Mao C (2017) LincRNA-p21 inhibits invasion and metastasis of hepatocellular carcinoma through miR-9/E-cadherin cascade signaling pathway molecular mechanism. *Oncotargets Ther* 10:3241–3247
- Enari M, Sakahira H, Yokoyama H, Okawa K (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391:43–50
- Fang L, Sun J, Pan Z, Song Y, Zhong L, Zhang Y, Liu Y, Zheng X, Huang P (2017) Long non-coding RNA NEAT1 promotes hepatocellular carcinoma cell proliferation through the regulation of miR-129-5p-VCP-IκB. *Am J Physiol Gastrointest Liver Physiol* 313:G150–G156
- Fitton JH (2011) Therapies from fucoidan; multifunctional marine polymers. *Mar drugs* 9:1731–1760
- Gomes AQ, Nolasco S, Soares H (2013) Non-coding RNAs: multi-tasking molecules in the cell. *Int J Mol Sci* 14:16010–16039
- Han EKH, Ng SC, Arber N, Begemann M, Weinstein IB (1999) Roles of cyclin D1 and related genes in growth inhibition, senescence and apoptosis. *Apoptosis* 4:213–219
- Hsu HY, Lin TY, Hwang PA, Tseng LM, Chen RH, Tsao SM, Hsu J (2013) Fucoidan induces changes in the epithelial to mesenchymal transition and decreases metastasis by enhancing ubiquitin-dependent TGFβ receptor degradation in breast cancer. *Carcinogenesis* 34:874–884
- Hsu HY, Lin TY, Wu YC, Tsao SM, Hwang PA, Shih YW, Hsu J (2014) Fucoidan inhibition of lung cancer in vivo and in vitro: role of the Smurf2-dependent ubiquitin proteasome pathway in TGFβ receptor degradation. *Oncotarget* 5:7870–7885
- Hung YL, Hwang PA, Wu CH (2010) Evaluation of antioxidative and immune activity of water extracts from three brown seaweeds. *J Fish Soc Taiwan* 18:95–107
- Hwang PA, Chien SY, Chan YL, Lu MK, Wu CH, Kong ZL, Wu CJ (2011) Inhibition of lipopolysaccharide (LPS)-induced inflammatory responses by *Sargassum hemiphyllum* sulfated polysaccharide extract in RAW 264.7 macrophage cells. *J Agric Food Chem* 59:2062–2068
- Hwang PA, Yan MD, Lin HTV, Li KL, Lin YC (2016) Toxicological evaluation of low molecular weight fucoidan in vitro and in vivo. *Mar drugs* 14:121–135
- Hwang PA, Yan MD, Kuo KL, Phan NN, Lin YC (2017) A mechanism of low molecular weight fucoidans degraded by enzymatic and acidic hydrolysis for the prevention of UVB damage. *J Appl Phycol* 29:521–529
- Kantari C, Walczak H (2011) Caspase-8 and Bid: caught in the act between death receptors and mitochondria. *BBA Mol Cell Res* 1813:558–563
- Kato A, Miyazaki M, Ambiru S, Yoshitomi H, Ito H, Nakagawa K, Shimizu H, Yokosuka O, Nakajima N (2001) Multidrug resistance gene (MDR-1) expression as a useful prognostic factor in patients with human hepatocellular carcinoma after surgical resection. *J Surg Oncol* 78:110–115
- Kew MC (2010) Epidemiology of chronic hepatitis B virus infection, hepatocellular carcinoma, and hepatitis B virus-induced hepatocellular carcinoma. *Pathol Biol* 58:273–277
- Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* 14:5579–5588
- Kung JT, Colognori D, Lee JT (2013) Long noncoding RNAs: past, present, and future. *Genetics* 193:651–669
- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier G, Earnshaw W (1994) Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371:346–347
- Li SP, Xu HX, Yu Y, He JD, Wang Z, Xu YJ, Wang CY, Zhang HM, Zhang RX, Zhang JJ, Yao Z, Shen ZY (2016) LncRNA HULC enhances epithelial-mesenchymal transition to promote tumorigenesis and metastasis of hepatocellular carcinoma via the miR-200a-3p/ZEB1 signaling pathway. *Oncotarget* 7:42431–42446
- MacLeod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, Vogelstein B, Jacks T (1995) p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev* 9:935–944
- Min EY, Kim IH, Lee J, Kim EY, Choi YH, Nam TJ (2014) The effects of fucoidan on senescence are controlled by the p16INK4a-pRb and p14Arf-p53 pathways in hepatocellular carcinoma and hepatic cell lines. *Int J Oncol* 45:47–56
- Naeini MM, Ardekani AM (2009) Noncoding RNAs and cancer. *Avicenna J Med Biotechnol* 1:55–70
- Nagasue N, Uchida M, Makino Y, Takemoto Y, Yamanoi A, Hayashi T, Chang YC, Kohno H, Nakamura T, Yukaya H (1993) Incidence and factors associated with intrahepatic recurrence following resection of hepatocellular carcinoma. *Gastroenterology* 105:488–494
- Nigam N, Prasad S, George J, Shukla Y (2009) Lupeol induces p53 and cyclin-B-mediated G2/M arrest and targets apoptosis through activation of caspase in mouse skin. *Biochem Biophys Res Commun* 381:253–258
- Park HY, Kim GY, Moon SK, Kim WJ, Yoo YH, Choi YH (2014) Fucoidan inhibits the proliferation of human urinary bladder cancer T24 cells by blocking cell cycle progression and inducing apoptosis. *Molecules* 19:5981–5998
- Park HY, Park SH, Jeong JW, Yoon D, Han MH, Lee DS, Choi G, Yim MJ, Lee JM, Kim DH (2017) Induction of p53-independent apoptosis and G1 cell cycle arrest by fucoidan in HCT116 human colorectal carcinoma cells. *Mar Drugs* 15:154
- Phuah NH, Nagoor NH (2014) Regulation of microRNAs by natural agents: new strategies in cancer therapies. *Biomed Res Int* 2014:804510
- Sehgal L, Mathur R, Braun FK, Wise JF, Berkova Z, Neelapu S, Kwak LW, Samaniego F (2014) FAS-antisense 1 lncRNA and production of soluble versus membrane Fas in B-cell lymphoma. *Leukemia* 28:2376–2387
- Tang SS, Zheng BY, Xiong XD (2015) LincRNA-p21: implications in human diseases. *Int J Mol Sci* 16:18732–18740
- Tran UM, Rajarajacholan U, Soh J, Kim TS, Thalappilly S, Sensen CW, Riabowol K (2015) LincRNA-p21 acts as a mediator of ING1b-induced apoptosis. *Cell Death Dis* 6:e1668
- Villamizar O, Chambers CB, Riberdy JM, Persons DA, Wilber A (2016) Long noncoding RNA Saf and splicing factor 45 increase soluble Fas and resistance to apoptosis. *Oncotarget* 7:13810–13826
- Wang Y, Chen F, Zhao M, Yang Z, Li J, Zhang S, Zhang W, Ye L, Zhang X (2017) The long noncoding RNA HULC

- promotes liver cancer by increasing the expression of the HMG2 oncogene via sequestration of the microRNA-186. *J Biol Chem* 292:15395–15407
- Xiong Y, Fang JH, Yun JP, Yang J, Zhang Y, Jia WH, Zhuang SM (2010) Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. *Hepatology* 51:836–845
- Yan MD, Hong CC, Lai GM, Cheng AL, Lin YW, Chuang SE (2005) Identification and characterization of a novel gene *Saf* transcribed from the opposite strand of *Fas*. *Hum Mol Genet* 14:1465–1474
- Yan MD, Yao CJ, Chow JM, Chang CL, Hwang PA, Chuang SE, Jacqueline WP, Lai GM (2015) Fucoidan elevates microRNA-29b to regulate DNMT3B-MTSS1 axis and inhibit EMT in human hepatocellular carcinoma cells. *Mar Drugs* 13:6099–6116
- Yang L, Wang P, Wang H, Li Q, Teng H, Liu Z, Yang W, Hou L, Zou X (2013) Fucoidan derived from *Undaria pinnatifida* induces apoptosis in human hepatocellular carcinoma SMMC-7721 cells via the ROS-mediated mitochondrial pathway. *Mar Drugs* 11:1961–1976
- Yu FJ, Zheng JJ, Dong PH, Fan XM (2015) Long non-coding RNAs and hepatocellular carcinoma. *Mol Clin Oncol* 3:13–17

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