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Fucoidan에 의한 ID-1 억제를 통한 in vitro와 in vivo에서의 간세포암 침습 억제에 대한 연구

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A thesis of the Degree of Doctor of Philosophy

Fucoidan–induced ID–1 suppression inhibits the in vitro and in vivo invasion of hepatocellular carcinoma cells

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ABSTRACT

**Introduction:** Hepatocellular carcinoma (HCC) is a fast growing tumor associated with a high tendency for vascular invasion and distant metastasis. Fucoidan displays inhibitory effect on proliferation of cancer cells and protective effects on hepatocyte. In this study, we investigated the anti-metastatic effect of fucoidan on HCC cells, the key signal that modulates metastasis and the hepatoprotective effect over bile acid (BA)–induced apoptosis.

**Methods:** The anti-metastatic effect of fucoidan was evaluated in vitro using an invasion assay with human HCC cells (Huh−7, SNU−761, and SNU−3085) under both normoxic (20% O₂ and 5% CO₂, at 37 °C) and hypoxic (1% O₂, 5% CO₂, and 94% N₂, at 37 °C) conditions. Complementary DNA (cDNA) microarray analysis was performed to find the molecule which is significantly suppressed by fucoidan. In vivo study using a distant metastasis model by injecting HCC cells into spleen via portal vein was performed to confirm the inhibitory effect by fucoidan or small interfering RNA (siRNA) transfection. Immunoblot analyses were used to investigate the signaling
pathway. Primary hepatocyte culture was performed to evaluate fucoidan’s hepatoprotective effect.

**Results:** Fucoidan significantly suppressed the invasion of human HCC cells (Huh-7, SNU-761, and SNU-3085). Using cDNA microarray analysis, we found the molecule, ID-1, which was significantly suppressed by fucoidan treatment. Downregulation of ID-1 by siRNA significantly decreased invasion of HCC cells, both in vitro and in vivo (both \( P < 0.05 \)) in a NDRG/CAP43-dependent manner. In immunoblot assay, downregulation of ID-1 by siRNA decreased the expressions of epithelial–mesenchymal transition markers including CK19, vimentin, MMP2, and fibronectin. Immunofluorescence study also revealed that actin rearrangement was inhibited when ID-1 was down-regulated in HCC cells. Interestingly, in SNU-761 cells, the ID-1 expressions under hypoxic conditions were lower as compared to those under normoxic conditions. Under hypoxic conditions, HIF-1α up-regulated NDRG-1/CAP43, while HIF-2α down-regulated ID-1, which might be a compensatory phenomenon against hypoxia-induced HCC invasion. Furthermore, fucoidan decreased BA-induced hepatocyte apoptosis as shown by the attenuation of caspase-8,
and -7 cleavages, and suppressed the mobilization of caspase-8 and FADD into the death-inducing signaling complex.

**Conclusions:** In conclusion, NDRG/CAP43-dependent down-regulation of ID-1 suppressed HCC invasion both in vitro and in vivo, which was modulated by fucoidan treatment, suggesting ID-1 is a novel therapeutic target for the treatment of metastatic HCC. Moreover, the compensatory down-regulation of ID-1 against hypoxia-induced HCC invasion was observed. Fucoidan protected hepatocytes from BA-induced apoptosis and preferentially inhibited invasion of HCC cells suggesting it may be a potent suppressor of tumor invasion with hepatoprotective effects.

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**Keywords:** hepatocellular carcinoma, metastasis, ID-1, fucoidan, hypoxia, NDRG/CAP43

**Student number:** 2014-30605
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<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
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<tr>
<td>BA</td>
<td>bile acid</td>
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<tr>
<td>EMT</td>
<td>epithelial–mesenchymal transition</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<td>WME</td>
<td>William's medium E</td>
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<td>MTS</td>
<td>3,4-(5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<td>DISC</td>
<td>death–inducing signaling complex</td>
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<td>GP</td>
<td>glypican</td>
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INTRODUCTION

HCC is associated with a high potential for vascular invasion, metastasis, and recurrence, leading to poor prognosis\textsuperscript{1,2}. Intrahepatic and extrahepatic metastases occur in more than half of patients after resection of HCC, with intrahepatic metastasis occurring more frequently\textsuperscript{3}. Tumor metastasis is a complex process by which cancer cells enter the vasculature, migrate and invade to distant organ. Epithelial–mesenchymal transition (EMT) is a key event in the process of tumor invasion. Epithelial cell layers lose polarity and cell-to-cell contacts. Finally, they undergo a dramatic remodeling of the cytoskeleton\textsuperscript{4}. Matrix metalloproteinases (MMP) have been reported as major molecules associated with extracellular matrix (ECM) degradation and tumor invasion\textsuperscript{5,6}. However, the molecular events aggravating HCC cell invasion are still hardly known and routine biomarkers for HCC metastasis are not yet available.

Fucoidan is a family of sulphated polyfucose polysaccharides purified from brown algae whose main sugar unit is sulfated fucose\textsuperscript{7}. Fucoidan exhibits a wide range of biological effects, including anti-tumor\textsuperscript{8}, anti-thrombotic\textsuperscript{9}, anti-inflammatory\textsuperscript{10},
and anti-viral activities. It has been reported that fucoidan inhibits HCC cell proliferation through down-regulation of chemokine ligand (CXCL)–12. Fucoidan displays inhibitory effect on invasion of HCC cells both \textit{in vitro} (Huh–7 and SNU–761) and \textit{in vivo} (C3H mouse). Fucoidan inhibited HCC cell invasion through up-regulation of p42/44 MAPK–dependent NDRG–1/CAP43, and partly through up-regulation of p42/44 MAPK–dependent VMP–1 expression under normoxic conditions.

Fucoidan also has been reported to have a protective property on hepatocytes, showing anti-oxidative effects against acute liver injury or liver fibrosis in many studies. Recently, a hepatoprotective effect of fucoidan against acetaminophen–associated liver injury \textit{in vivo} was reported. However, it has not yet been investigated whether fucoidan has hepatoprotective effect over bile acid (BA)–induced apoptosis.

In this study, we assessed gene–specific changes in HCC cells by fucoidan using a microarray. We also evaluated the role of key molecule in suppressing invasion of HCC cells and the hepatoprotective effect over BA.
MATERIALS AND METHODS

1. Cell Lines and Cell Culture Conditions

In this study, three human HCC cell lines were used: Huh-7, a well-differentiated HCC cell line\(^{17}\); SNU-761, a poorly differentiated HCC cell line\(^{18}\); and SNU-3058, a hypovascular HCC cell line which was deposited to the Korea Cell Line Bank. Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM: Huh-7) or in RPMI 1640 (SNU-761 and SNU-3058) supplemented with 10% fetal bovine serum (FBS), 100,000 U/L penicillin, and 100 mg/L streptomycin with or without 100 nmol/L insulin. In all experiments, cells were treated with overnight serum starvation to avoid confounding variables related to serum-induced signaling. Cells were incubated under either normoxic conditions (20% O\(_2\) and 5% CO\(_2\) at 37°C) or hypoxic conditions (1% O\(_2\), 5% CO\(_2\), and 94% N\(_2\) at 37°C). Fucoidan from Fucus vesiculosus (Sigma-Aldrich Co. LLC., Seoul, South Korea) was used in this study.

2. Cell Proliferation Analysis (MTS Assay)

With the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI), cell proliferation was measured
on the basis of cellular conversion of the colorimetric reagent
3,4-((5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-
2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) into soluble
formazan by dehydrogenase enzyme found in metabolically
proliferating cell. Following each treatment, 20 μL of dye
solution was added into each well in 96-well plate and
incubated for 2 hours. Subsequently, the absorbance was
recorded at a wavelength of 490 nm using an enzyme-linked
immunosorbent assay (ELISA) plate reader (Molecular Devices,
Sunnyvale, CA, USA).

3. Cell Invasion Assay

In vitro invasion assays were performed using 24-well
chambers to study invasion of HCC cells (Huh-7, SNU-761,
and SNU-3058) under either normoxic or hypoxic conditions.
Inserts were transferred into wells and coated with Matrigel
(BD Biosciences, Billerica, MA, USA) for 30 min at 37°C. HCC
cells were suspended in serum-free medium and implanted on
the Matrigel-coated upper chambers (5 × 10^4 cells/chamber),
and DMEM containing 10% fetal bovine serum was added to the
lower chambers. The cells were incubated for 6 hours at 37°C.
The cells were then incubated in the presence or absence of fucoidan or ID–1 small interfering RNA (siRNA) for 24 hours and stained with 4 \( \mu \text{g/mL} \) calcein AM (BD Biosciences, Billerica, MA, USA) in HANK’s Balanced Salt Solution (HBSS) at 37° C for 90 min. Invasion ability was determined as relative fluorescence units measured at an excitation of 494 nm and an emission of 517 nm using a multifunctional plate reader (EnVision Multilabel Reader; PerkinElmer Inc., Waltham, MA, USA).

4. cDNA microarray analysis

In order to compare the relative gene expression profiles in Huh–7 cells with fucoidan treatment, total RNA from Huh–7 cells treated with fucoidan or control was extracted and purified. Microarray analysis was performed according to the Macrogen Rat Bead Chip technical manual (Macrogen, Seoul, Korea) used by Illumina RatRef–12 Expression Bead Chip (Illumina, Inc., San Diego, CA). Biotinylated cRNAs were prepared from 0.55 \( \mu \text{g} \) quantities of total RNA using the Illumina Total Prep RNA Amplification Kit (Ambion, Austin, TX). Following fragmentation, cRNA was hybridized to the Illumina RatRef–12
Expression Beadchip in 0.75 μg quantities using protocols provided by the manufacturer. Arrays were scanned using the Illumina Bead Array Reader Confocal Scanner. Array data export processing and analysis were performed using Illumina Bead Studio v 3.1.3 (Gene Expression Module v 3.3.8).

5. Immunoblot Analysis

Cells were lysed for 20 min on ice with lysis buffer and centrifuged at 14,000 g for 10 min at 4° C. Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes, blotted with appropriate primary antibodies at a dilution of 1:1000, and treated with peroxidase–conjugated secondary antibodies (Biosource International, Camarillo, CA, USA). Bound antibodies were visualized using chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA) and exposed to Kodak X–OMAT film (Kodak, New Haven, CT, USA). Primary antibodies included anti-N–myc downstream–regulated gene (NDRG)–1/CAP43 from Invitrogen Corporation (Camarillo, CA, USA), anti–caspase 8 from BD Biosciences (San Jose, CA, USA), anti–caspase 9,
anti-caspase 7 (cleaved), and anti-MMP2 from Cell Signaling Technology (Danvers, MA, USA), anti-CK19, anti-vimentin from BioGenex (San Ramon, CA, USA), anti-LOX, anti-fibronectin from Abcam (Cambridge, UK). Anti-ID-1, anti-HIF-1 α, anti-HIF-2 α, and anti-actin antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

6. **Real-time Polymerase Chain Reaction (PCR) Analysis**

Total ribonucleic acids (RNAs) were extracted from HCC cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Complementary deoxyribonucleic acid (cDNA) templates were prepared using oligo(dT) random primers and Moloney Murine Leukemia Virus (MoMLV) reverse transcriptase. ID-1 was quantitated by real-time PCR (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany) using SYBR green as the fluorophore (Molecular Probes, Eugene, OR, USA). The 5' and 3' PCR primers were AGGTGGTGCGCTGTCTGTCT and TAATTCCTCTTTGCCGCCCTGG for ID-1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as a control. The level of ID-1 mRNA expression was calculated as the relative intensity of the PCR product bands.
compared with that from the GAPDH gene using the $2^{-\Delta\Delta Ct}$ method. All PCR experiments were performed in triplicate.

7. **SiRNA Transfection**

Cells were seeded in a 6-well culture plate (2 × 10$^5$ cells/well) in 2 mL antibiotic-free medium supplemented with 10% FBS. At 60–80% confluence, the cells were transfected with small interfering RNA (siRNA) using the siRNA Transfection Reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) according to the manufacturer’s instructions. The cells were treated with siRNA for 6 hours at 37°C and then growth medium containing 20% FBS and antibiotics was added. After 18 hours, the medium was replaced with fresh medium containing 10% FBS and antibiotics. At 24 hours after the transfection, the cells were ready to used in the experiments.

8. **In Vivo, Intrahepatic via Portal Vein Metastasis Model (Splenic Injection Model)**

Five-week-old male C3H mice from Orient Bio Inc. were anesthetized with ether. Using a 31-gauge needle, 1 × 10$^5$
MH134 cells (mouse hepatocellular carcinoma cell line) were injected into the lower pole of the spleen. Eight days after splenic injection, the number of macroscopic liver metastasis and the sum of the maximal diameter for each nodule were counted. Mice were treated with intraperitoneal injection of normal saline (control group, n = 4) or 10 mg/kg of fucoidan (fucoidan treatment group, n = 4) every two days. The intraperitoneal dose of fucoidan was determined by reference to the previous study which examined the antitumor effect of fucoidan in vivo.

Five-week-old male BALB/c nude mice were anesthetized with ether. A splenic injection model was used as previously described. To expose the spleen, an upper quadrant incision was performed. Using a 31-gauge needle, $5 \times 10^7$ SNU-761 cells treated with control siRNA (n = 4) or SNU-761 cells treated with ID-1 siRNA (n = 4) were injected into the lower pole of the spleen. Three weeks after splenic injection, the number of macroscopic liver metastasis was counted. Tumor specimens were fixed in 4% formaldehyde and embedded in paraffin. For immunohistochemical (IHC) analyses, specimens were cut into 4 $\mu$m sections. IHC staining was performed using
the anti-mouse CD31 antibody (Vector Laboratories, Inc., Burlingame, USA) at a 1:300 dilution rate, anti-ID-1 antibody at a 1:200 dilution rate, and anti-glypican (GP)-3 antibody (Novus Biologicals, Littleton, USA) at a 1:200 dilution.

9. Primary Hepatocyte Isolation and Culture

Primary hepatocyte isolation and culture were performed as previously described\textsuperscript{22}. Primary hepatocytes were isolated from five-week-old C3H mice. The use of mice was approved by the animal experimental committees. Mice were housed and handled under specific pathogen-free conditions. HANKS solution I was produced as follows: BASAL HANKS solution with 2.5 mmol/L EGTA, 0.1% glucose and penicillin/streptomycin at a dilution of 1:100. HANKS solution II was produced as follows: BASAL HANKS solution with 0.3 mg/mL collagenase CLSII and 5 mmol/L CaCl\textsubscript{2}. Hanks solutions were prewarmed upto 42° C. Prior to liver perfusion, collagenase was added immediately. After shaving the abdomen, abdominal cavity was opened. The portal vein was cannulated with a 25-gauge catheter, and a silicon tube was connected to the catheter. HANKS solution I was perfused for using a
peristaltic infusion pump at a flow rate of 8 mL/min. After the infusion pump was started, inferior vena cava was incised to permit sufficient outflow. The liver was perfused with HANKS solution I for 2 min followed by HANKS solution II for 5 min. Efficient perfusion via portal vein was evidenced by the color change in liver from dark red–brown to a light brown. Followed by perfusion, the liver was transferred to a sterile Petri dish. The gallbladder and liver capsule were removed using a pincette.

The perfused liver was disintegrated by gentle shaking. The suspension was passed through a 100 mm cell strainer by gravity filtration, and was transferred to a 50 mL Falcon tube. Washing with William's medium E (WME) at room temperature 37.5° C was performed twice. Cells were centrifuged for 2 min, and were resuspended in WME. Hepatocytes were plated on collagen–coated culture dishes with fetal calf serum (FCS) cell culture medium. Cells were kept in a humidified cell culture incubator (37° C and 5% CO₂). After 4 hours, hepatocytes were attached in the collagen–coated dish. And then, the FCS medium was removed and replaced by serum–free cell culture medium. After 14–20 hours in serum–free cell culture medium,
washing three times with starvation medium was performed. In starvation medium, hepatocytes were then incubated for the indicated time periods.

10. Death-Inducing Signaling Complex Immunoprecipitation (DISC IP)

Cell pellets were treated with lysis buffer (100 mmol/L Tris-HCL, 5 μmol/L EDTA, and 1% NP-40) containing protease inhibitor and phosphatase inhibitor cocktails. Total protein (500 μg) was incubated with rat anti-death receptor (DR) 5 antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), mouse anti-Fas-associated death domain (FADD) antibody from BD Biosciences (San Jose, CA, USA), mouse anti-cellular FLICE inhibitory protein (cFLIP) antibody from Enzo Life Science (Farmingdale, NY, USA), or mouse anti-caspase 8 antibody from Upstate (Lake Placid, NY, USA) at 4°C for 1 hour. Protein A/G beads (Santa Cruz, San Diego, CA) were added to the lysate and incubated overnight with gentle rocking at 4°C. The beads were washed five times by repeated suspension in 500 μL lysis buffer followed by centrifugation at 10,000 g for 1 min. After the last wash, 35 μL Laemmli buffer
(4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 mol/L Tris HCl, pH 6.8) was added to the pellet and heated at 100° C for 5 min. Centrifugation was performed again to collect supernatant and then the samples were subjected to immunoblot analysis as described above.

11. Statistical Analysis

Statistical analyses were performed using PASW version 22.0 (IBM, Chicago, IL, USA). All experimental results were obtained from three independent experiments using cells from three separate isolations and are presented as the mean ± standard deviation (SD). For comparisons between groups, data were analyzed by the Mann–Whitney U test or one-way ANOVA. For all tests, $P < 0.05$ was regarded as statistically significant.

12. Ethics Statement

Ethics approval from the ethics committee at Seoul National University Hospital was obtained. We carried out this study in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes
of Health. *In vivo* study protocol was approved by the Institutional Animal Care and Use Committee (IACUC No. 15-0259-S1A1) of Seoul National University Hospital, Central South University. All *in vivo* surgical procedures were performed under anesthesia with isoflurane and all efforts were made to minimize suffering.
RESULTS

Decreased proliferation and invasion of HCC cells by fucoidan

Fucoidan treatment decreased Huh–7 and SNU–761 cell proliferation in a dose–dependent manner (Fig. 1A). Moreover, 1 mg/mL fucoidan enhanced apoptosis of HCC cells as compared to control as shown by the expression of caspase–8, −7, and −9 cleavages (Fig. 1B). Invasion assays were performed using a concentration of 0.1 mg/mL fucoidan that did not inhibit proliferation of HCC cells (both Huh–7 and SNU–761). As shown in Fig. 1C, fucoidan significantly suppressed the invasion of human HCC cells.

Figure 1. The effect of fucoidan on proliferation and invasion of HCC cells. (A) HCC cell growth following treatment with fucoidan at various concentrations (0, 0.1, 0.25, 0.5, 1, and 2 mg/mL) was evaluated by MTS assays. Data are expressed as mean ± SD of percent changes of triplicate optical densities.
Figure 1. The effect of fucoidan on proliferation and invasion of HCC cells. (B) Fucoidan enhanced apoptosis of HCC cells. (C) Fucoidan significantly decreased HCC cell invasion as compared to control.

Suppressed mRNA and protein expressions of ID-1 in HCC cells by fucoidan

First, we tried to find molecules which are significantly changed by fucoidan treatment. Using cDNA microarray analysis, we found the molecule, ID-1, which was significantly suppressed by 8.2 log-odds in fucoidan-treated HCC cells as
compared to control. ID−1 mRNA expression was also significantly decreased by fucoidan treatment (0.1 mg/mL) in HCC cells (Huh−7, SNU−761, and SNU−3085) (Fig. 2A). Suppressed ID−1 protein expression by fucoidan was confirmed by immunoblot assay on Huh−7, SNU−761, and SNU−3085 cells (Fig. 2B).

Figure 2. Fucoidan−induced ID−1 suppression inhibited invasion of HCC cells. (A) Real−time PCR revealed that fucoidan decreased ID−1 mRNA expressions on HCC cells. (B) Immunoblot analyses revealed that ID−1 protein expression was suppressed by fucoidan.
Decreased invasion of HCC cells by ID−1 siRNA transfection, *in vitro*

First, we examined the effective ID−1 siRNA transfection with RT−PCR. As shown in Fig. 2C, ID−1 siRNA transfection significantly suppressed ID−1 mRNA expression as compared to control siRNA, on both SNU−761 and SNU−3058 cells (*P* < 0.05). Then, we performed MTS assay to evaluate whether ID−1 modulates HCC cell proliferation. There was no significant change in HCC cell (SNU−761, SNU−3058) proliferation following ID−1 siRNA transfection as compared to control siRNA (Fig. 2D). However, down−regulation of ID−1 by siRNA significantly decreased the invasion of HCC cells, *in vitro* (Fig. 2E, *P* < 0.05). While the fucoidan treatment significantly decreased control siRNA transfected−HCC cell invasion, ID−1 siRNA transfected−HCC cells showed an attenuated degree of decrease in HCC cell invasion by fucoidan treatment (Fig. 2F).

We previously found that fucoidan inhibits invasion of HCC cells by up−regulating p42/44 MAPK−dependent NDRG/CAP43. Therefore, we confirmed whether ID−1 is modulated in a NDRG/CAP43−dependent manner. As shown in Fig. 2G, ID−1 protein expression increased when the activity of NDRG/CAP43
was suppressed by siRNA transfection as compared to control siRNA at 0, 8, and 24 hr time points in SNU-761 cells (24, 32, and 48 hr after siRNA transfection, respectively) and at 0 and 8 hr time points in SNU-3058 cells (24 and 32 hr after siRNA transfection, respectively), which means the expression of ID-1 is modulated by NDRG/CAP43.

**Figure 2.** Fucoidan–induced ID-1 suppression inhibited invasion of HCC cells. (C) ID-1 siRNA transfection suppressed ID-1 mRNA expression (vs. control siRNA). (D) No significant change on HCC proliferation was noted between the two groups.
Figure 2. Fucoidan-induced ID-1 suppression inhibited invasion of HCC cells. (E) Downregulation of ID-1 by siRNA significantly decreased the invasion of HCC cells (vs. control siRNA). (F) ID-1 siRNA transfected–HCC cells showed an attenuated degree of decrease in HCC cell invasion by fucoidan treatment. (G) ID-1 protein expression enhanced by NDRG/CAP43 siRNA transfection (vs. control siRNA).

Decreased invasion of HCC cells by ID-1 siRNA, in vivo

We examined the in vivo anti-metastatic effect of fucoidan using a distant metastasis model. The number of intrahepatic metastases (Fig. 3A) and the sum of the maximal diameter of
each nodule (Fig. 3B) in the fucoidan treatment group was significantly lower than that of the control group ($P < 0.05$).\(^{13}\)

**Figure 3. Fucoidan inhibits liver metastasis in the distant metastasis model.** (A) The number of intrahepatic metastases in the fucoidan treatment group compared to the control group. (B) The sum of the maximal diameter of each nodule in the fucoidan treatment group compared to the control group.

**ID−1 siRNA inhibited *in vivo* liver metastasis**

ID−1 siRNA transfection also significantly inhibited liver metastasis in an intrahepatic portal vein metastasis model (Fig. 4A, $P < 0.05$). ID−1 siRNA transfection attenuated the nuclear grade of SNU−761 cells as determined by hematoxylin and eosin (H&E) staining, the expression of ID−1, and the angiogenesis of SNU−761 cells as determined by decreased CD31 expression by IHC staining (Fig. 4B).
Figure 4. ID-1 siRNA inhibited in vivo liver metastasis and suppressed the expression of EMT markers compared to control siRNA. (A) ID-1 siRNA transfection significantly inhibited liver
metastasis (vs. control siRNA). (B) ID-1 siRNA transfection attenuated the nuclear grade, the expression of ID-1, and the angiogenesis (CD31) of HCC cells. (C) Downregulation of ID-1 by siRNA decreased the expressions of EMT markers on HCC cells (vs. control siRNA). (D) Forty-eight hours after ID-1 siRNA transfection, HCC cells were stained with FITC–phalloidin solution and anti-ID-1 antibody. The spreading of si-ID-1 transfected cells was remarkably inhibited.

Change in EMT markers and actin rearrangement by ID-1 siRNA transfection

In immunoblot assay, down-regulation of ID-1 by siRNA decreased the expressions of EMT markers including CK19, MMP-2, vimentin, and fibronectin on HCC cells (SNU-761, SNU-3058) as compared to control siRNA transfection (Fig. 4C). To investigate the role of ID-1 in actin reorganization involved in the formation of microfilaments, control siRNA–transfected cells and ID-1 siRNA transfected–cells were stained with FITC–conjugated phalloidin and anti-ID-1 antibody. Immunofluorescence study revealed that actin rearrangement was remarkably inhibited when ID-1 was down-regulated in HCC cells by siRNA transfection as compared to control siRNA transfection (Fig. 4D). Control siRNA transfected–cells presented a longer appearance with a
thicker and denser F–actin stress fiber network as compared with ID–1 siRNA transfected cells, which indicates that ID–1 plays a role in regulating HCC cell adhesion and migration, as well as actin rearrangement.

**Modulation of ID–1 by HIF–2 α under hypoxic conditions**

We additionally investigated whether ID–1 expressions is modulated by hypoxic conditions, which is crucial in survival signal in HCC\textsuperscript{23}. Interestingly, especially in SNU–761 cells, the ID–1 expressions under hypoxic conditions were lower as compared to those under normoxic conditions (Fig. 5A). Under hypoxic conditions, NDRG–1/CAP43 protein expression was suppressed by HIF–1 α siRNA transfection in SNU–761 cells (Fig. 5B). While ID–1 protein expression increased by disulfiram (Fig. 5C) which is a HIF–2 α suppressor under hypoxic conditions in SNU–761 cells (Fig. 5D). However, there was no significant change in ID–1 protein expression on SNU–3085 by disulfiram under hypoxic conditions. It means that HIF–2 α down–regulates ID–1 in SNU–761 cells, which might be a compensatory phenomenon against hypoxia–induced HCC invasion. As shown in Fig. 5E, disulfiram, the hypoxia–induced
ID-1 enhancer, increased HCC cell invasion under hypoxic conditions only for SNU-761 cells and not for SNU-3058 cells. This might be a possible difference obtained using the two different cell lines.

![Image of Western Blot Experiments](image.png)

**Figure 5.** Hypoxic conditions suppress ID-1 protein expression in a HIF-2α-dependent pathway as a compensatory mechanism to hypoxia. (A) In SNU-761 cells, the ID-1 protein expressions under hypoxic conditions were lower as compared to those under normoxic conditions. (B) Under hypoxic conditions, NDRG-1/CAP43 protein expression was suppressed by HIF-1α siRNA transfection in SNU-761 cells.
Figure 5. Hypoxic conditions suppress ID-1 protein expression in a HIF-2α-dependent pathway as a compensatory mechanism to hypoxia. (C) The ID-1 expression in SNU-761 cells enhanced by disulfiram. (D) Disulfiram is a HIF-2α suppressor under hypoxic conditions in SNU-761 cells. (E) Disulfiram, the hypoxia-induced ID-1 enhancer, increased HCC cell invasion under hypoxic condition, only for SNU-761.
**Fucoidan suppressed BA−induced apoptosis**

We investigated whether fucoidan decreases BA−induced hepatocyte apoptosis. First, we evaluated whether fucoidan has a hepatoprotective effect on Huh−BAT cells which have been stably transfected with a BA transporter and have been previously used to examine BA−induced hepatocyte apoptosis$^{24−26}$. As shown in Fig. 6A, fucoidan significantly decreased BA−induced Huh−BAT cell apoptosis as shown by the attenuation of caspase−8, −9, −3, and −7 cleavages, p−JNK, and bid as compared to BA−treated cells. We performed DISC−IP to evaluate changes in DISC assembly induced by fucoidan.

As shown in Fig. 6B, fucoidan suppressed the mobilization of caspase−8, cFLIP, DR−5, and FADD into DISC as compared to BA−treated cells. Decreased recruitment to DISC led to suppression of autocatalytic processes of the procaspases within DISC. Finally, following primary mouse hepatocyte culture, immunoblotting was performed with BA. Fucoidan significantly decreased BA−induced hepatocyte apoptosis as shown by the attenuation of caspase−8, and −7 cleavages as compared to BA−treated cells (Fig. 6C).
**Figure 6.** Fucoidan suppresses BA−induced hepatocyte apoptosis. (A) Fucoidan treatment suppressed BA−induced Huh-BAT cell apoptosis as shown by the attenuation of caspase−8, −9, −3, and −7 cleavages, p−JNK, and bid as compared to control. (B) In DISC−IP, fucoidan suppressed the mobilization of caspase−8, cFLIP, and FADD into DISC. (C) Fucoidan significantly decreased BA−induced primary cultured−hepatocyte apoptosis as compared to BA−treated cells.
DISCUSSION

In the present study, we first demonstrated that fucoidan-induced ID−1 suppression inhibits the *in vitro* and *in vivo* invasion of HCC cells. Down-regulation of ID−1 by siRNA transfection decreased the expressions of EMT markers, and inhibited actin rearrangement on HCC cells. Interestingly, the ID−1 expressions under hypoxic conditions were lower in SNU−761 cells, as compared to those under normoxic conditions. Under hypoxic conditions, HIF−1α up-regulated NDRG−1/CAP43, while HIF−2α down-regulated ID−1, which might be a compensatory phenomenon against hypoxia-induced HCC invasion. Fucoidan also exerts a hepatoprotective effect by decreasing BA-induced hepatocyte apoptosis.

Fucoidan, a sulphated polysaccharide purified from brown algae, possesses anti-metastatic effects on HCC both *in vitro* (Huh−7 and SNU−761) and *in vivo* (C3H mouse) through up-regulation of p42/44 MAPK-dependent NDRG−1/CAP43. We previously reported that fucoidan may represent an ideal therapeutic agent for HCC patients as a potent suppressor of tumor invasion\textsuperscript{13}. In this study, we found the key molecule which is modulated by fucoidan treatment, the ID−1.
ID-1 (inhibitor of differentiation/DNA synthesis) is a member of the helix-loop-helix transcription factor family which lacks a basic domain for DNA binding\textsuperscript{27}. Therefore, it acts as a dominant inhibitor of the basic helix-loop-helix transcription factors by forming heterodimers. ID-1, an inhibitor of basic helix-loop-helix transcription factors, has recently been suggested to be a key regulator of metastatic potential of cancers. ID-1 was overexpressed in highly aggressive cancer cells, but not in nonaggressive cancer cells\textsuperscript{28}. There have been some reports that ID-1 overexpression is correlated with HCC cell proliferation and metastasis\textsuperscript{29}. HCC metastases lead to poor prognosis for HCC patients due to limited efficacy of systemic chemotherapy including sorafenib or 5-fluorouracil-based therapy\textsuperscript{30,31}. Therefore, finding a targeting molecule to inhibit HCC metastasis is crucial to overcome the limited efficacy of sorafenib for advanced HCC patients.

Our results showed that silencing ID-1 effectively suppressed EMT markers and actin rearrangement, which are crucial in metastasis of cancer cells. A recent study reported that ID-1 promoted metastasis by modulating PI3K/Akt
dependent expression of MMP-9 in esophageal cancer. Another study reported that knockdown of ID-1 significantly suppressed the secretion of MMPs and cancer metastasis in xenograft models. Taken together, ID-1 has an important role in governing invasive properties of cancer cells, especially in early times. In this study, we found that the protein level of ID-1 decreases at 24–48 hr time points. This phenomenon might implicate that ID-1 expression is attenuated as HCC cells proliferate. As HCC cells proliferate, NDRG-1/CAP43 is up-regulated, especially at 48 hr time point as shown in Fig. 4B as a compensatory mechanism against HCC proliferation. Up-regulated CAP43 expression might attenuate ID-1 expression at 24–48 hr time points.

In a hypoxic microenvironment, HCC cells express survival signals to proliferate more efficiently even than HCC cells in a normoxic microenvironment. Moreover, HIF provokes adaptation to hypoxic stress in rapidly growing cancer cells. In this study, the compensatory down-regulation of ID-1 by HIF-2α against hypoxic stress was noted, which is a novel finding.

Surprisingly, fucoidan demonstrated protective effect on
hepatocyte by decreasing BA–induced apoptosis, while enhancing apoptosis of HCC cells. The dual effects of fucoidan may represent an ideal anti-cancer strategy, preserving hepatic functional reservoir. The cytotoxic effects of hydrophobic BAs have been well documented in normal hepatocytes. Previous studies have shown that both mitochondrial dysfunction, caused by oxidative stress, and the non-mitochondrial nitric oxide–dependent pathway can cause BA–induced apoptosis in hepatocytes\textsuperscript{35,36}. Procaspases are recruited to DISC via homotypic interactions between death effector domains (DED) of FADD and initiator caspases\textsuperscript{37}. Once recruited to DISC, these procaspases undergo autocatalytic processing within DISC\textsuperscript{38}. The cFLIP regulates both recruitment and processing of procaspases within DISC\textsuperscript{39}. A potential mechanism which toxic BA may promote death receptor cytotoxic signaling is by modulating the composition of DISC \textsuperscript{40}. Our results following DISC IP suggest that fucoidan suppresses these cascades. 

In conclusion, NDRG/CAP43–dependent down-regulation of ID–1 suppressed HCC invasion both \textit{in vitro} and \textit{in vivo}, which was modulated by fucoidan treatment. Moreover, the
compensatory down-regulation of ID-1 against hypoxia-induced HCC invasion was observed. Down-regulation of ID-1 is a novel therapeutic target for the treatment of metastatic HCC. Moreover, fucoidan may represent an ideal therapeutic agent for HCC patients as a potent suppressor of tumor invasion with hepatoprotective effects.
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국문 초록

서론: 간세포암은 혈관 전이 및 원격 전이를 하는 경향이 많은 빠르게 성장하는 종양이다. 후코이단은 암세포의 증식은 억제하면서 간세포는 보호하는 역할을 하는 것으로 알려져 있다. 본 연구에서는 후코이단의 간세포암에 대한 항전이 효과 및 이를 조절하는 핵심신호를 확인하며 담즙에 의한 간세포의 사멸에 대한 보호 효과를 확인하고자 한다.


결과: 후코이단은 간세포암 세포의 침습을 유의하게 감소시켰다. 마이크로어레이 데이터를 통하여 후코이단 치료에 의하여 ID-1이라는 유전자가 억제되는 것을 확인하였다. ID-1 의 하향 조절은 간세포
포암의 침습을 시험 관내 및 생체 내에서 NDRG/CAP43 에 대하여 의존적인 방식으로 감소시켰다. 면역블롯검사에서 ID-1 에 대한 작은 간섭 RNA 를 처리한 경우 상피-중간엽 이행의 지표인 CK19, vimentin, MMP2, 및 fibronectin 의 발현이 감소하는 것을 확인하였다. 또한 면역형광검사를 시행하였을 때에 ID-1 이 간세포암 세포에서 하향 조절되었을 때에 actin 의 재배열이 역제되는 것을 확인하였다. SNU-761 세포에서는 ID-1 의 발현이 저산소 환경에서 정상산소 환경에 비교하여 더 억제되는 경향이 확인되었다. 저산소 환경에서는 HIF-1α 가 NDRG-1/CAP43 을 상향 조절하였는데, 반면 HIF-2α 는 ID-1 을 하향 조절하였다. 이는 간세포암의 저산소 환경으로 인한 침습 증가에 대한 보상 현상으로 판단된다. 또한 후코이단은 담즙에 의한 간세포의 사멸을 감소시켰는데 이는 후코이단이 caspase-8 과 caspase-7 의 활성도를 감소시키고 caspase-8 과 FADD 의 세포사멸 유도 신호복합체 내로의 진입이 감소되는 것에서 확인할 수 있다.

결론: 결론적으로 NDRG/CAP43 의존적 ID-1 의 하향 조절이 시험 관내 및 생체 내에서 간세포암의 침습을 역제하였고 이는 후코이단에 의하여 조절이 되었다. 이는 ID-1 이 전이성 간세포암에서 새로운 치료의 표적이 될 수 있음을 시사한다. 또한 본 연구는 간세포암의 저산소 환경으로 인한 침습 증가에 대한 보상 현상으로 ID-1 이 하향조절되는 현상을 밝혔다. 후코이단은 담즙에 의한 간세포 사멸

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을보호하며간세포암의침습을억제하는물질로서간세포암치료제개발에크게기여할수있을것이다.

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주요어: 간세포암, 전이, ID-1, 후코이단, 저산소중
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