의학석사 학위논문

Role of Bile Acid-induced Fibroblast Growth Factor 19 Expression in Hepatocellular Carcinoma Cell Proliferation

담즙산으로 유도된 fibroblast growth-19가 간세포암의 증식에 미치는 영향

2017년 2월

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A thesis of the Master's degree

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Feburary 2017

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동의서

Abstract

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Background: Fibroblast growth factor 19 (FGF19) is produced by enterocytes and acts on FGF receptor 4 (FGFR4) to down-regulate cholesterol 7α-hydroxylase (CYP7A1), the key gene involved in bile acid synthesis. Abundant bile acid increases the production of FGF19, resulting in a negative feedback cycle for bile synthesis. We hypothesized that bile acid-induced FGF19 expression also may promote hepatocellular carcinoma (HCC) cell proliferation.

Methods: Huh-BAT and SNU761 HCC cell lines were treated with control small interfering RNA (siRNA) or FGF19-specific siRNA with or without chenodeoxycholic acid (CDCA, bile acid) or obeticholic acid (OCA). Cell viability, gene expression, and protein expression were assessed by MTS cell proliferation assay, RT-qPCR, and immunoblotting.

Results: HCC cells treated with 25 μM CDCA exhibited increased cell proliferation and elevated levels of FGF19 mRNA. Cell transfection with FGF19- or FGFR4-specific siRNA attenuated bile acid-induced HCC cell proliferation, and FGF19-specific siRNA reduced the phosphorylation of p42/44 mitogen-activated protein kinase. These results suggest that bile acid-induced HCC cell proliferation is mediated by autocrine FGF19 signaling.

Conclusions: This study demonstrated that inhibition of FGF19/FGFR4 signaling suppresses bile acid-induced HCC cell proliferation. Therefore, suppression of FGF19 or FGFR4 expression may be a therapeutic strategy for attenuating the growth of HCC with cholestasis.

Key words: Fibroblast growth factor 19, Hepatocellular carcinoma, Bile acid

Student Number: 2015-21972

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Introduction

Fibroblast growth factors (FGFs) are polypeptide growth factors that mediate a variety of cellular responses including proliferation, differentiation, tissue repair and healing, and tumor angiogenesis.[1,2] Human FGF19 was first identified based on the amino acid similarity with murine FGF15 and its human ortholog.[3] FGF19 was shown to increase metabolic rate, reduce adiposity, and reverse diet-induced diabetes.[4,5] Recent studies reported that FGF19 has an important role in regulation of bile acid biosynthesis mediated by fibroblast growth factor receptor 4 (FGFR4) by repressing cholesterol 7α-hydroxylase (CYP7A1).[6,7]

Bile acid levels need to be tightly regulated to prevent hepatic toxicity from excess bile acid. The farnesoid X receptor (FXR), a transcription factor activated by bile acid,[8-10] is expressed in tissues that are exposed to bile acids including intestine, gall bladder, and liver.[11,12] FXR directly regulates FGF19 expression.[6] FGF19 is detected in human sera, and FGF19 levels exhibit diurnal variations in accordance with serum 7α-hydroxy-4-cholesten-3-one levels, which is a product of CYP7A1.[13] FGF19 also inhibits CYP7A1 expression in primary human hepatocytes and HepG2 cells.[6] These combined results indicate that serum FGF19 is released from the intestine via FXR activation by transintestinal bile acid flux, and subsequently transported to hepatocytes to inhibit CYP7A1. FGF19 is induced in human hepatocytes by treatment with chenodeoxycholic acid (CDCA, bile acid), indicating that liver as well as intestine are sources of serum FGF19 in humans.[7]

The investigators suggested that hepatocyte bile acids activate the liver FGF19 signaling pathway in a paracrine or autocrine manner to prevent accumulation of toxic bile acid in human liver.

Several recent studies have reported that FGF19 is involved in hepatocellular carcinoma (HCC). FGF19 overexpression in an ectopic site in transgenic mice promotes hepatocyte proliferation, hepatocellular dysplasia, and neoplasia.[14] Neutralizing monoclonal antibody that blocks the interaction of FGF19 with FGFR4 effectively reduces the growth of colon tumors and prevents HCC development *in vivo*.[15] Miura *et al.* reported that FGF19 expression increases tumor progression, and FGF19 levels are an independent prognostic factor for overall and disease-free survival in HCC patients.[16]

We hypothesized that bile acids induce FGF19 overexpression in HCC cells, and bile acid-induced FGF19 promotes HCC cell proliferation. In this study, we performed small interfering RNA (siRNA) transfection in HCC cell lines and monitored cell viability and molecular responses using MTS cell proliferation assays, RT-PCR, and immunoblotting.

Materials and methods

Cell lines and cell culture

Two human HCC cell lines were used in this study. Huh-BAT is a well-differentiated HCC cell line, which contains Huh-7 cells transfected with a bile acid transporter [Na(+)-dependent taurocholic contransporting polypeptides (NTCPs)],[17] whereas SNU-761 is a poorly-differentiated HCC cell line.[18] Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Huh-BAT) or Roswell Park Memorial Institute 1640 medium (RPMI-1640, SNU-761) supplemented with 10% fetal bovine serum (FBS), 100 mg/L of streptomycin, and 100,000 U/L of penicillin. Cells were incubated under either normoxic (20% O_2 and 5% CO_2 at 37°C) or hypoxic (1% O_2, 5% CO_2, and 94% N_2 at 37°C) conditions. Cells were starved overnight in all experiments to prevent confounding factors with respect to serum-induced cellular signaling.

RNA isolation and real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Complimentary DNA (cDNA) was obtained from total RNA by reverse transcription and amplified by polymerase chain reaction (PCR) according to the manufacturer's protocols for the RNA PCR kit (Promega, Madison, WI). Quantitative real-time PCR (qRT-PCR) was performed with the ABI prism 7300

sequence detection system (Applied Biosystems, Foster City, CA) using the SYBR Green PCR Master Mix (Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene was used as a control. FGF19 gene expression levels were calculated as relative intensity of the FGF19 PCR product compared with the reference intensity of the GAPDH gene using the delta-delta crossing threshold (ΔΔCt) method. All PCR experiments were performed in triplicate.

Cell transfection with small interfering RNAs

Cells were transfected with FGF19-specific or FGFR4-specific siRNAs and control (non-targeting) siRNA using siRNA Transfection Reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) according to the manufacturer's instructions. Cells were treated with siRNAs for 6 h at 37°C, and then growth medium containing 20% FBS and antibiotics was added. The growth medium was changed with fresh medium containing 10% FBS and antibiotics after 18 h. Cells were ready for experimental use at 24 h after transfection.

MTS assay for cell proliferation analysis

The Cell Titer 96 Aqueous One Solution Cell Proliferation assay (Promega, Madison, WI) was used to measure cell proliferation. In this assay, dehydrogenase enzyme activity converts the colorimetric reagent [3(4,5-dimethylthiazol-2-yl)-5-

(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS)] into soluble formazan only in metabolically active proliferating cells. The reactions contained 20 μL of reagent dye solution and cells in each well of a 96-well plate, and then the cells were incubated for 2 h . The absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Sunnyvale, CA, USA).

Immunoblot assay

Cells were lysed for 20 min on ice using lysis buffer (50 mM Tris-HCl pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl, 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 μg/L each of aprotinin, leupetin, and pepstatin; 1mM Na3VO4; and 1 mM NaF), and centrifuged at 14,000 g for 10 min at 4℃. Samples were resolved by electrophoresis on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), transferred to nitrocellulose membranes, blotted with appropriate primary antibodies , and then treated with peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA, USA). Bound antibodies were visualized using the ECL chemiluminescent substrate (Amersham, Arlington Heights, IL, USA) according to the manufacturer's instructions, and then exposed to Kodak X-OMAT film (Kodak, New Haven, CT, USA).

Statistical analysis

All experimental data were obtained from at least three independent experiments and are presented as the mean ± standard deviation (SD). The Mann-Whitney U-test and one-way analysis of variance (ANOVA) were used to compare differences between groups. All statistical analyses were computed with SPSS version 21.0 for Windows (SPSS, Inc., Chicago, IL, USA). A P value of <0.05 was regarded as statistically significant for all tests.

Results

Bile acids induce FGF19 mRNA expression in HCC cells

To identify whether bile acids induce FGF19 in HCC cells, we treated the Huh-BAT and SNU761 HCC lines with control or 25 μM of CDCA under both normoxic and hypoxic conditions. Quantitative RT-PCR assays were performed to measure the FGF19 mRNA expression levels at 0, 3, and 6 h. Treatment with CDCA dramatically increased the FGF19 mRNA levels compared with those of the controls in both cell lines (Figure 1A and B).

Bile acids induce FGF19 expression and promote HCC cell proliferation

To demonstrate the effect of bile acid-induced FGF19 expression on HCC cells, we transfected Huh-BAT and SNU761 cells with control or FGF19 siRNA and then cultured with or without 25 μM CDCA under either normoxic or hypoxic conditions. Cell proliferation was evaluated by MTS assay after incubating for 24 and 48 h.

Under normoxic conditions for 24 h, Huh-BAT cells transfected with control siRNA and cultured in the presence of CDCA displayed marginally enhanced cell proliferation (P=0.052). Under the same normoxic conditions for 24 h, Huh-BAT cells transfected with FGF19 siRNA and cultured in the presence or absence of CDCA did not display any significant differences in cell proliferation (P=0.876). In

the presence of CDCA, cell proliferation in Huh-BAT cells transfected with FGF19 siRNA was significantly lower than that in cells transfected with control siRNA (P=0.004) (Figure 2A). Under hypoxic conditions in the presence of CDCA for 48 h as well, cell proliferation was significantly increased by CDCA treatment (P=0.001) and attenuated by FGF19 siRNA transfection (P<0.001) (Figure 2B).

The SNU761 cell line showed similar results as the Huh-BAT cell line. After 24 h under hypoxic conditions, cells treated with CDCA displayed significantly higher levels of cell proliferation than cells without CDCA treatment (P=0.003). SNU761 cells transfected with FGF19 siRNA displayed similar levels of cell proliferation in the presence or absence of CDCA (P=0.076). In the presence of CDCA, SNU761 cells transfected with FGF19 siRNA displayed significantly lower levels of cell proliferation than cells transfected with control siRNA (P=0.037) (Figure 2C). Under normoxic conditions for 48 h, SNU761 cells transfected with FGF19 siRNA had significantly lower cell proliferation levels than those transfected with control siRNA (P<0.001) (Figure 2D).

These combined results indicate that bile acids strongly promote HCC proliferation, and FGF19 has a major role in this cell proliferation induced by bile acid.

FGF19 mediates bile acid-induced HCC cell proliferation via the FGF19/FGFR4 autocrine/paracrine pathway

The importance of FGF19 in tumor growth was examined further by investigating the FGF19/FGFR4 signaling pathway. Both HCC cell lines were transfected with FGFR4 siRNA to target the receptor tyrosine kinase specifically activated by FGF19 or control siRNA, and then cultured with or without 25 μM CDCA for 24 h. The MTS cell proliferation assay results were very similar to those from the preceding FGF19 siRNA experiments. Transfection with FGF19 siRNA significantly suppressed CDCA-induced cell proliferation in both Huh-BAT (P=0.002; Figure 3A) and SNU761 (P=0.032; Figure 3B) cell lines. These combined results suggest that FXR-mediated FGF19 induction proceeds via FGF19/FGFR4 signaling by an autocrine/paracrine pathway, which promotes HCC cell proliferation

Obeticholic acid enhances HCC cell proliferation via the FGF19 signaling pathway

We repeated the Huh-BAT experiments described above using 1 μM obeticholic acid (OCA), a potent synthetic FXR ligand,[19] in place of CDCA. After incubating for 24 h, OCA-treated cells showed significantly higher cell proliferation levels than cells cultured in the absence of OCA under both normoxic and hypoxic conditions (P=0.032 and P=0.024, respectively). By contrast, there were no significant differences in cell proliferation levels after siRNA-mediated FGF19 knockdown in the presence or absence of OCA under normoxic (P=0.940) or

hypoxic (P=1.000) conditions. In the presence of OCA, the cell proliferation level was significantly lower in cells transfected with FGF19 siRNA than in control cells under both normoxic (P=0.008) and hypoxic (P=0.006) conditions (Figure 4).

FGF19 mediates bile acid-induced HCC cell proliferation via the p42/44 mitogen-activated protein kinase signaling pathway

To identify the downstream signaling pathway of bile acid-induced HCC cell proliferation, we performed western blot analysis to determine the expression and phosphorylation of selected proteins involved in the p42/44 mitogen-activated protein kinase (MAPK) signaling pathway. SNU761 cells transfected with either control or FGF19-specific siRNA were cultured with or without 25 μM CDCA, and then lysed and prepared for immunoblotting. Phosphorylation of p42/44 MAPK was strongly induced by CDCA. By contrast, after siRNA-mediated FGF19 knockdown, p42/44 MAPK phosphorylation in response to CDCA treatment was markedly reduced (Figure 5A and B). Similar results were observed in Huh-BAT cells treated with 1 μM OCA under normoxic conditions (Figure 5C).

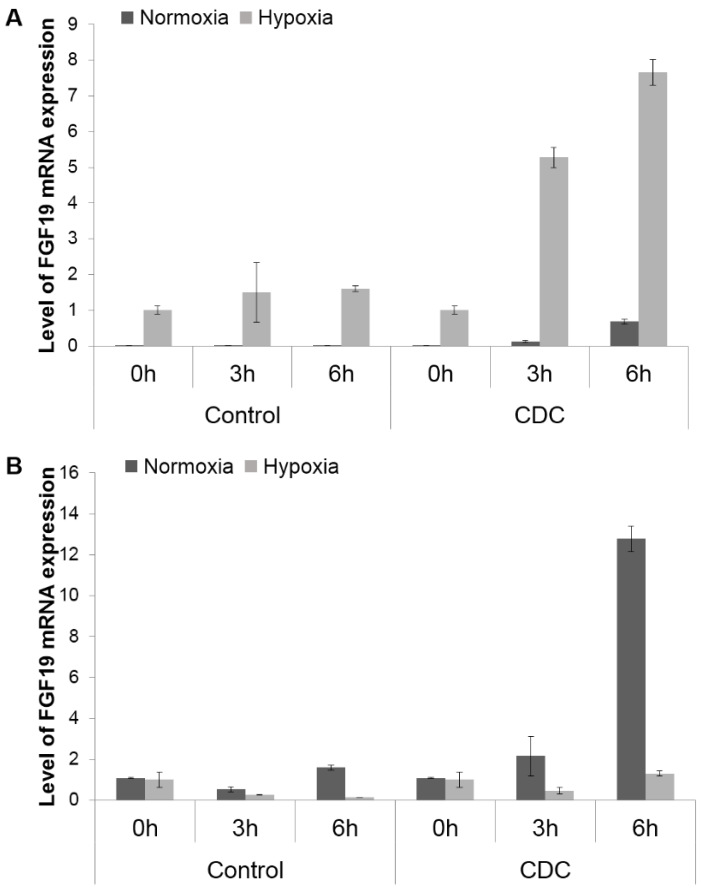
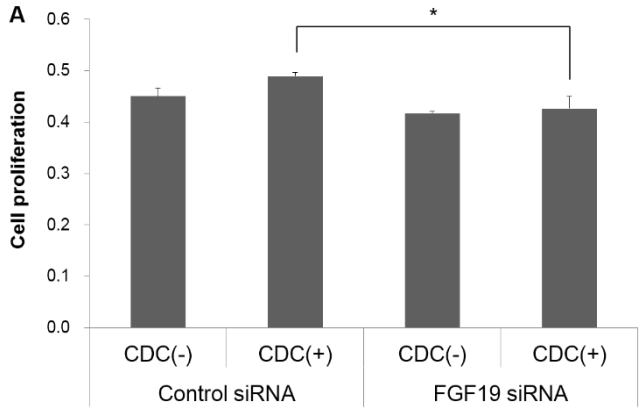


Figure 1. CDCA treatment increases FGF19 expression. (A) Huh-BAT cells and (B) SNU761 cells were treated with or without 25 μM CDCA under both normoxic and hypoxic conditions for up to 6 h. Total RNA was extracted from cell lysates and RT-qPCR was performed on total RNA from cell lysates. Higher levels of FGF19 mRNA expression were observed in Huh-BAT and SNU761 cells treated with CDCA than in those treated with placebo control under either normoxic or hypoxic conditions.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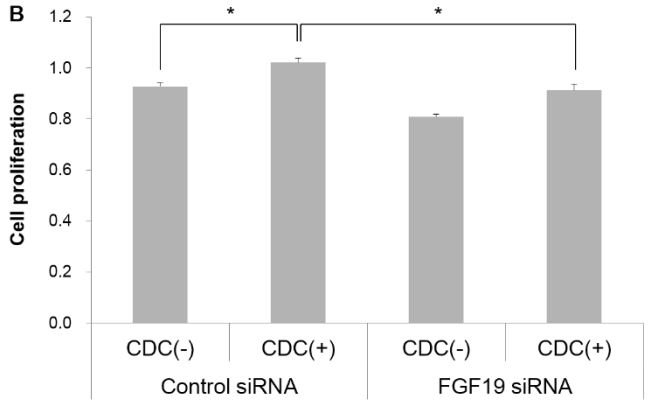
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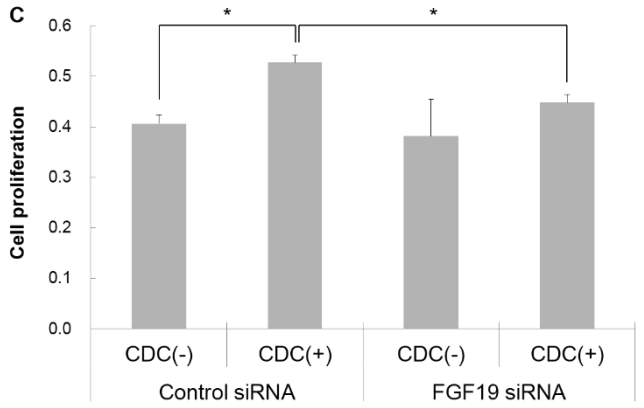
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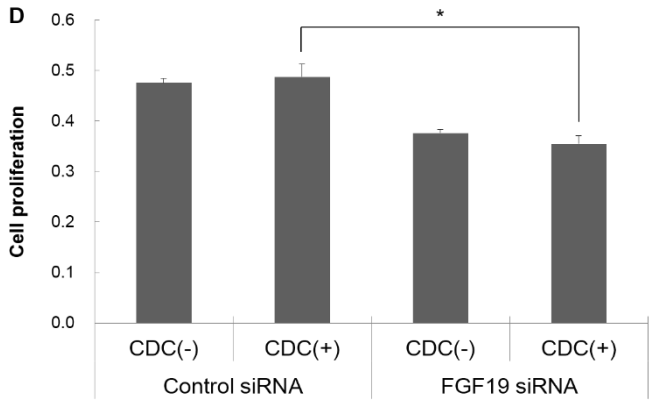

Figure 2. FGF19 depletion selectively inhibits CDCA-induced HCC cell proliferation. Huh-BAT and SNU761 cells were transfected with control (non-targeting) or FGF19-specific siRNA and cultured with or without 25 μM CDCA under either normoxic or hypoxic conditions. MTS assays were performed to measure cell proliferation after incubating for 24 and 48 h. FGF19 knockdown inhibited CDCA-induced cell proliferation in both Huh-BAT and SNU761 cell lines regardless of oxygen concentration. (A) Huh-BAT cells incubated for 24 h under normoxic conditions. (B) Huh-BAT cells incubated for 48 h under hypoxic conditions. (C) SNU761 cells incubated for 24 h under hypoxic conditions. (D) SNU761 cells incubated for 48 h under normoxic conditions.

Data were expressed as the mean ± SD. *, $P<0.05$

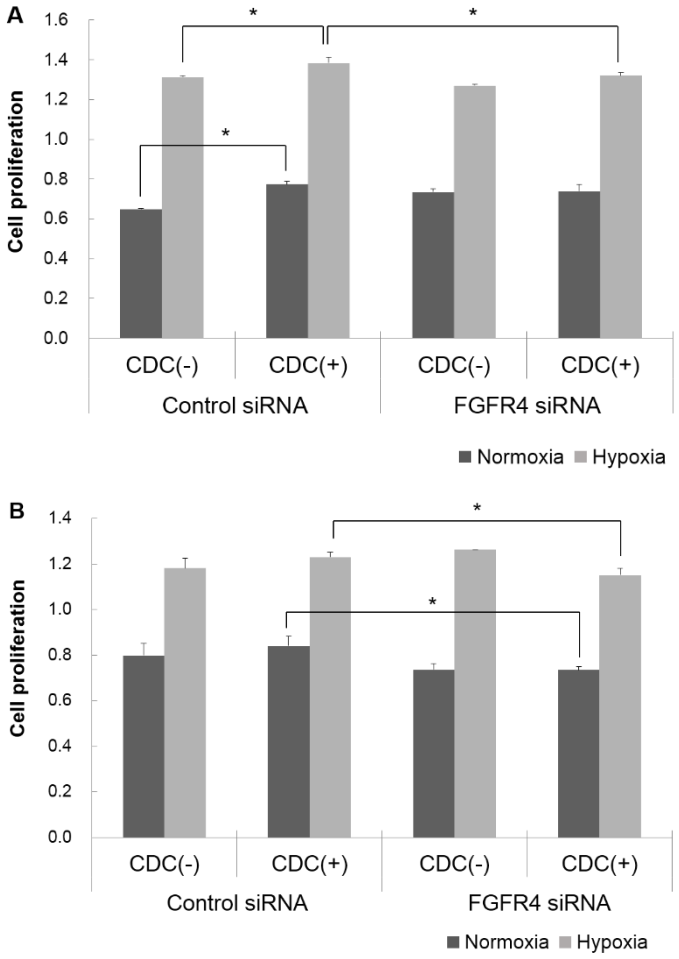

Figure 3. Depletion of endogenous FGFR4 inhibits CDCA-induced HCC cell proliferation. (A) Huh-BAT and (B) SNU761 cells transfected with control (non-targeting) or FGFR4-specific siRNA were cultured with or without 25 μM CDCA under either normoxic or hypoxic conditions. The MTS assay for cell proliferation was performed after incubating for 24 h. FGFR4 depletion selectively inhibited CDCA-induced cell proliferation in both Huh-BAT and SNU761 cell lines.

Data were expressed as the mean ± SD. *, $P<0.0$

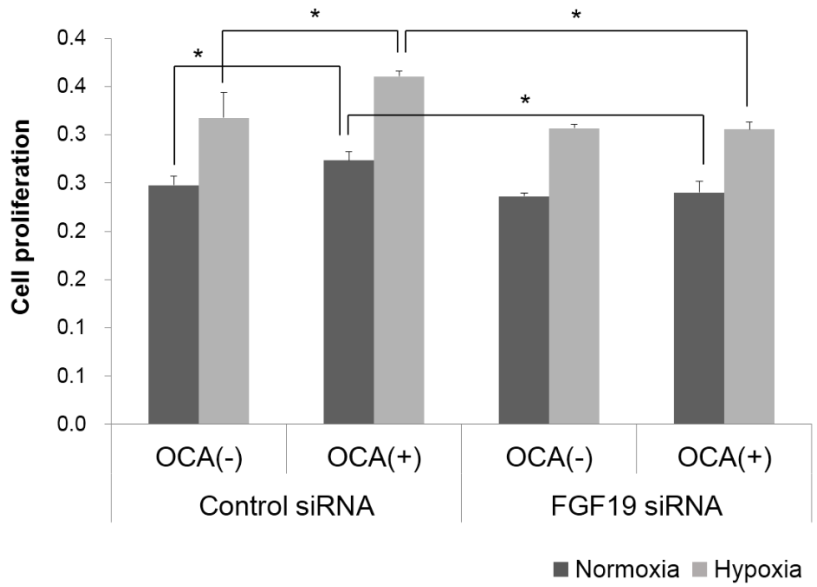

Figure 4. FGF19 depletion inhibits OCA-induced HCC cell proliferation. Huh-BAT cells transfected with control (non-targeting) or FGF19-specific siRNAs and cultured with or without 1 μM OCA under either normoxic or hypoxic conditions. The MTS assay for cell proliferation was performed after incubating for 24 h. OCA enhanced cell proliferation, whereas FGF19 depletion inhibited OCA-induced cell proliferation.

Data were expressed as the mean ± SD. *, $P<0.05$

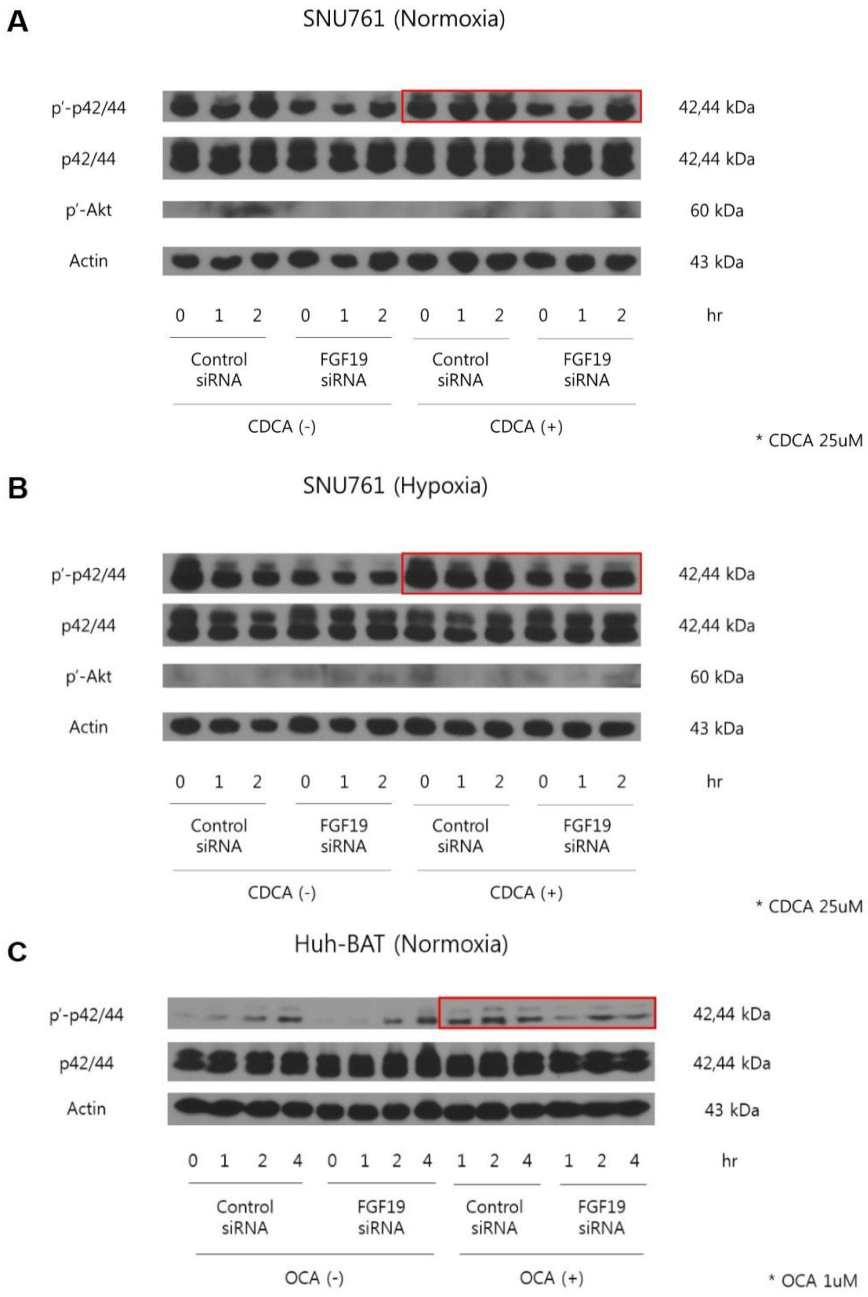

Figure 5. FGF19 depletion reduces bile acid-induced ERK1/2 phosphorylation.

SNU761 cells transfected with control (non-targeting) or FGF19-specific siRNA

were cultured with or without 25 μM CDCA under either normoxic or hypoxic conditions. Cell lysates were collected 24 h after transfection and analyzed by western blotting to determine the expression and phosphorylation of selected proteins. Actin was used as the loading control. FGF19 depletion attenuated CDCA-induced AKT and ERK1/2 phosphorylation in SNU761 cells under both (A) normoxic and (B) hypoxic conditions. (C) Similar results were obtained in OCA-treated Huh-BAT cells.

Discussion

The endogenous ligands of the FXR nuclear receptor are bile acids.[8-10] FXR is strongly expressed in liver and enterocytes, and the main target gene in humans is FGF19.[20] FGF19 and FGF19 subfamily members function as endocrine factors, although most FGFs perform their functions in either paracrine or autocrine pathways.[21] Previous studies report that endocrine FGFs require Klotho protein family members as co-receptors to support binding to the corresponding FGFRs.[22,23] FGF19 can bind and activate FGFR4 without Klotho beta (KLB), but KLC is crucial for FGF19-mediated FGFR4 activation due to the low concentration of plasma FGF19 under physiological conditions.[24] KLB is only expressed in a few organs, whereas FGF4 is widely expressed in numerous organs. FGFR4 and KLB are strongly co-expressed exclusively in the liver.[25] These combined results suggest that liver is the main target organ for FGF19. Bile acids entering the intestine stimulate FXR in the enterocyte to secrete FGF19 into the portal blood vessels. The secreted FGF19 reaches the liver via blood circulation. FGF19 is strongly induced in primary human hepatocytes and the gut via bile acid-induced FXR activation in either autocrine or paracrine manner.[7]

FGF19 regulates various liver functions in both the physiological and pathological state, including bile acid metabolism, cell proliferation, cell differentiation, and cell migration. In the neoplastic state, FGF19 expression has been suggested to promote HCC in mice and humans,[14,15] and is associated with poor prognosis of HCC

patients.[16] Several studies have attempted to elucidate the FGF19 signaling pathway that mediates HCC proliferation. Rama *et al.* reported that FGF19 increased β-catenin activation, which caused the loss of β-catenin-E-cadherin binding, whereas FGF19 inhibition reduced tumor growth by modulating β-catenin signaling.[26] FGF19 activates fibroblast growth factor receptor substrate 2 (FRS2) and extracellular signal-regulated kinase (ERK)[15]. These combined results suggest that more than one signaling pathway (including β-catenin and ERK signaling) may be involved in FGF19-mediated HCC proliferation; cross-regulation of Wnt signaling by MAPK also may be considered based on the results of earlier studies.[27,28]

We hypothesized that bile acid-induced FGF19 enhances HCC cell proliferation based on the following three established facts: bile acids induce FGF19 expression, FGF19 promotes HCC cell proliferation, and the MAPK/ERK signaling pathway is downstream from HCC cell proliferation. In this study, we showed that bile acids increased FGF19 mRNA expression in human HCC cell lines. We also proved that bile acids promoted HCC cell proliferation by amplifying downstream signaling such as ERK phosphorylation, and FGF19 had a key role in this process. The FGF19 or FGFR4 knockdown experiments demonstrated that CDCA-mediated cell proliferation and ERK phosphorylation were markedly attenuated to the levels observed in the absence of CDCA. These results are consistent with those of several other studies, which suggest that FGF19 inactivation is a potential therapeutic strategy for HCC.[15,16,26] Our results suggest more specifically that FGF19 inhibition is a promising treatment option for patients with HCC and cholestasis.

OCA, the 6α-ethyl derivative of CDCA, is the most potent selective FXR agonist with approximately 100-times greater potency in activating FXR rather than CDCA.[19] A number of preclinical and clinical trials indicate that OCA improves FXR-activated hepatic steatosis, fibrosis, and insulin sensitivity.[29-32] Recent studies report that OCA shows beneficial effects in patients with primary biliary cholangitis.[33,34] Moreover, OCA has also been suggested as a therapeutic agent for diverse diseases such as non-alcoholic fatty liver disease, type 2 diabetes, and primary biliary cholangitis. Recognized adverse effects of OCA are pruritus, lipid changes, GI trouble, and other minor events.[32,34] We presumed that OCA and CDCA could show similar effects on FGF19, and found that the results from OCA and CDCA experiments did not significantly differ. In the neoplastic state, OCA significantly increased FGF19 mRNA expression, ERK phosphorylation, and HCC cell proliferation. The siRNA-mediated FGF19 knockdown experiments indicated that FGF19 primarily mediated OCA-induced cell proliferation, similarly as observed for CDCA-mediated cell proliferation. These results may have important precautionary implications for the use of OCA as a therapeutic agent. Further *in vivo* studies are warranted to confirm the relationship between OCA and HCC cell proliferation.

In conclusion, our present results suggest that inhibition of FGF19/FGFR4 signaling could provide considerable therapeutic benefit for patients with HCC, especially when accompanied by cholestasis.

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국문초록

서론: Fibroblast growth factor-19는 장관내 세포에서 분비되어 간세포에 작용하는 물질로 담즙산 합성을 조절하는 역할을 하는 것으로 알려져 있다. 담즙산의 과도한 분비는 FGF19의 생성을 증가시킴으로써 담즙산의 합성을 억제하게 된다. 최근의 연구에 따르면 FGF19은 간암세포에서 발현이 증가되어 있으며 증가된 FGF19이 간세포암 환자들의 예후에 영향을 미치는 것이 밝혀진 바 있다. 이에 본 논문에서는 인간 간세포암 세포주에서 담즙산이 FGF19의 발현을 증가시키고, 담즙산에 의해 유도된 FGF19이 간세포암의 증식에 영향을 미친다는 것을 밝히고자 하였다.

방법: 간암세포주로 Huh-BAT과 SNU761을 사용하였고, 각각의 세포주를 FGF19 혹은 FGFR4 특이 siRNA로 처리를 하여 FGF19, FGFR4를 중성화시킴으로써 대조군과 비교하여 그 기능을 밝히고자 하였다. 또한 각각의 경우에서 담즙산(chenodoxycholic acid 혹은 obeticholic acid) 처리를 하여 FGF19/FGFR4가 작용하는 기전에 담즙산이 미치는 영향을 확인하였다. 세포의 증식은 MTS assay, 유전자 발현은 RT-qPCR, 단백질 발현은 immunoblotting 기법을 사용하였다.

결과: 담즙산이 간세포암에서의 FGF19 발현을 증가시켰고, 담즙산에 의해 증가된 FGF19은 간세포암의 증식을 촉진시키고, 담즙산에 의해 유

도된 FGF19을 siRNA로 불활성화 시켰을 때 간세포암의 증식이 억제하였다. 또한 담즙산을 obeticholic acid로 하여 동일한 과정으로 실험을 반복했을 때 FGF19가 유도되어 간세포암의 증식을 촉진시키는 결과를 확인하였다.

결론: FGF19/FGFR4 신호 기전을 차단함으로써 담즙산으로 유도되는 간암세포의 증식을 억제할 수 있으므로 향후 간암세포의 치료제로 활용될 수 있을 것이다.