February 2010
Doctor's Thesis

Ursodeoxycholic acid induces apoptosis in human gastric cancer cells

Graduate School of Chosun University

Department of Bio New Drug Development

Duong Hong Quan

Ursodeoxycholic acid induces apoptosis in human gastric cancer cells

February 2010

Graduate School of Chosun University

Department of Bio New Drug Development

Duong Hong Quan

Ursodeoxycholic acid induces apoptosis in human gastric cancer cells

Ursodeoxycholic acid에 의한 위암세포 고사유도

Advisor: Prof. Sung-Chul Lim

Thesis submitted for the degree of Doctor of Science

December 2009

Graduate School of Chosun University

Department of Bio New Drug Development

Duong Hong Quan

Thesis submitted in partial fulfillment of the requirements for

the degree of Doctor of Science in the Graduate School

of Chosun University, Chosun University

Approved by the Guidance Committee:

Prof. Cheol-Hee Choi
Chosun University _____

Prof. Sung-Heui Shin
Chosun University _____

Prof. Sung-Haeng Lee
Chosun University _____

Prof. Song-Iy Han
Chosun University _____

Prof. Sung-Chul Lim
Chosun University _____

December 2009

Graduate School of Chosun University

February 2010 Doctor's Thesis

Ursodeoxycholic acid induces apoptosis in human gastric cancer cells

Duong Hong Quan

TABLE OF CONTENT

LIST OF FIGURES

ABBREVIATIONS

TNFR	Tumor necrosis factor receptor
FADD	Fas-associated death domain
DCFH-DA	2',7'-dichlorofluorescein diacetate
TNF	Tumor necrosis factor
HO/PI	Hoechst 33342/Propidium Iodide
ERK1/2	Extracellular signal-regulated kinase
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
MEK1/2	Mitogen-activated and extracellular regulated kinase kinase
PI-3K	Phosphatidylinositol-3 kinase
DR4	Death receptor 4
DR5	Death receptor 5
ROS	Reactive oxygen species
PARP	Poly (ADP ribose) polymerase
DISC	Death-inducing signaling complex
RIP1	Receptor-interacting protein 1
MBCD	Methylbetacyclodextrin
DPI	diphenylene iodonium

Z-DEVD-fmk	Benzyloxycarbonyl-Asp(ome)-Glu(ome)-Val-Asp(ome)-fluoromethylketone
Z-VAD-fmk	Benzyloxycarbonyl-val-ala-asp (ome) - fluoromethylketone
BHA	Butylated hydroxyanisole
NAC	N-acetylcystein

ABSTRACT

Ursodeoxycholic acid induces apoptosis in human gastric cancer cells

Duong Hong Quan

Advisor: Prof. Sung-Chul Lim, PhD

Department of Bio New Drug Development

Graduate School of Chosun University

Gastric cancer is the major cause of cancer death in the world. Since the efficacy of therapeutic approaches such as surgery, radio- and chemotherapy is limited, the novel chemotherapeutic agents are needed to treat gastric cancer. We showed that ursodeoxycholic acid (UDCA) mainly induced apoptosis in two different human gastric cancer cells SNU-601 and SNU-638 cells. UDCA-induced apoptosis was dependent on caspases-3, -6, -9 and -8 but not caspase-4, indicating that UDCA induced apoptosis through directly death receptor and indirectly mitochondrial pathway but not endoplasmic reticulum stress pathway. UDCA induced expression of DR5 and knock-down of expression of DR5 significantly suppressed UDCA-induced apoptosis. UDCA activated the translocation of protein kinase C delta (PKC δ), and inhibition of PKC δ significantly suppressed UDCA-induced expression of DR5 to trigger apoptosis. Moreover, UDCA stimulated ROS production, and ROS scavengers significantly prevented UDCA-induced expression of DR5 to trigger apoptosis. In addition, NAC blocked UDCA-activated the

translocation of PKC δ from cytosol to membrane. Interestingly, lipid raft depleting agent, methyl-β-cyclodextrin (MBCD) completely suppressed ROS production and the translocation of PKC δ from cytosol to membrane as well as expression of DR5 and apoptosis by UDCA in both cancer cell lines. On the other hand, UDCA activated phosphorylation status of MEK/ERK and inhibition of MEK1/2 prevented UDCA-induced expression of DR5 to trigger apoptosis in SNU-601 cells but not in SNU-638 cells. In addition, siRNA ERK1 but not siRNA ERK2 partially prevented UDCA-induced expression of DR5 to trigger apoptosis. Moreover, UDCA-activated phosphorylation status of MEK/ERK was completely blocked by MBCD in SNU-601 cells.

Overall, for the first time, our results suggest that UDCA-induced apoptosis is significantly regulated by lipid raft/ROS/PKC δ pathway in both cancer cell lines and partially regulated by lipid raft/MEK/ERK1 pathway in SNU-601 cells.

Taken together, UDCA can be a potential chemotherapeutic agent to develop for the new promising strategy to treat gastric cancer at clinical trial in the future.

Key words: Ursodeoxycholic acid, apoptosis, DR5, lipid raft, ROS, PKC δ, MEK, ERK

국문초록

Ursodeoxycholic acid 에 의한 위암세포 고사유도

Duong Hong Quan

지도교수: 임 성 철

조선대학교 대학원

바이오신약개발학과

위암은 전세계적으로 흔한 종양이지만 기존의 외과적 수술, 방사선요법 및 화학요법은 치료효율이 제한적이어서 새로운 치료법의 개발이 필요한 상황이다. 저자들은 ursodeoxycholic acid (UDCA)가 위암세포주 SNU-601 과 SNU-638 에서 주로 apoptosis 를 일으키는 것을 알아냈다. UDCA 에 의한 apoptosis 는 death receptor 를 직접 경유하며 mitochondria 경로는 간접적으로 경유하지만 ER 스트레스 경로는 거치지 않는 것으로 확인되었다. UDCA 에 의한 apoptosis 유도는 두 세포주 모두에서 lipid raft-ROS-PKC δ-DR5 경로를 통하여 일어나지만 lipid raft/MEK/ERK 경로는 SNU-601 세포에서만 작용됨을 알아냈다.

따라서 UDCA 는 위암의 효과적인 치료를 위한 새로운 치료법 개발에 희망적인 항암치료제 또는 항암치료보조제로 사용될 가치가 있는 것으로 판단된다.

Key words: Ursodeoxycholic acid, apoptosis, DR5, lipid raft, ROS, PKC δ, MEK, ERK

CHAPTER I: INTRODUCTION

Bile acids are a group of molecular species of acidic steroids with peculiar physical, chemical and biological characteristics. At high concentrations, they become toxic to mammalian cells, and their presence is pertinent in the pathogenesis of several liver diseases and colon cancer [1]. Hydrophobic bile acids include deoxycholic acid (DCA), chenodeoxycholic acid (CDCA) and lithocholic acid (LCA). Hydrophilic bile acids include ursodeoxycholic acid (UDCA), taurodeoxycholic acid (TUDCA), taurohyodeoxycholic acid (THDCA) and cholic acid (CA). Hydrophobic bile acids have been accommodated within the hepatocyte to induce hepatic cell death during cholestasis and colorectal cancer development [2, 3] especially toxic bile acids have been shown to induce apoptosis involving the death receptor, mitochondrial and endoplasmic reticulum (ER) stress pathway in hepatocytes and colon cancer cells [4-8]. However, UDCA exerts cytoprotective, anti-apoptotic and immunomodulatory activities, and is widely used for the treatment of certain cholestatic liver diseases such as primary biliary cirrhosis and cystic fibrosis-related cholestasis [9, 10]. In addition, UDCA inhibits toxic bile acid-induced apoptosis by preventing formation of reactive oxygen species (ROS) and modulating mitochondrial membrane perturbation, pore formation, Bax translocation, cytochrome C release, caspase activation and subsequent substrate cleavage [11, 12]. Interestingly, the use of UDCA as an agent to treat non-liver diseases associated with increased levels of apoptosis, such as neurodegenerative disorder [13] and tumor suppressive activity of UDCA is believed to be linked to the induction of apoptosis and cell cycle arrest, and the

inhibition of oncogenic factors including Ras and COX-2 in human gastric cancer cell lines [14-16].

Gastric cancer is one of the most common types of solid tumor, and it is estimated to be the fourth most common in terms of mortality, and the second most frequent cause of cancer death in the world [17]. Gastric cancer is particularly common in Asia, Eastern Europe, and in South America, where the preservation of food is most performed by submerging it into salt, and where the detection rate of *Helicobactor pylori* is considerably high [18]. Recently, the efficacy of therapeutic approaches such as surgery, radio- and chemotherapy is limited [19]. Thus, new therapeutic approaches are needed.

Apoptotic cell death is a highly regulated mechanism, which can be viewed as a program of cell suicide vital for a wide variety of biological process. Apoptosis is an integral part of normal embryonic development and tissue homeostasis during adulthood and apoptosis has also been documented as a prominent player in immune regulation and tissue homeostasis, as well as in pathological and therapeutic settings [1]. Excessive apoptosis can lead to T cell depletion, neurodegenerative diseases, or hepatocellular degeneration, while impaired apoptosis contributes to oncogenesis, autoimmune diseases and infections [20]. The apoptotic pathway includes the mitochondrial or intrinsic pathway and the death receptor or extrinsic pathway. The mitochondrial pathway of apoptosis is triggered by intracellular stresses that converge on mitochondria, leading to membrane permeabilization, release of apoptogenic proteins, and disruption of the mitochondrial membrane potential. These changes culminate in activation of caspases and ultimately cell death [21]. The death receptor pathway of apoptosis is triggered by ligand-induced

activation of death receptors at the cell surface, followed by recruitment and oligomerization of intracellular adaptor molecules [22]. Death receptors are type 1 transmembrane proteins, belonging to the tumor necrosis factor (TNF) receptor super-family, and include TNFR-1, CD95/Fas, TNF-related apoptosis-inducing ligand (TRAIL) receptors -1 (DR4) and -2 (DR5), and death receptors 3 and 6. TRAIL binds to DR4 and DR5 or Fas ligand binds to CD95/Fas, which in turn recruit intracellular Fas-associated death domain (FADD). Through its death effector domain, FADD recruits caspase-8 to the receptors for the assembly of death-inducing signaling complex (DISC) [23]. In the DISC, caspase-8 is activated and cleaves caspase-3 directly or indirectly through cleavage of Bcl-2-inhibitory BH3 domain protein (Bid). The Bid then induces mitochondrial release of cytochrome C into the cytosol, where caspase-9 is activated and cleaves downstream caspase-3 [24, 25].

Protein kinase C (PKC) is a family of serine-threonine kinase isoenzyme, subclassified as classical PKC, novel PKC or atypical PKC depending on cofactor requirements [26]. Inactive PKC isoenzymes are located predominantly in the cytosol. Activated PKC isoenzymes translocate to a variety of intracellular sites including cell membrane, nucleus and membrane associated cytoskeleton [26]. PKC δ is a member of the novel PKC subfamily. PKC δ activation and translocation are induced by a variety of apoptotic stimuli in different cellular systems [27]. PKC δ translocates to and activates specific pathways in the plasma membrane, mitochondria and nucleus that eventually converge to the activation of caspase-3 and subsequent apoptosis [27]. Bile acids are reported to activate and translocate PKC δ from cytosol to membrane in colon cancer cells [28]. However, UDCA are reported to inhibit the translocation of PKC δ from cytosol to

membrane in these cells [28]. Indeed, the effects of UDCA on PKC δ activation and translocation from cytosol to membrane have not been reported.

ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), have been shown to induce various biological process, including apoptosis [29]. This implies that redox state of a cell is a crucial factor in deciding its susceptibility to apoptotic stimuli [30]. ROS at low concentrations play a role as an intracellular messenger in many molecular events, including cell proliferation and apoptosis, while the production of large amounts of ROS contributes to apoptosis [31]. Although, some reports have suggested that bile acids, especially hydrophobic bile acid can be generated ROS through a multi-factorial mechanism involving NADPH oxidase activation and interaction of bile acids with mitochondria [32, 33]. But UDCA has been shown to inhibit DCA-induced ROS production [34]. However, the effects of UDCA to induce ROS production have not been reported.

Mitogen-activated protein kinase (MAPK) cascades are involved in the signaling of various cellular responses such as cell death, proliferation, and inflammation [35, 36]. The extracellular signal-regulated kinase 1/2 (ERK1/2) cascade is activated through receptor-mediated signaling stimuli and is associated with cell proliferation, differentiation and survival [37, 38]. However, in some cases, ERK activation contributes to cell death [39, 40]. Studies have shown that the p38/ERK/PI3K pathways are involved in protection of TUDCA against GCDCA-induced apoptosis in rat hepatocytes [41]. In addition, DCA-induced ERK, which is mediated to activate Ap-1 activator that control cell growth and are

involved in tumorigenesis [42]. However, the effect of on ERK induction has not been reported.

Cholesterol is an abundant component of the plasma membrane of eukaryotic cells that plays a pivotal role in the regulation of membrane fluidity, permeability, receptor function and ion channel activity [43-46]. The lateral distribution of cholesterol in the membranes is not uniform and its content is particularly high in the submicroscopic areas also enriched in gangliosides and sphingolipids. These microdomains known as lipid rafts, act as molecular platforms that spatially organize membrane receptor molecules [45, 47]. Lipid raft plays an important role in cellular signaling, functioning as physical platforms to concentrate and assemble the signal transduction machinery [48], and regulate signal transduction by activating or suppressing the phosphorylation caspases related to cell growth, survival and death and other physiological processes [49]. Numerous studies show that expression of DR5 is regulated through ROS production [50-52], ERK activation [53], c-Jun N-terminal kinase activation (JNK) [53], and NF-kB and p53 activation [54]. some reports show that toxic bile acids enhance TRAIL-R2/DR5 expression and aggregation also promoting a death receptor-dependent apoptosis [5] and bile acids up-regulate DR5 to trigger apoptosis via JNK pathway involving Sp1 [53]. In addition, it is known that UDCA can selectively induce apoptosis of HepG2 and Bel7402 cell lines by blocking cell cycle and regulating the expression of Bax/bcl-2 gene. But the effects and molecular mechanism of UDCA remain unclear.

The overall objective of this study was to examine the molecular mechanism of UDCA on induction of apoptosis in human gastric cancer cells. To address this objective,

we formulated the following questions. (I) Do UDCA induce apoptosis in human gastric cancer cells? (II) What are the UDCA-induced apoptotic pathways? (III) What is the most crucial death receptor to trigger UDCA-induced apoptosis? (IV) What are the molecular mechanisms of UDCA-induced expression of DR5 to trigger apoptosis? In this study, we showed that UDCA induced apoptosis involving in directly death receptor and indirectly mitochondrial pathway. We also showed that UDCA-induced expression of DR5 to trigger apoptosis was regulated by lipid raft/ROS/PKC δ and lipid raft/MEK/ERK1 pathway. For the first time, our findings provided a novel function of UDCA on induction of apoptosis and the molecular mechanism of apoptosis by UDCA in human gastric cancer cells.

CHAPTER II: MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Cell culture

Human gastric cancer cell lines (SNU-601 and SNU-638 cells) were provided by Korea cell line bank, Seoul National University, South Korea. These cells were grown in RPMI 1640 medium (Invitrogen, Carlbad, CA) supplemented with heat-inactivated 10 % fetal bovine serum (FBS; Invitrogen, Carlbad, CA) and 1 % penicillin-streptomycin (Welgene, Seoul, Korea) in a 37^0 C humidified incubator in an atmosphere of 5 % CO_2.

The cells were seeded at a density of 5 x 10^6 cells/ 20 cm^2 dish, 1.5 x 10^6 cells/ 10 cm^2 dish, 5 x 10^5 cells/ 6 cm^2 dish, 2 x 10^5 cells/ 3.5 cm^2 dish and 5 x 10^4 cells/ well of 24-well plate then used for the drug exposure experiments after being culture over night

2.1.2. Reagents

Ursodeoxycholic acid (UDCA)	ICN Biomedicals
Deoxycholic acid (DCA)	Sigma-Aldrich
Z-DEVD-FMK (caspase-3 inhibitor)	Calbiochem
Z-VEID-FMK (caspase-6 inhibitor)	Calbiochem
Z-IETD-FMK (caspase-8 inhibitor)	Calbiochem
Z-LEHD-FMK (caspase-9 inhibitor)	Calbiochem
Z-VAD-FMK (poly-caspase inhibitor)	Calbiochem

Ac-LEVD-CHO (caspase-4 inhibitor)	Calbiochem
Methyl-β-Cyclodextrin (MBCD)	Sigma-Aldrich
N-acetyl-L-cysteine (NAC)	Roche
Butylated hydroxyanisole (BHA)	Roche
1,2-dihydroxybenzene-3,5-disulforic acid (Tiron)	Roche
Catalase	Sigma-Aldrich
DPI	Sigma-Aldrich
UO126	A.G. Scientific
PD98059	A.G. Scientific
Rottlerin	A.G. Scientific
GF109203x	A.G. Scientific
GO6976	A.G. Scientific

2.1.3. Antibodies

Cleaved caspase-3 (Asp175) antibody	Cell signaling
Cleaved caspase-6 (Asp315) antibody	Cell signaling
p-ERK1/2 antibody	Santa Cruz Biotechnology
t-ERK1/2 antibody	Santa Cruz Biotechnology
Anti-PARP antibody	BD pharmingen

p-MEK1/2 antibody	Cell signaling
t-MEK1/2 antibody	Cell signaling
p-EGFR antibody	Cell signaling
t-EGFR antibody	Cell signaling
FADD antibody	Santa Cruz Biotechnology
RIP1 antibody	BD pharmingen
DR4 antibody	ProSci
DR5 antibody	ProSci
PKC α	BD pharmingen
PKC δ	BD pharmingen
PKC θ	BD pharmingen
PKC ε	BD pharmingen
Tubulin	BioGenex Laboratories
Caveolin-1	Santa Cruz Biotechnology
Goat anti-mouse IgG-HRP antibody	Santa Cruz Biotechnology
Goat anti-rabbit IgG-HRP antibody	Santa Cruz Biotechnology

2.2. METHODS

2.2.1. Morphological detection of apoptosis and necrosis by Hoechst 33342 (HO) and Propidium Iodide (PI) assay

Human gastric cancer cells (SNU-601 and SNU-638 cells) were seeded with 2 x 10^5 cells in 3.5 cm^2 dishes per 1 ml of medium. After the overnight incubation, the indicated drugs were treated at various time points. Cells were stained with Hoechst 33342 (1 μg/ml) and PI (5 μg/ml) to the culture medium and incubated at 37^0 C with 5 % CO_2 for 15 minutes. After that, cells were harvested by trypsinization with 1X trypsin at 37^0 C with 5 % CO_2 for 15 minutes. Some apoptotic and necrotic cells detached from the culture substratum into the medium. All cells were collected by centrifugation at 1500 rpm at 4^0 C for 10 minutes. The cell pellets were also washed with 1X cold PBS and centrifuged again as described above. The cell pellets were suspended in 500 μl of 3.7 % paraformaldehyde and incubated for 5 minutes. The fraction of suspension was centrifuged in cytospinner (Cellspin, Hanil). Slides were immediately fixed with mounted gel after washed with water, dried and covered surely with a glass cover-slip. Slides were examined using a DM5000 fluorescence microscopy (Leica, Wetzal, Germany) at respective excitation/emission wavelengths of 340/425 nm (HO) and 580/630 nm (PI). Morphological assessments of apoptosis and necrosis were performed; intact blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei, and intact pink nuclei were considered viable, early apoptosis, late apoptotic (secondary necrotic), and necrotic cells, respectively. A total of 500 cells or 800 cells from several the randomly chosen fields were counted. The

number of apoptotic or necrotic cells was counted and calculated into the percentage of the total number of counted cells.

2.2.2. Cell viability assay

Cell viability was assessed by the MTT assay. In MTT assay, 5 x 10^4 cells of human gastric cancer cells were seeded (triplicate) into 24 well plates per 500 µl of RPMI medium. After the overnight of incubation in 37^0 C in 5 % CO_2 contained humidified incubator overnight, drugs were treated at various time points. After 4 h of incubation with MTT (0.5 µg/ml), plate was centrifuged at 1000 rpm at room temperature for 5 minutes. Then medium was removed and added 750 µl DMSO to dissolve formazin crystals. Absorbance was measured at 540 nm using an ELISA microplate reader (Perkin-Elmer). Absorbance of untreated cells was designated as 100 % and cell survival expressed as a percentage of this value.

2.2.3. Lactate dehydrogenase (LDH) release assay

This assay was used for the measurement of cytotoxicity of plasma membrane damage. The LDH is a stable enzyme, present in all cell types, and rapidly released into the cell culture medium upon damage of the plasma membrane. 5 x 10^4 cells of human gastric cancer cells were seeded (triplicate) into the 24 well plate per well per 500 µl of RPMI medium. After the overnight of incubation in 37^0 C in 5 % CO_2 contained humidified incubator overnight, drugs were treated at various time points. 50 µl LDH lysis buffer was added into the high control samples before 30 minutes of completed of time points. After the 30 minutes of lysis of cells, plates were centrifuged (600 g for 10 minutes) to precipitate the cells. 10 µl aliquot of supernatant was dispensed into several well of 24-well

micro titer plate and combined with 100 µl LDH reaction mix (mix 200 µl of WST substrate mix with 10.5 ml of LDH assay buffer) to each. Plate was read after 30 minutes of shaking at room temperature using a plate reader (Bio-Rad, Hercules, CA, USA) with primary wavelength 450 nm and reference wavelength 650 nm. The percentage of specific LDH release was calculated by the formula: % cytotoxicity = [(experimental LDH release) – (spontaneous LDH release by effector and target)/(maximum LDH release)-(spontaneous LDH release)] x 100 %. The spontaneous release of LDH activity from control cells was less than 2 % of the maximal release of LDH activity which was determined from the complete lysis by adding lysis buffer.

2.2.4. SDS-PAGE and Western blot analysis

For the detection of various kinds of protein expression, 5 x 10^5 cells of human gastric cancer cells were plated into the 6 cm^2 dish in 3 ml RPMI medium and incubated at 37^0 C in 5 % CO_2 contained humidified incubator overnight. At various time points after incubated with drug treatment, human gastric cancer cells and rat gastric normal cells were collected and washed with 1X cold PBS. And then, the collected cell pellets were lysed in lysis buffer (50 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 20 mM NaF, 50 mM PMSF, 1 mM Na_3VO_4, 100 ug/ml Leupeptin, and 10ug/ml Aprotinin). Cell debris was removed by centrifugation (13,000 rpm, 15 min, 4^0 C). Protein concentration was determined by using the Bio-Rad protein assay, and the samples were boiled for 10 min. The boiled samples were loaded onto a 7 %, 8 %, 10 %, 12 % or 15 % SDS-PAGE gel in accordance with the molecular weight of proteins, and electrophoresis was fixed at 100 V and run for 2.5 h. Protein was electrophoretically transferred onto 0.22 µM

nitrocellulose transfer membrane. The transferred membrane was blocked in 5 % nonfat dry milk in 1X TBST for 1 hour and incubated at 4^0 C overnight with the various primary antibodies against different proteins. Immunobilized proteins were incubated with horseradish peroxidase-linked secondary antibodies and visualized by enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA) and signals were acquired by image analyzer (Image station 4000MM, Kodak, NY, USA).

2.2.5. Small interference RNA (siRNA) transfection

To determine the role of FAS, RIP1, FADD, DR5, DR4, c-Jun, NF-kB (p65), ERK1 and PKC δ study, 1 x 10^6 of human gastric cancer cells were prepared and 8 μg siRNAs (**siRNA FAS** *5'-GCU UAU ACA UAG CAA UGG U (dtdt)-3' (S), 5'-ACC AUU GCU AUG UAU AAG C (dtdt)-3' (AS)* ; **siRNA RIP1** *5'-CAC ACA GUC UCA GAU UGA U (dtdt)-3' (S), 5'-AUC AAU CUG AGA CUG UGU G (dtdt)-3' (AS)*; **siRNA FADD** *5'-CCA AGA UCG ACA GCA UCG A (dtdt)-3' (S), 5'-UCG AUG CUG UCG AUC UUG G (dtdt)-3' (AS)*; **siRNA DR4** *5'-CUG GAA AGU UCA UCU ACU U (dtdt)-3' (S), 5'-AAG UAG AUG AAC UUU CCA G (dtdt)-3' (AS)*; **siRNA DR5** *5'-CAG ACU UGG UGC CCU UUG (dtdt)-3' (S), 5'-UCA AAG GGC ACC AAG UCU G (dtdt)-3' (AS)*; **siRNA c-Jun** *5'-ACU GUA GAU UGC UUC UGU A (dtdt)-3' (S), 5'-UAC AGA AGC AAU CUA CAG U (dtdt)-3' (AS)*; **siRNA NF-kB (p65)** *5'-CCU GAG CAC CAU CAA CUA U (dtdt)-3' (S), 5'-AUA GUU GAU GGU GCU CAG G (dtdt)-3' (AS)*; **siRNA ERK1** *5'-CUC UCU AAC CGG CCC AUC U (dtdt)-3' (S), 5'-AGA UGG GCC GGU UAG AGA G (dtdt)-3' (AS)*; **siRNA PKC δ** *5'-CUC AUG GUA CUU CCU CUG U (dtdt)-3' (S), 5'-ACA GAG GAA GUA CCA UGA G (dtdt)-3' (AS)*; and **SiRNA Control** *5'-CCU ACG CCA CCA AUU UCG*

U (dtdt)-3' (S), 5'-ACG AAA UUG GUG GCG UAG G (dtdt)-3' (AS)) were used to be transfection by using AMAXA method according to manufacturer's protocol. After transfection, the transfected cells were incubated in 37^0 C in 5 % CO_2 contained humidified incubator for 40 h and treated by drugs with various concentration and time points. And then, transfected cells lysed was subjected to Western blot for detection of specific proteins or performed for the caspase-8 activity assay, MTT assay, ROS production and HO/PI assay.

2.2.6. Caspase-8 activity assay

Caspase-8 activity assay was carried out using a FADD-like IL-1β-converting enzyme (FLICE)/caspase-8 colorimetric assay kit (BioVision) according to the manufacturer's protocol. 5 x 10^5 cells of human gastric cancer cells were seeded in 5 cm^2 dishes per 3 ml medium. After the time point of drug treatment, cells were collected by centrifugation. The collected cell pellets were lysed by 50 µl of chilled cell lysis buffer, incubated on ice for 10 minutes and centrifuged the samples in microcentrifuge at 10,000 g for 1 minute at 4^0C. The supernatant was transferred to a next tube and kept in ice. Protein concentration was measured by Bio-Rad protein assay. Protein samples were diluted at 150 µg to 50 µl by cell lysis buffer for each assay. 50 µl of 2 x reaction buffers (containing 10 mM DTT) and 5 µl the 4 mM IETD-pNA substrate were added to each sample to produce a final concentration of 200 µM, and then incubated at 37^0C for two h. Absorbance was measured at 405 nm in a plate reader. Fold increase in FLICE activity was determined by comparing the results of treated samples with the level of the untreated control.

2.2.7. Assay for ROS

To determine production of intracellular ROS, 5 x 10^4 cells of human gastric cancer cells were seeded (duplicate) into the 24 well plate per well per 500 μl RPMI medium. After the overnight of incubation in 37^0 C in 5 % CO_2 contained humidified incubator, drugs were treated at various time points. Cells were further incubated in 37^0 C with in 5 % CO_2 contained humidified incubator and loaded with 50 μM 2'-7'-dichlorofluorescein diacetate (DCFH-DA, Molecular probes) to measure ROS generation and 0.5 μg HO to quantify cell number for 1 hour after the indicated times. After rinsing, fluorescence measurements were obtained by Fluorocount model MQM200 plate reader (Packard Instrument, Meriden, CT, USA) with excitation at 490 nm and emission at 530 nm for DCFH-DA or excitation at 340 nm and emission at 425 nm for HO. The values of ROS production were obtained by determining the ratio of DCFH-DA/HO signals per well.

2.2.8. Colony formation assay

For the colony formation assay, 5 x 10^5 cells of human gastric cancer cells were seeded in 5 cm^2 dish per 3 ml medium. After the overnight of incubation in 37^0 C in 5 % CO_2 contained humidified incubator, drugs were treated at various concentration points of UDCA. Cells were further incubated in 37^0 C with in 5 % CO_2 contained humidified incubator for 24 h. After treatment, cells were reseeded with 2 x 10^3 in 5 cm^2 per 3 ml medium (duplicate). Each culture dish was incubated for 15 days at 37^0 C with in 5 % CO_2 contained humidified incubator and photographed after staining with 0.5 % crystal violet in PBS including 25 % methanol. Colonies were examined under a light microscope and counted after capturing images by scanner.

2.2.9. Subcellular protein fractionation

For subcellular protein fractionation, human gastric cancer cells were plated in 10 cm^2 cultural dishes with 1.2 x 10^6 cells per 10 ml RPMI medium. After the overnight of incubation in 37^0 C in 5 % CO_2 contained humidified incubator overnight, drugs were treated at various time points. Cytosolic protein (Fraction 1-C) and membrane/organelle protein (Fraction 2-M) was extracted by using ProteoExtract subcellular proteome extraction kit (S-PEK) according to the manufacture's instructions. Cytosolic and membrane fraction were then analyzed by Western blot.

2.2.10. Protein quantification

As following the Bio-Rad protein assay, the standard curve was set up with BSA dilution in water as final concentration of 0, 1.25, 2.5, 5, 7.5 and 10 µg/ µl. 2 µl of each sample and 798 µl sterilized water were added with 200 µl of Bio-Rad protein assay reagent, vortexed and mixed well. And then, take 100 µl of each sample mixture and move into 96 well plate. Light absorbance was read at 595 nm, compared to the standard curve to determine protein concentration of the samples.

2.2.11. Data analysis

Comparison of the effects of various treatments was performed by using one way analysis of variance and a two-tailed t test. Differences with a P value <0.05 were considered statistically significant. Experiment results shown are the means of multiple individual points (±standard error)

CHAPTER III: RESULTS

The present studies were designed to investigate the novel effect of UDCA on induction of apoptosis. Two different human gastric cancer cells (SNU-601 and SNU-638 cells were used for most studies.

3.1. UDCA induces mainly apoptosis in human gastric cancer cells

To investigate whether UDCA could be used as a chemotherapeutic agent in gastric cancer, as the first step, two different gastric cancer cells (SNU-601 and SNU-638 cells) were treated with UDCA as a function of concentration (250, 500 or 1000 μM) and 1000 μM UDCA as a function of time (12, 24, 36 or 48 h). Its effect was analyzed by using the MTT assay. The MTT assay demonstrated that UDCA significantly induced cytotoxic effect as a function of concentration and time with a similar pattern in both cancer cell lines (Fig. 1A, B). To determine the long-term effect of UDCA to inhibit the colony-forming ability of tumor cells, both cancer cell lines were treated with UDCA as a function of concentration (250, 500 or 1000 μM) for 12 h, incubated at 37^0 C for 15 days and analyzed by colony formation assay. As expected, UDCA significantly inhibited the colony-forming ability of these tumor cells, especially 1000 μM UDCA completely inhibited the colony-forming ability of these tumor cells in both human gastric cancer cells (Fig. 1C, D).

To examine which type of cell death is induced by UDCA, we used HO/PI double staining to identify UDCA-induced cell damage in terms of cell death. HO stains the nuclei of all cells with blue fluorescence, while PI only penetrates and fluoresces in cells with

damages membranes. Thus, the HO/PI double staining can show intact blue (viable), condensed/fragmented blue nuclei (early apoptotic), condensed/fragmented pink nuclei (late apoptotic, secondary necrotic), and intact pink nuclei (necrotic cells). The result of HO/PI double staining showed that UDCA mainly induced apoptotic cell death but scarcely necrotic cell death as a function of concentration (250, 500 or 1000 μM) in both cancer cell lines (Fig. 2A, B). Next, UDCA did not have the effect to induce necrotic cell death that was also confirmed by evidence of release of LDH into medium. LDH release at early stage of cell death is indicated by the cell membrane rupture, a typical feature of necrotic cell death. However, LDH could be released from secondary necrosis in cell culture. The result of LDH assay showed that UDCA did not induce release of LDH into medium in both cancer cell lines (Fig. 2C). It has been demonstrated that caspase activation and cleavage of PARP (poly (ADP-ribose) polymerase) are biochemical event during occurring of apoptosis. Moreover, the result of Western blot showed that UDCA induced an activation of caspase-3, caspase-6 and PARP cleavage as a function of in both cancer cell lines (Fig. 2D). In addition, to investigate whether UDCA induced autophagy that is other type of cell death beside of apoptosis and necrosis. As expected, UDCA did not induce the translocation from LC-I to LC-II (the autophagosome-association form of LC3) as a function of concentration in both cancer cell lines that is molecular marker of autophagy (Fig. 2E). Collectively, these results suggest that UDCA induces mainly apoptosis but not necrosis or autophagy in human gastric cancer cells.

It is well established that DCA induces apoptosis in several cell lines, especially hepatocyte and colon cancer cells. Therefore, to investigate the effect of DCA on induction of apoptosis like UDCA in gastric cancer, both cancer cell lines were treated with DCA as

a function of concentration (250, 500 or 1000 μM). The MTT assay showed that DCA reduced strongly cell survival as a function of concentration and cytotoxic effect of DCA in a similar pattern in both cancer cell lines (Fig. 3A). Next, we used HO/PI double staining to identify DCA induced apoptotic cell death or necrotic cell death. In contract of UDCA, DCA induced mainly necrotic cell death but less apoptotic cell death as a function of concentration (Fig. 3B, C), and DCA induced necrotic cell death as a function of concentration in a similar pattern with increasing effect depending on the concentration in both cancer cell lines. DCA induced mainly necrotic cell death that was also confirmed by evidence of release of LDH into medium. Similarly, DCA increased release of LDH into medium as a function of concentration in both cancer cell lines (Fig. 3D). Taken together, these results suggest that UDCA induces mainly apoptosis whereas DCA induced mainly necrosis in human gastric cancer cells.

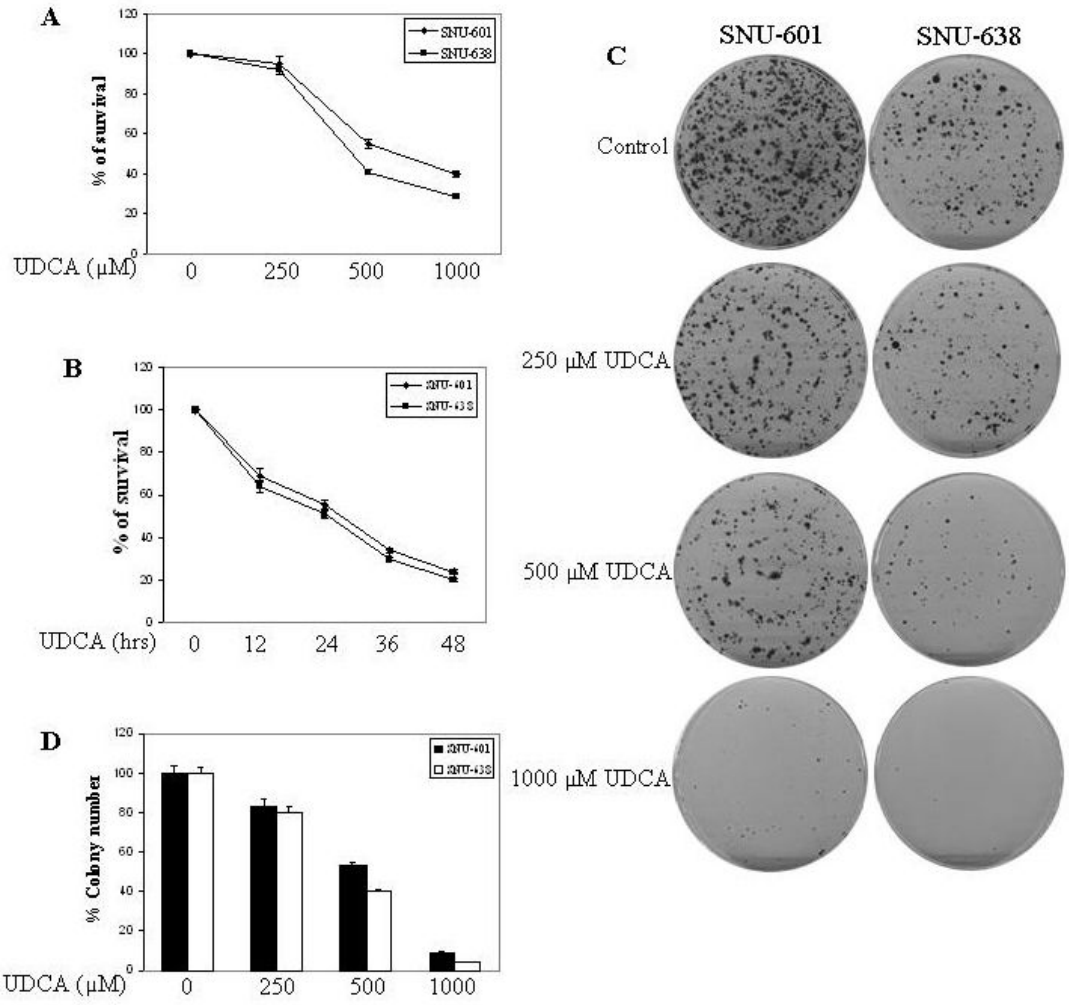

A
SNU-601
SNU-638
% of survival
UDCA (μM)
0
250
500
1000
B
% of survival
UDCA (hrs)
0
12
24
36
48
C
SNU-601
SNU-638
Control
250 μM UDCA
500 μM UDCA
1000 μM UDCA
D
% Colony number
UDCA (μM)
0
250
500
1000

Fig. 1: UDCA induced cell death in human gastric cancer cells. (**A**) Treated with UDCA as a function of concentration (250, 500 or 1000 μM) for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Cell viability was determined by the MTT assay. (**B**) SNU-601 and SNU-638 cells were treated with 1000 μM UDCA as a function of time (12, 24, 36 or 48 h). Cell viability was determined by the MTT assay. (**C, D**) SNU-601 and SNU-638 cells were treated with UDCA as a function of concentration (250, 500 or 1000 μM) for 12 h. The cells were then reseeded in 60-mm petri dishes and allowed to form colonies for 15 days after which they were stained with crystal violet. Colony numbers are calculated according to percentage of the untreated cells.

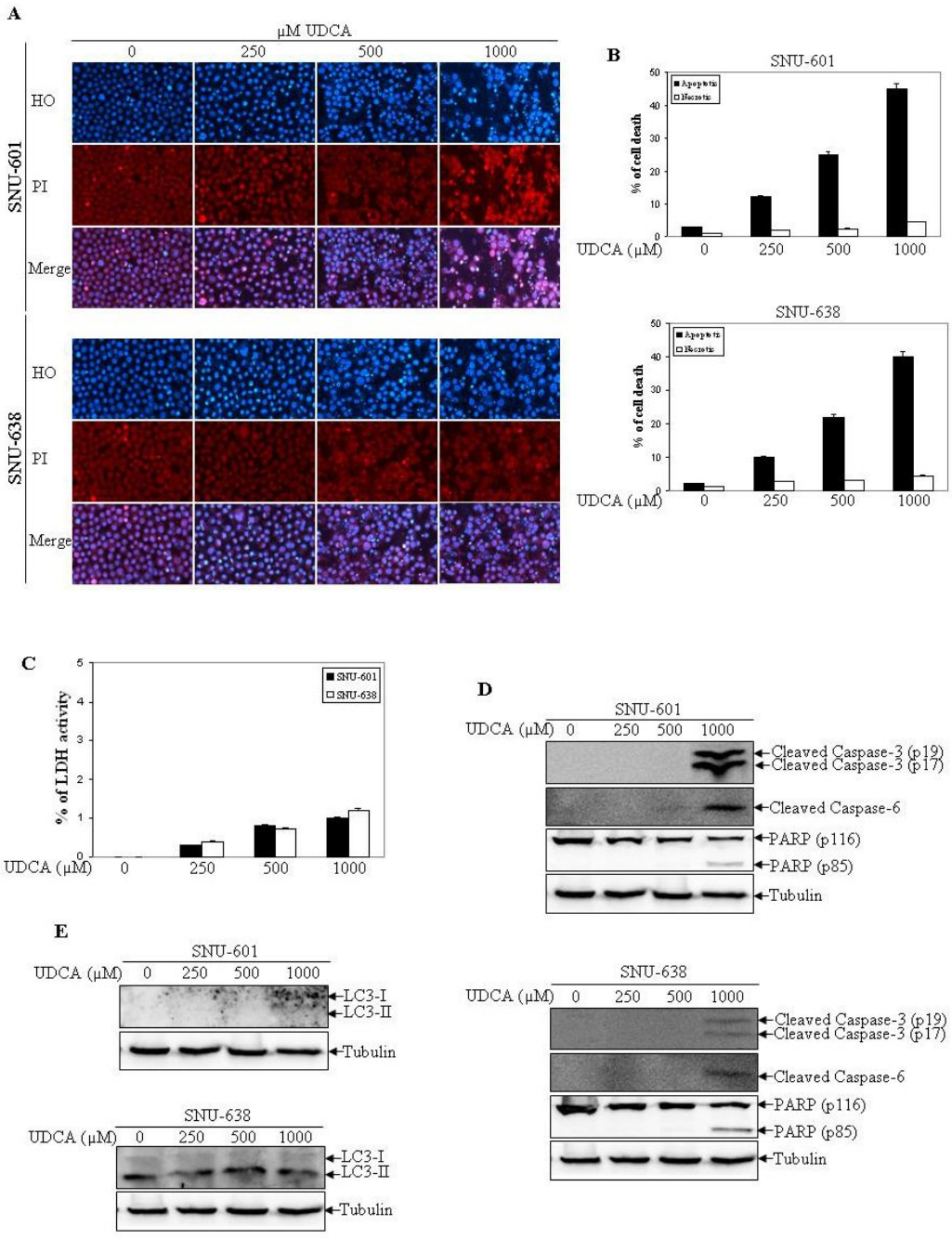

A
μM UDCA
0
250
500
1000
SNU-601
HO
PI
Merge
SNU-638
HO
PI
Merge
B
SNU-601
Apoptotic
Necrotic
% of cell death
UDCA (μM)
0
250
500
1000
SNU-638
Apoptotic
Necrotic
% of cell death
UDCA (μM)
0
250
500
1000
C
SNU-601
SNU-638
% of LDH activity
UDCA (μM)
0
250
500
1000
D
SNU-601
UDCA (μM)
0
250
500
1000
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PARP (p116)
PARP (p85)
Tubulin
SNU-638
UDCA (μM)
0
250
500
1000
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PARP (p116)
PARP (p85)
Tubulin
E
SNU-601
UDCA (μM)
0
250
500
1000
LC3-I
LC3-II
Tubulin
SNU-638
UDCA (μM)
0
250
500
1000
LC3-I
LC3-II
Tubulin

Fig. 2: UDCA induced mainly apoptosis in human gastric cancer cells. (**A, B**) Treated with UDCA as a function of concentration (250, 500 or 1000 μM) for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Cells were then stained with HO/PI double staining to identify apoptotic and necrotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells after staining with HO/PI, and cells with intact pink nuclei were counted as necrotic cells after staining with PI under fluorescence microcopy. (**C**) Treated with UDCA as a function of concentration (250, 500 or 1000 μM) for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. LDH release into the medium was determined by using LDH-cytotoxicity assay Kit. (**D, E**) Treated with UDCA as a function of concentration (250, 500 or 1000 μM) for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Protein levels of cleaved caspase-3, cleaved caspase-6, cleaved PARP, LC3 and tubulin were measured by Western blot analysis.

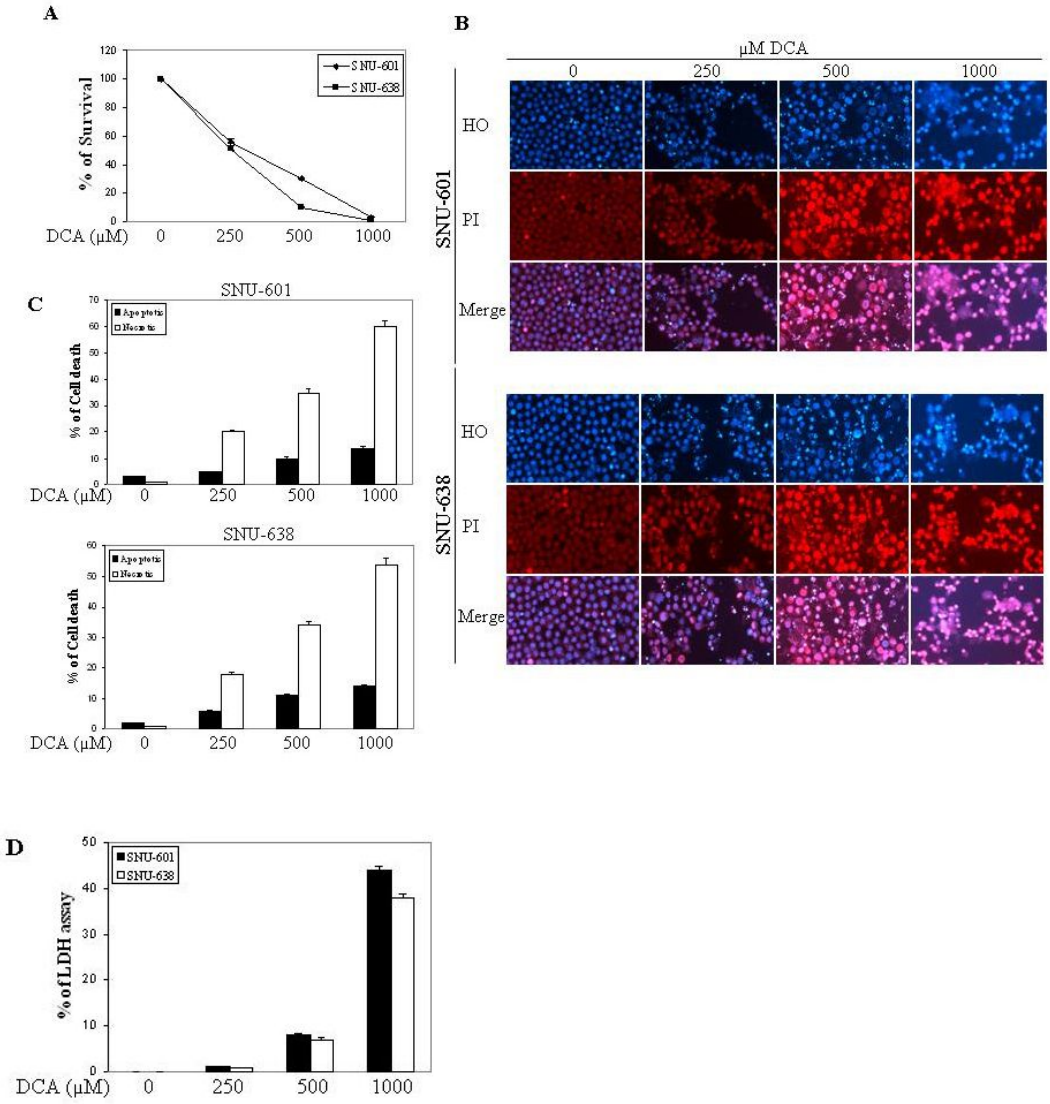

A
% of Survival
SNU-601
SNU-638
DCA (μM) 0 250 500 1000
B
μM DCA
0 250 500 1000
SNU-601
HO
PI
Merge
SNU-638
HO
PI
Merge
C
SNU-601
Apoptosis
Necrosis
% of Cell death
DCA (μM) 0 250 500 1000
SNU-638
Apoptosis
Necrosis
% of Cell death
DCA (μM) 0 250 500 1000
D
SNU-601
SNU-638
% of LDH assay
DCA (μM) 0 250 500 1000

Fig. 3: DCA induced necrosis in human gastric cancer cells. (**A**) Treated with DCA as a function of concentration (250, 500 or 1000 μM) for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Cell viability was determined by a MTT assay. (**B, C**) Treated with DCA as a function of concentration (250, 500 or 1000 μM) for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Cells were then stained with HO/PI double staining to identify apoptotic and necrotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells after staining with HO/PI, and cells with intact pink nuclei were counted as necrotic cells after staining with PI under fluorescence microcopy. (**D**) Treated with DCA as a function of concentration (250, 500 or 1000 μM) for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. LDH release into the medium was determined by using LDH-cytotoxicity assay kit.

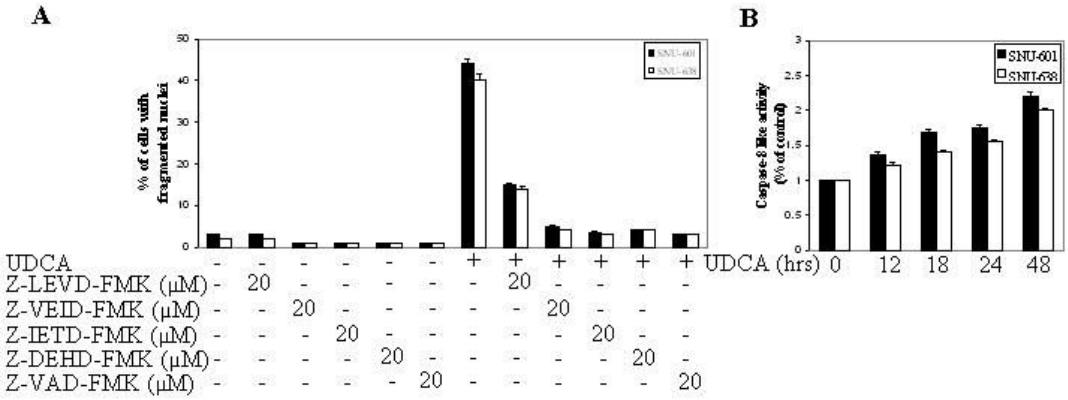
3.2. UDCA-induced apoptosis is dependent on caspase-3, -6, -9 and -8 but not caspase-4

To further understand the molecular mechanism of UDCA-induced apoptosis in human gastric cancer cells, we used specific caspase inhibitors including Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor), Z-VEID-FMK (caspase-6 inhibitor) and Z-VAD-FMK (pan-caspase inhibitor) to examine their involvement in UDCA-induced apoptosis. The results showed that UDCA-induced apoptosis was completely suppressed by Z-VAD-FMK, Z-IETD-FMK, Z-DEVD-FMK or Z-VEID-FMK, and also significantly suppressed by Z-LEHD-FMK in both cancer cell lines (Fig. 4A). However, the inhibitory effect of Z-LEHD-FMK was lower than those of the other specific caspase inhibitors (Fig. 4A). Next, both cancer cell lines were treated with 1000 μM UDCA as a function of time (12, 18, 24 or 48 h) and then measured for activation of caspase-8 through IETD-pNA substrate cleavage. As expected, the result showed that activation of caspase-8 was induced by UDCA as a function of time in both cancer cell lines (Fig. 4B). Furthermore, to investigate the important role of caspase-8 on regulation of UDCA-induced apoptosis, SNU-601 cells were pretreated with Z-IETD-FMK for 1 h and treat with 1000 μM UDCA for 24 h and SNU-638 cells for 36 h. Interestingly, Z-IETD-FMK completely suppressed UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage (Fig. 4C), indicating that caspase-8 activation was required for regulation of apoptosis by UDCA in human gastric cancer cells. In addition, caspase-8 is a crucial downstream mediator of death receptor signaling, so UDCA induced apoptosis through the death receptor pathway. It is well established that p53 is mutated in both cancer cell lines (SNU-601 and SNU-638 cells). Therefore, UDCA-induced apoptosis

was independent of p53. However, the result of HO/PI double staining showed that Z-LEHD-FMK significantly suppressed UDCA-induced apoptosis in both cancer cell lines (Fig. 4A), indicating that beside of death receptor pathway, UDCA induced apoptosis through mitochondrial pathway. Collectively, the results demonstrate that UDCA-induced apoptosis is dependent on activation of caspases-3, -6, -9 and -8.

In order to demonstrate whether UDCA-induced apoptosis involves ER stress through activation of caspase-4. It is well established that caspase-4 is a potential homolog of murine caspase-12 that locates at the cytoplasmic side of ER, and is activated after ER stress. We used specific inhibitor of caspase-4 to examine its involvement in UDCA-induced apoptosis. The result of MTT assay showed that Ac-LEVD-CHO (specific caspase-4 inhibitor) did not have an effect to suppress UDCA-induced cell death in both cancer cell lines. The ratio of cell viability after treating with UDCA in the presence or absence of Ac-LEVD-CHO was similar (Fig. 5A). Next, we also investigated that UDCA-induced apoptosis was not suppressed by Ac-LEVD-CHO by analysis of HO/PI double staining (Fig. 5B). Furthermore, UDCA-induced apoptosis is independent of activation of casapse-4 that is confirmed by Western blot analysis of activation of caspase-3, caspase-6 and PARP cleavage. As expected, UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage was not suppressed by Ac-LEVD-CHO in both cancer cell lines (Fig. 5C), indicating that UDCA-induced apoptosis is independent of activation of caspase-4 that involves ER stress. Taken together, these results suggest that UDCA-induced apoptosis is dependent on activation of caspase-3, -6, -9, and -8 but not activation of caspase-4, suggesting UDCA induces apoptosis through directly death receptor and indirectly

mitochondrial pathway but not ER stress pathway. Moreover, caspase-8 is the upstream initiator caspase of both these apoptosis pathway in human gastric cancer cells.


A
% of cells with fragmented nuclei
SNU-601
SNU-638
UDCA - - - - - - + + + + + +
Z-LEVD-FMK (μM) - 20 - - - - - 20 - - - -
Z-VEID-FMK (μM) - - 20 - - - - - 20 - - -
Z-IETD-FMK (μM) - - - 20 - - - - - 20 - -
Z-DEHD-FMK (μM) - - - - 20 - - - - - 20 -
Z-VAD-FMK (μM) - - - - - 20 - - - - - 20
B
Caspase-8 like activity (% of control)
SNU-601
SNU-638
UDCA (hrs) 0 12 18 24 48

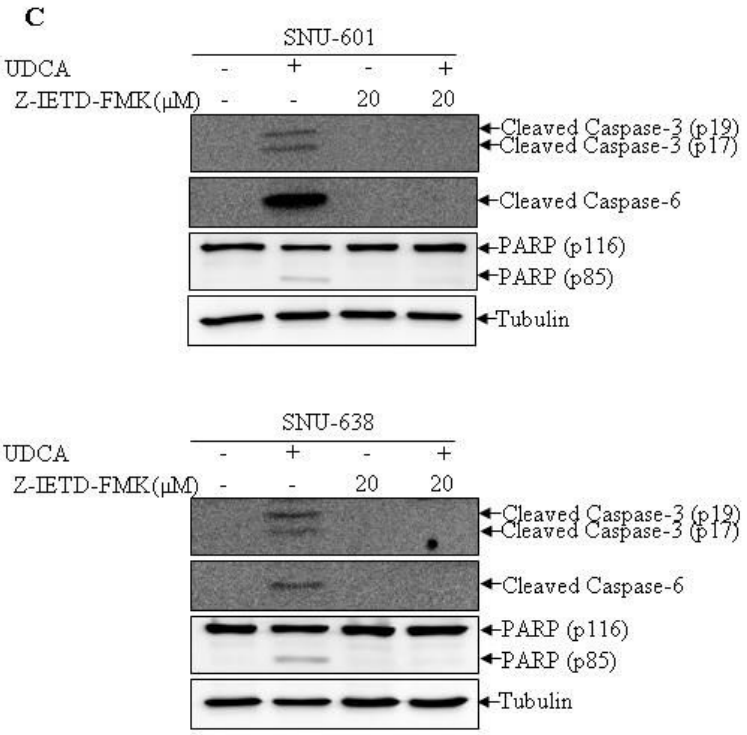

C
SNU-601
UDCA - + - +
Z-IETD-FMK(μM) - - 20 20
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PARP (p116)
PARP (p85)
Tubulin
SNU-638
UDCA - + - +
Z-IETD-FMK(μM) - - 20 20
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PARP (p116)
PARP (p85)
Tubulin

Fig. 4: UDCA-induced apoptosis was dependent on caspases-3, -6, -9 and -8. (**A**) Pretreated with 20 μM caspase inhibitors (Z-DEVD-FMK, Z-VEID-FMK, Z-IETD-FMK, Z-LEHD-FMK or Z-VAD-FMK) for 1 h, and treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Cells were then stained with HO/PI double staining to identify apoptotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells under fluorescence microcopy. (**B**) SNU-601 and SNU-638 cells were treated with 1000 μM UDCA as a function of time (12, 18, 24 or 48 h). Caspase-8 activation was then assessed by measuring IETD-pNA substrate cleavage by FLICE/Caspase-8 colorimetric assay kit. (**C**) Pretreated with 20 μM Z-IETD-FMK for 1 h, and treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Protein levels of cleaved caspase-3, cleaved caspase-6, cleaved PARP, and tubulin were measured by Western blot analysis.

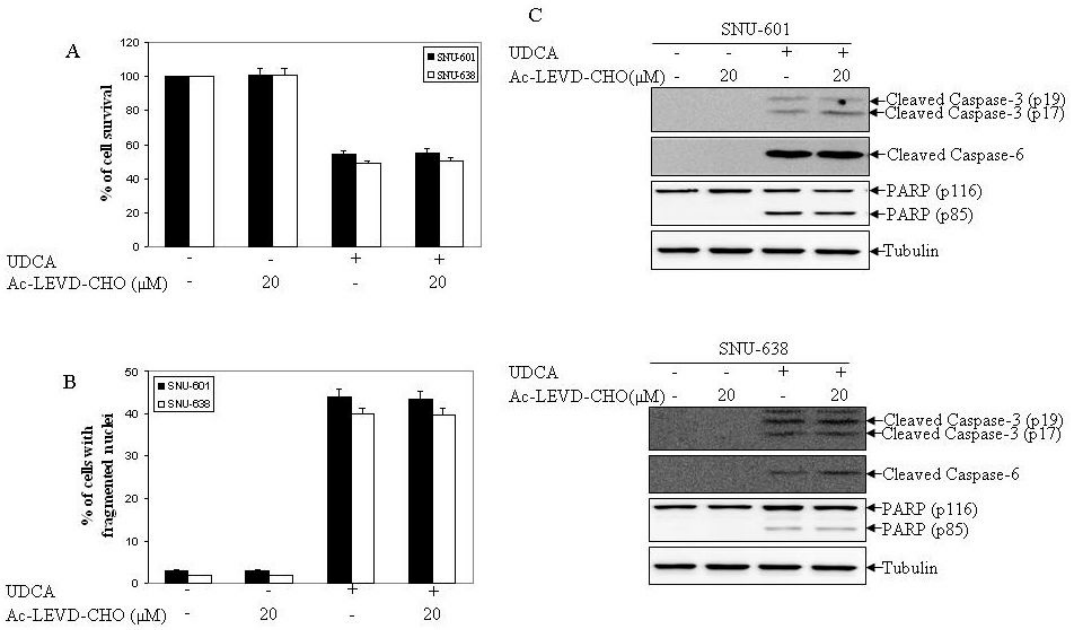


Fig. 5: UDCA-induced apoptosis was independent of caspase-4. (**A, B, C**) Pretreated with 20 μM Ac-LEVD-CHO for 1 h and treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. (**A**) Cell viability was then determined by the MTT assay. (**B**) Cells were then stained with HO/PI double staining to identify apoptotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells under fluorescence microcopy. (**C**) Protein levels of cleaved caspase-3, cleaved caspase-6, cleaved PARP and tubulin were measured by Western blot analysis.

3.3. UDCA induces DR5 expression, which is responsible for trigger apoptosis

It is well established that cell surface death receptor DR4, DR5 and FAS which play an important role in death of tumor cells. To determine whether UDCA-induced apoptosis is regulated by DR4, DR5 and/or FAS in human gastric cancer cells, we used siRNA specific to DR4, DR5 and FAS to knock-down the expression of these receptors. To do this, both cancer cell lines were transfected with siRNA DR4, siRNA DR5, siRNA FAS or siRNA Control respectively by using AMAXA method, incubated at 37^0 C in 5 % CO_2 contained humidified incubator for 42 h and then treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. We investigated that UDCA-induced apoptotic cell death was reduced by siRNA DR5 or siRNA FAS but not by siRNA DR4 in SNU-601 cells and siRNA DR4 or siRNA DR5 but not by siRNA FAS in SNU-638 cells by using HO/PI double staining to identify condensed or fragmented nuclei like apoptotic cell death (Fig. 6A). The expression of DR5 was completely suppressed in both cancer cell lines treated with specific siRNA to DR5 when compared with both cancer cell lines treated with siRNA Control (Fig. 6B). Next, by analysis of Western blot, we investigated that UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage was reduced by siRNA DR5 or siRNA FAS but not siRNA DR4 in SNU-601 cells and siRNA DR4 or siRNA DR5 but not by siRNA FAS in SNU-638 cells (Fig. 6B). To confirm UDCA-induced apoptosis is regulated by DR5 and FAS in SNU-601 cells, and DR4 and DR5 in SNU-638 cells and to determine the relationship between the death receptors and caspase-8, we used FLICE/caspase-8 colorimetric assay kit to measure caspase-8 activation. As expected, UDCA-induced activation of caspase-8 was reduced by siRNA DR5 or siRNA FAS but not by siRNA DR4 in SNU-601 cells and siRNA DR4 or SiRNA DR5 but not by

siRNA FAS in SNU-638 cells (Fig. 6C). Taken together, the results suggest that UDCA-induced apoptosis is regulated by DR5 and FAS in SNU-601 cells, and DR4 and DR5 in SNU-638 cells. More importantly, DR5 was a common regulator to trigger UDCA-induced apoptosis in both human gastric cancer cells.

In order to determine the significant role of DR5 in regulating of UDCA-induced apoptosis, both cancer cell lines were treated with UDCA as a function of concentration (250, 500, 750 or 1000 μM) and expression of DR5 was analyzed by Western blot. Expression of DR5 was dramatically induced by UDCA as a function of concentration, with optimum induction occurring at around 750 to 1000 μM (Fig. 7A). On the other hand, we also investigated that UDCA dramatically induced expression of DR5 as a function of time in both cancer cell lines, with optimum induction occurring at around 18 to 24 h in SNU-601 cells and 24 to 48 h in SNU-638 cells (Fig. 7B). Next, to determine whether UDCA induced expression of DR5 at cell membrane, both cancer cell lines were treated with 1000 μM UDCA as a function of time (1, 2, 4, 8 or 12 h) and analyzed by Western blot after extraction of protein fraction including cytosolic protein and membrane protein by using Subcellular Proteome Extraction Kit. As expected, we investigated that UDCA induced dramatically expression of DR5 as a function of time at membrane fraction but not cytosolic fraction in both cancer cell lines (Fig. 7C). Taken together, these results suggest that UDCA induces expression of DR5, which is responsible for trigger apoptosis in human gastric cancer cells.

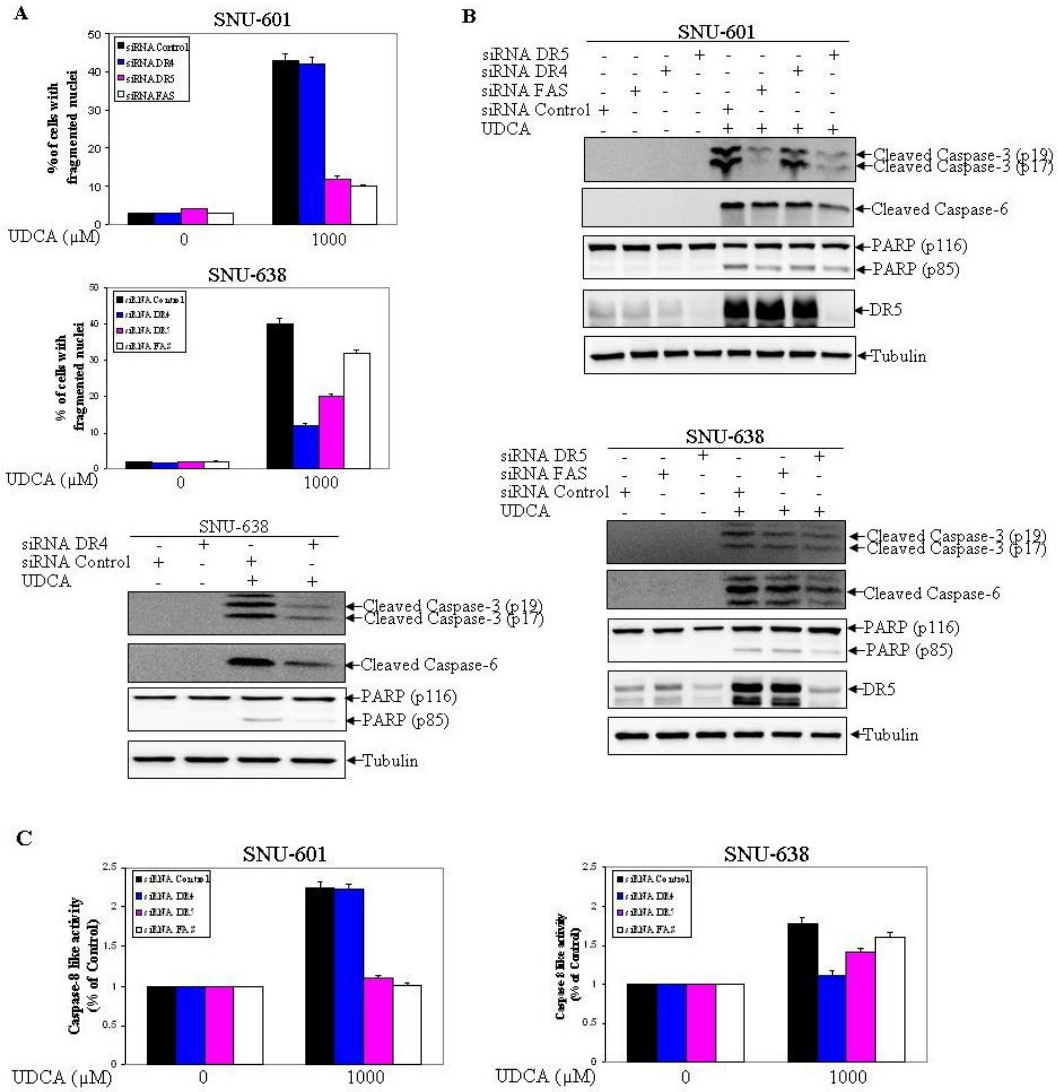

A
SNU-601
siRNA Control
siRNA DR4
siRNA DR5
siRNA FAS
% of cells with fragmented nuclei
UDCA (μM)
0
1000
SNU-638
B
SNU-601
siRNA DR5
siRNA DR4
siRNA FAS
siRNA Control
UDCA
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PARP (p116)
PARP (p85)
DR5
Tubulin
SNU-638
C
SNU-601
SNU-638
Caspase-8 like activity (% of Control)

Fig. 6: UDCA-induced apoptosis was regulated by DR5 and FAS in SNU-601 cells, and DR4 and DR5 in SNU-638 cells. (**A, B, C**) SNU-601 and SNU-638 cells were transiently transfected with siRNA FAS, siRNA DR4, siRNA DR5 or siRNA Control by using AMAXA method. siRNA-transfected cells (siRNA FAS, siRNA DR4, siRNA DR5 or siRNA Control) were treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h. (**A**) Cells were stained with HO/PI double staining to identify apoptotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells under fluorescence microcopy. (**B**) Protein levels of cleaved caspase-3, cleaved caspase-6, cleaved PARP, DR5 and tubulin were measured by Western blot analysis. (**C**) Caspase-8 activation was assessed by measuring IETD-pNA substrate cleavage by FLICE/Caspase-8 colorimetric assay kit.

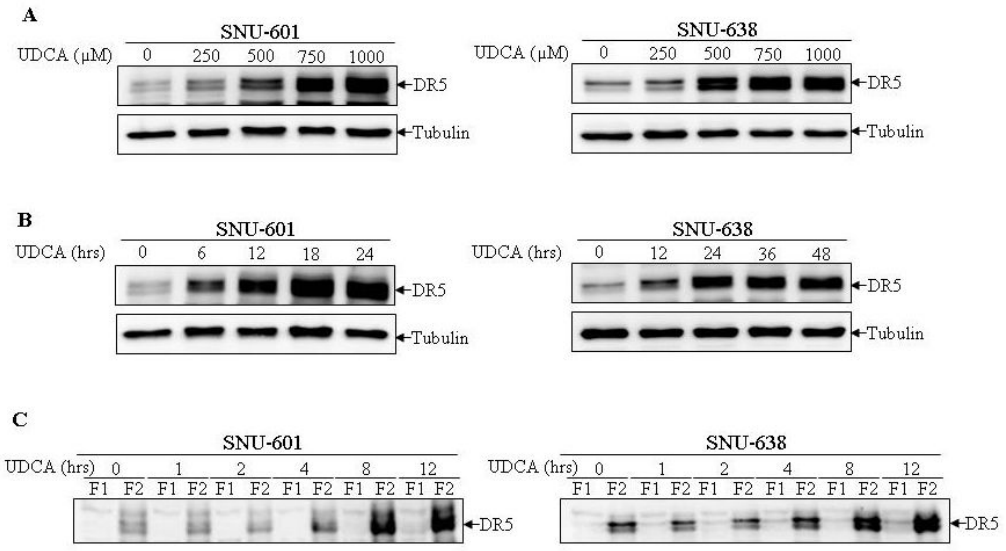


Fig. 7: UDCA induced expression of DR5 in human gastric cancer cells. (**A**) Treated with UDCA as a function of concentration (250, 500, 750 or 1000 μM) for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Protein levels of DR5 and tubulin were measured by Western blot analysis. (**B**) Treated with 1000 μM UDCA as a function of time (6, 12, 18 or 24 h) in SNU-601 cells and (12, 24, 36 or 48 h) in SNU-638 cells. Protein levels of DR5 and tubulin were measured by Western blot analysis. (**C**) SNU-601 and SNU-638 cells were treated with 1000 μM UDCA as a function of time (0, 1, 2, 4, 8 or 12 h). Cytosolic protein (fraction 1-C) and organelle/membrane protein (fraction 2-M) were then extracted by using ProteoExtract Subcellular Proteome Extraction kit. Protein level of DR5 was measured by Western blot analysis.

3.4. UDCA-induced apoptosis is mediated through FADD and RIP1

It is well established that TRAIL binds to death receptor DR4 and DR5, and FAS ligand binds to FAS, which in turn recruit intracellular FADD. Through its death effector domain, FADD recruits caspase-8 to the receptors for the assembly of a death-inducing signaling complex. To determine whether UDCA-induced apoptosis is mediated through FADD and RIP1 in human gastric cancer cells, we used siRNA specific to FADD and RIP1 to knock-down the expression of these adaptors. Both cancer cell lines were transfected with siRNA FADD, siRNA RIP1 or siRNA Control by using AMAXA method, incubated at 37^0 C in 5 % CO_2 contained humidified incubator for 42 h and then treated with 1000 μM UDCA. We investigated that UDCA-induced apoptotic cell death was reduced by siRNA RIP1 and slightly reduced by siRNA FADD in SNU-601 cells by using HO/PI double staining to identify condensed or fragmented nuclei like apoptotic cell death (Fig. 8A). In contrast, UDCA-induced apoptotic cell death was reduced by siRNA FADD and slightly reduced by siRNA RIP1 in SNU-638 cells by using HO/PI double staining to identify condensed or fragmented nuclei like apoptotic cell death (Fig. 8A). The expression of FADD and RIP1 were significantly reduced in both cancer cell lines treated with specific siRNA FADD and siRNA RIP1 respectively when compared with both cancer cell lines treated with siRNA Control (Fig. 8B). Next, by analysis of Western blot, we investigated that UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage were reduced by siRNA RIP1 and slightly reduced by siRNA FADD in SNU-601 cells. However, UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage were reduced by siRNA FADD and slightly reduced by siRNA RIP1 in SNU-638 cells (Fig. 8B). To confirm UDCA-induced apoptosis is mediated through FADD and RIP1 in human

gastric cancer cells, we used LICE/caspase-8 colorimetric assay kit to measure caspase-8 activation. As expected, we investigated that UDCA-induced activation of caspase-8 was reduced by siRNA RIP1 and slightly reduced by siRNA FADD in SNU-601 cells, but reduced by siRNA FADD and slightly reduced by siRNA RIP1 in SNU-638 cells (Fig. 8C), indicating that both FADD and RIP1 were the important adaptors to recruit caspase-8 to death receptor. Taken together, the results suggest that UDCA-induced apoptosis is mediated through FADD and RIP1 in human gastric cancer cells.

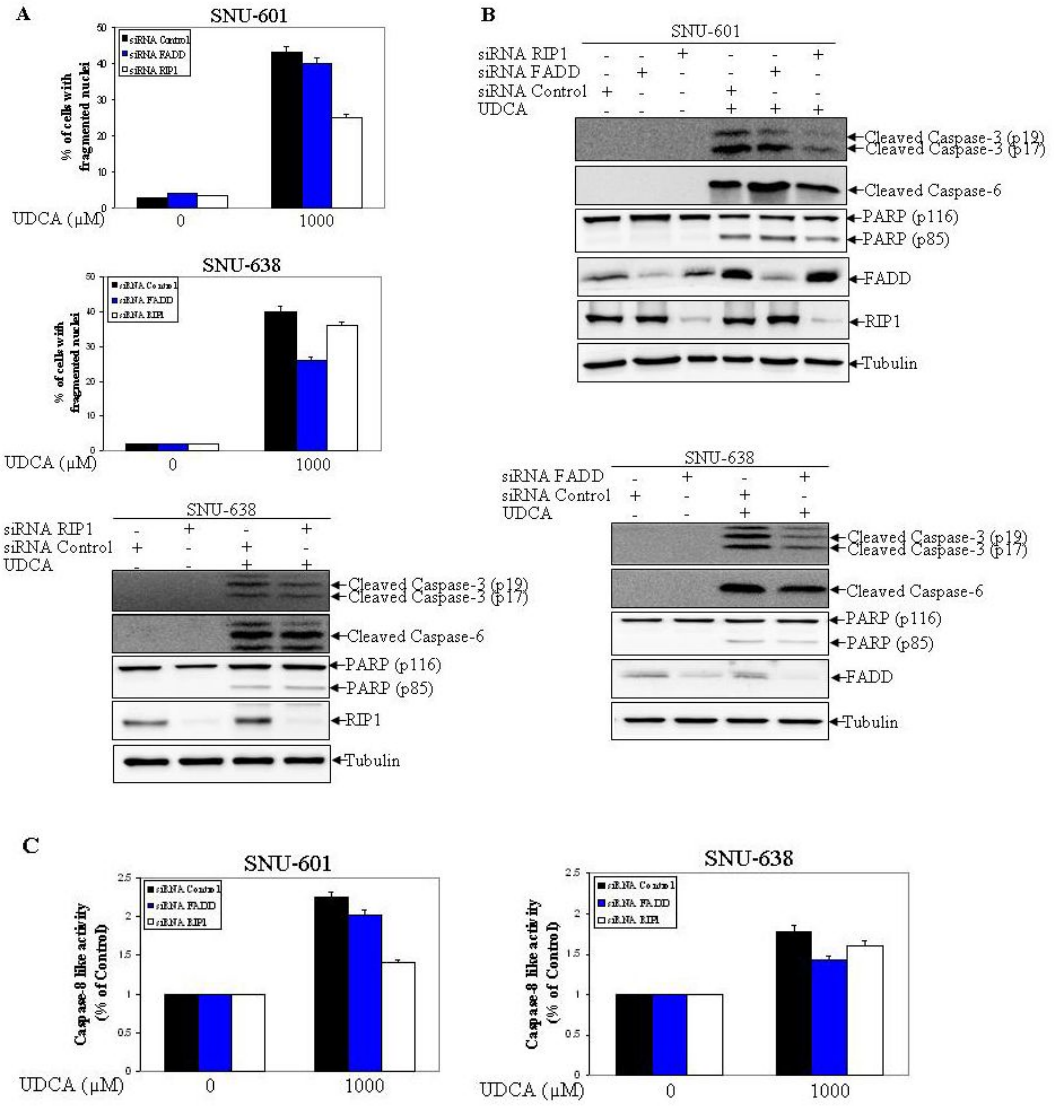

A
SNU-601
siRNA Control
siRNA FADD
siRNA RIP1
% of cells with fragmented nuclei
UDCA (μM)
0
1000
SNU-638
B
SNU-601
siRNA RIP1
siRNA FADD
siRNA Control
UDCA
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PARP (p116)
PARP (p85)
FADD
RIP1
Tubulin
SNU-638
C
SNU-601
SNU-638
Caspase-8 like activity (% of Control)

Fig. 8: UDCA-induced apoptosis was mediated through FADD and RIP1. (**A, B, C**) SNU-601 and SNU-638 cells were transiently transfected with siRNA FADD, siRNA RIP1 or siRNA Control by using AMAXA method. siRNA-transfected cells (siRNA FADD, siRNA RIP1 or siRNA Control) were treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. (**A**) Cells were stained with HO/PI double staining to identify apoptotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells under fluorescence microcopy. (**B**) Protein levels of cleaved caspase-3, cleaved caspase-6, cleaved PARP, FADD, RIP1 and tubulin were measured by Western blot analysis. (**C**) Caspase-8 activation was assessed by measuring IETD-pNA substrate cleavage by FLICE/Caspase-8 colorimetric assay kit.

3.5. UDCA-induced expression of DR5 to trigger apoptosis is regulated by PKC δ

PKC δ, a member of the novel PKC super-family, is actively involved in cell apoptosis in a stimulus and tissue specific manner. PKC δ translocates to the plasma membrane, mitochondria and nucleus and activates specific pathways that eventually converge to the activation of caspase-3 and subsequent apoptosis. It is well established that one of the possible manifestations of PKC activation is kinase redistribution between the cytosol and other intracellular compartments. For these reason, to determine whether UDCA activates intracellular PKC δ localization, which is responsible for expression of DR5 to trigger apoptosis, both cancer cell lines were treated with 1000 μM UDCA as a function of time (0, 1, 2, 4, 8 or 12 h). Cytosolic protein (fraction 1) and organelle/membrane protein (fraction 2) were then extracted by using ProteoExtract Subcellular Proteome Extraction Kit and translocation of PKC δ, PKC α, PKC θ and PKC ε from cytosol to membrane fraction were measured by Western blot. Interestingly, we investigated that UDCA activated significantly translocation of PKC δ, but not PKC α, and PKC ε from cytosol to membrane fraction as a function of time in both cancer cell lines (Fig. 9A). The result showed that UDCA activated markedly translocation of PKC δ from cytosol to membrane fraction after 2 h, sustained highly at 4 h and recovered to basal level in SNU-601 cells and after 4 h, sustained highly at 8 h and recovered to basal level in SNU-638 cells (Fig. 9A), indicating that UDCA-activated the translocation of PKC δ is earlier than UDCA-induced apoptosis in both cancer cell lines.

To investigate the possible association between UDCA-activated the translocation of PKC δ from cytosol to membrane and UDCA-induced apoptosis in human gastric

cancer cells, we used the specific inhibitor of PKC δ (rottlerin) to examine the involvement in UDCA-induced apoptosis. Both cancer cell lines were treated with 1000 μM UDCA in the presence or absence of rottlerin (PKC δ inhibitor) as a function of concentration (1, 2 or 5 μM). We investigated that UDCA-induced apoptotic cell death was markedly suppressed by rottlerin as a function of concentration in both cancer cell lines by using HO/PI double staining to identify condensed or fragmented nuclei like apoptotic cell death (Fig. 9B). Next, by analysis of Western blot, we also investigated that UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage was significantly suppressed by various concentrations of rottlerin, especially 5μM rottlerin (Fig. 9C). To confirm the significant role of PKC δ in regulating UDCA-induced apoptosis, we used siRNA specific for PKC δ to knock-down the expression of PKC δ. Both cancer cell lines were transfected with siRNA PKC δ or siRNA Control by using AMAXA methods, incubated at 37^0 C in 5 % CO_2 contained humidified incubator for 42 h and then treated with 1000 μM UDCA. The expression of PKC δ was significantly reduced in both cancer cell lines treated with specific siRNA PKC δ when compared with both cancer cell lines treated with siRNA Control to evaluate this transfection ability (Fig. 9D). By analysis of Western blot, we investigated that UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage was reduced by siRNA PKC δ in both cancer cell lines (Fig. 9D), indicating that PKC δ is an important regulator to regulate UDCA-induced apoptosis. Collectively, these results demonstrate that UDCA activates the translocation of PKC δ from cytosol to membrane, which is responsible for regulation of apoptosis in human gastric cancer cells.

We investigated that UDCA induced expression of DR5 which is responsible for trigger apoptosis and UDCA-induced apoptosis was significantly regulated by PKC δ

activation in human gastric cancer cells. To determine possible association between UDCA-activated the translocation of PKC δ from cytosol to membrane and UDCA-induced expression of DR5, both cancer cell lines were treated with 1000 μM UDCA in the presence or absence of rottlerin (PKC δ inhibitor) as a function of concentration (1-, 2 or 5 μM). By analysis of Western blot, we investigated that UDCA-induced expression of DR5 was reduced by 5 μM rottlerin in both cancer cell lines (Fig. 10A). To confirm the significant role of PKC δ to regulate UDCA-induced expression of DR5, we used siRNA specific to PKC δ to knock-down the activation of PKC δ. Both cancer cell lines were transfected with siRNA PKC δ or siRNA Control by using AMAXA method, incubated at 37^0 C in 5 % CO_2 contained humidified incubator for 42 h and then treated with 1000 μM UDCA. Similarly, by analysis of Western blot, we investigated that UDCA-induced expression of DR5 was significantly reduced by siRNA PKC δ in both cancer cell lines (Fig. 10B), indicating that UDCA-induced expression of DR5 is regulated by PKC δ activation. Taken together, these results suggest that UDCA-induced expression of DR5 to trigger apoptosis is regulated by PKC δ activation. Therefore, PKC δ is an important regulator to regulate UDCA-induced expression of DR5 to trigger apoptosis in human gastric cancer cells.

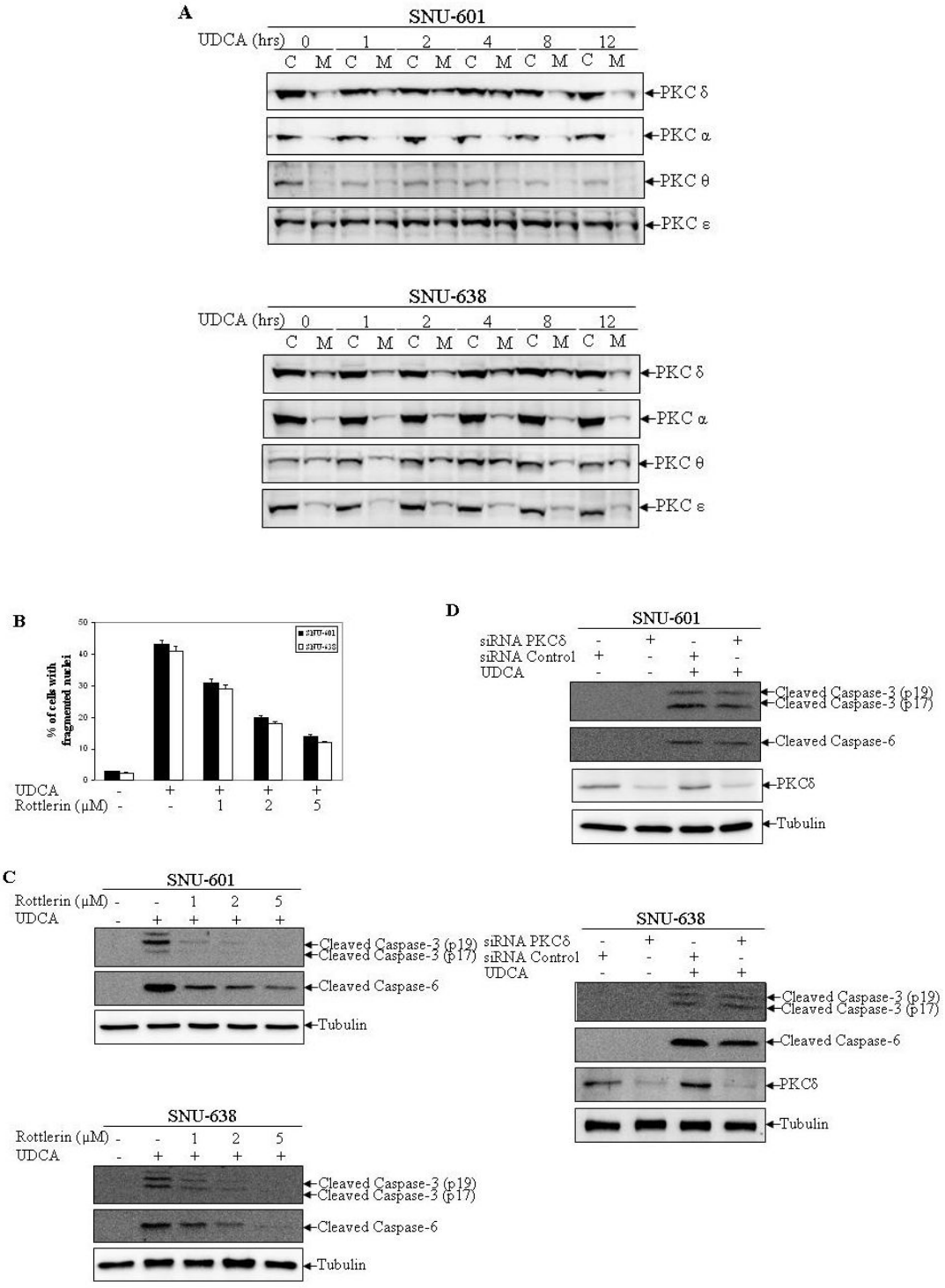

A
SNU-601
UDCA (hrs) 0 1 2 4 8 12
C M C M C M C M C M C M
PKC δ
PKC α
PKC θ
PKC ε
SNU-638
UDCA (hrs) 0 1 2 4 8 12
C M C M C M C M C M C M
PKC δ
PKC α
PKC θ
PKC ε
B
% of cells with fragmented nuclei
SNU-601
SNU-638
UDCA - + + + +
Rottlerin (μM) - - 1 2 5
D
SNU-601
siRNA PKCδ - + - +
siRNA Control + - + -
UDCA - - + +
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PKCδ
Tubulin
C
SNU-601
Rottlerin (μM) - - 1 2 5
UDCA - + + + +
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
Tubulin
SNU-638
Rottlerin (μM) - - 1 2 5
UDCA - + + + +
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
Tubulin
SNU-638
siRNA PKCδ - + - +
siRNA Control + - + -
UDCA - - + +
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PKCδ
Tubulin

Fig. 9: UDCA-induced apoptosis was regulated by PKC δ activation. (**A**) SNU-601 cells and SNU-638 cells were treated with 1000 μM UDCA as a function of time (0, 1, 2, 4, 8 or 12 h). Cytosolic protein (fraction 1-C) and organelle/membrane protein (fraction 2-M) were then extracted by using ProteoExtract Subcellular Proteome Extraction kit. Protein levels of PKC δ, PKC α, PKC θ, and PKC ε were measured by Western blot analysis. (**B, C**) Pretreat with Rottlerin as a function of concentration (1, 2 or 5 μM) for 1 h, and treat with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. (**B**) Cells were then stained with HO/PI double staining to identify apoptotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells under fluorescence microcopy. (**C**) Protein levels of cleaved caspase-3, cleaved caspase-6 and tubulin were measured by Western blot analysis. (**D**) SNU-601 and SNU-638 cells were transiently transfected with siRNA PKC δ or siRNA Control by using AMAXA method. siRNA-transfected cells (siRNA PKC δ or siRNA Control) were treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638. Protein levels of cleaved caspase-3, cleaved caspase-6, PKC δ and tubulin were measured by Western blot analysis.

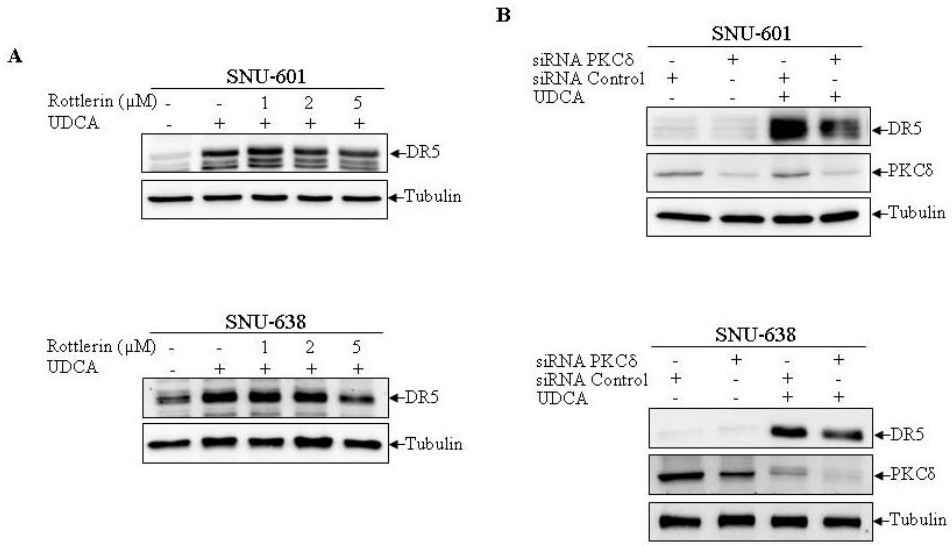


Fig. 10: UDCA-induced expression of DR5 was regulated by PKC δ activation. (**A**) Pretreat with Rottlerin as a function of concentration (1-, 2 or 5 μM) for 1 h, and treat with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Protein levels of DR5 and tubulin were measured by Western blot analysis. (**B**) SNU-601 and SNU-638 cells were transiently transfected with siRNA PKC δ or siRNA Control by using AMAXA method. siRNA-transfected cells (siRNA PKC δ or siRNA Control) were treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638. Protein levels of DR5, PKC δ and tubulin were measured by Western blot analysis.

3.6. UDCA-induced DR5 expression to trigger apoptosis is regulated by ROS production

It is well been demonstrated that ROS is the important or critical factor in the determination of cell death mode including apoptotic cell death and necrotic cell death. To determine the critical role of ROS involving in UDCA-induced apoptosis in human gastric cancer cells, we used assay for ROS to determine UDCA-induced ROS production by using DCFH-DA/HO. By analyzing of assay for ROS, we investigated that UDCA stimulated markedly ROS production as a function of time (3 or 5 h) in both cancer cell lines (Fig. 11A). The ROS production was dramatically stimulated at 3 h and up to maximal at 5 h. However, UDCA-stimulated ROS production in SNU-638 cells is lower than that in SNU-601 cells (Fig. 11A). To demonstrate the possible association between UDCA-stimulated ROS production and UDCA-induced apoptosis, both cancer cell lines were treated with UDCA in the presence or absence of various ROS scavengers including 10 mM NAC, 100 μM BHA, 10 mM Tiron, 2 μM DPI, or 1000 U catalase. By analyzing of HO/PI double staining to identify condensed or fragmented nuclei like apoptotic cell death, we investigated that two of five scavengers including NAC and BHA significantly suppressed UDCA-induced apoptotic cell death in both cancer cell lines (Fig. 11B). Moreover, by analyzing of HO/PI double staining, we also investigated that Tiron and DPI except catalase slightly reduced UDCA-induced apoptotic cell death in SNU-601 cells, and Tiron and catalase except DPI slightly reduced UDCA-induced apoptotic cell death in SNU-638 cells (Fig. 11B). To confirm the significant association between UDCA-stimulated ROS production and UDCA-induced apoptosis, both cancer cell lines were treated with UDCA in the presence or absence of 10 mM NAC, 100 μM BHA, 10 mM

Tiron, 2 μM DPI, or 1000 U catalase. Similarly, by analysis of Western blot, we investigated that NAC and BHA significantly suppressed UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage in both cancer cell lines. We also investigated that Tiron and DPI except catalase slightly reduced UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage in SNU-601 cells, but Tiron and catalase except DPI slightly reduced UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage in SNU-638 cells (Fig. 11C). The results suggest that UDCA-induced apoptosis is significantly regulated by ROS production in human gastric cancer cells.

We investigated that UDCA-induced expression of DR5, which is responsible for trigger apoptosis and UDCA-induced apoptosis is significantly regulated by ROS production in both cancer cell lines. It is well established that DR5 can be regulated via ROS production. To determine possible association between UDCA-stimulated ROS production and UDCA-induced expression of DR5, both cancer cell lines were treated with UDCA in the presence or absence of 10 mM NAC and 100 μM BHA (ROS scavengers). As expected, UDCA-induced expression of DR5 was completely blocked by NAC and slightly by BHA in both cancer cell lines (Fig. 12A, B), indicating that UDCA-induced expression of DR5 is significantly regulated by ROS production. Taken together, these results suggest that UDCA-induced expression of DR5 to trigger apoptosis is regulated by ROS production. Moreover, ROS is another important regulator to regulate UDCA-induced expression of DR5 to trigger apoptosis in human gastric cancer cells.

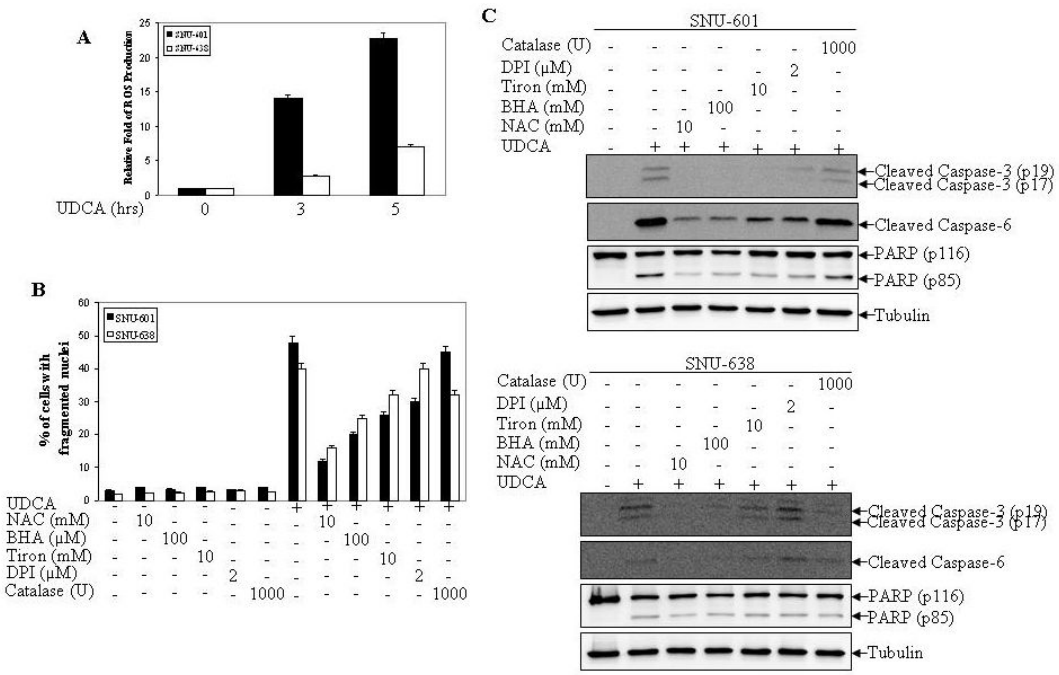


Fig. 11: UDCA-induced apoptosis was regulated by ROS production. (**A**) SNU-601 and SNU-638 cells were treated with 1000 μM UDCA as a function of time (3 or 5 h). ROS production was measured after incubation with 50 μM DCFH-DA and 100 μg HO for 1 h. The values of ROS production were obtained by determining the ratio of DCFH-DA/HO signals per well. (**B, C**) Pretreat with ROS scavengers including 10 mM NAC, 100 μM BHA, 10 mM Tiron, 2 μM DPI or 1000 U catalase for 1 h, and treat with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. (**B**) Cells were then stained with HO/PI double staining to identify apoptotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells under fluorescence microcopy. (**C**) Protein levels of cleaved caspase-3, cleaved caspase-6, cleaved PARP and tubulin were measured by Western blot analysis.

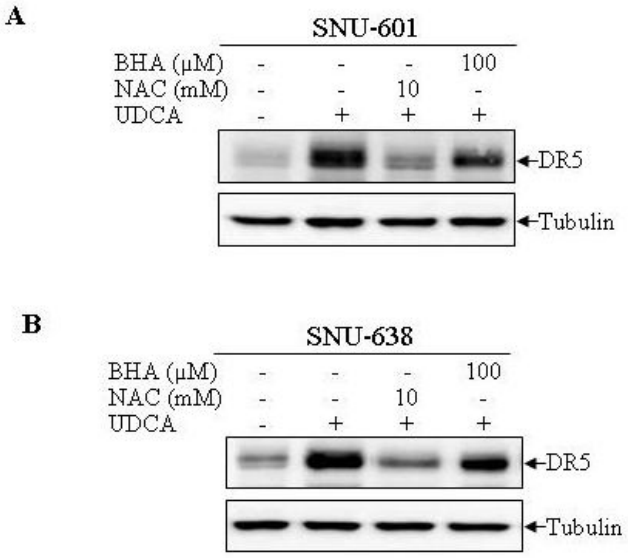


Fig. 12: UDCA-induced expression of DR5 was regulated by ROS production. (**A**) SNU-601 cells were treated with 1000 μM UDCA in the presence or absence of 10 mM NAC or 100 μM BHA for 24 h. Protein levels of DR5 and tubulin were measured by Western blot analysis. (**B**) SNU-638 cells were treated with 1000 μM UDCA in the presence or absence of 10 mM NAC or 100 μM BHA for 36 h. Protein levels of DR5 and tubulin were measured by Western blot analysis.

3.7. UDCA-activated translocation of PKC δ is mediated by ROS production

We investigated that UDCA-induced apoptosis is significantly regulated by ROS production and by PKC δ activation in human gastric cancer cells. Therefore, not only ROS but also PKC δ is significant regulator to regulate UDCA-induced apoptosis. It is well established that PKC δ is downstream event of ROS production. To examine the possible association between UDCA-activated the translocation of PKC δ from cytosol to membrane and UDCA-stimulated ROS production, both cancer cell lines were treated with 1000 μM UDCA in the presence or absence of 10 mM NAC. Cytosolic protein (fraction 1) and organelle/membrane protein (fraction 2) were then extracted by using ProteoExtract Subcellular Proteome Extraction Kit. By analysis of Western blot, we investigated that UDCA-activated translocation of PKC δ from cytosolic to membrane was blocked by NAC in both cancer cell lines (Fig. 13), indicating that UDCA-activated the translocation of PKC δ from cytosol to membrane is mediated by ROS production. These results suggest that PKC δ is downstream event of ROS in human gastric cancer cells.

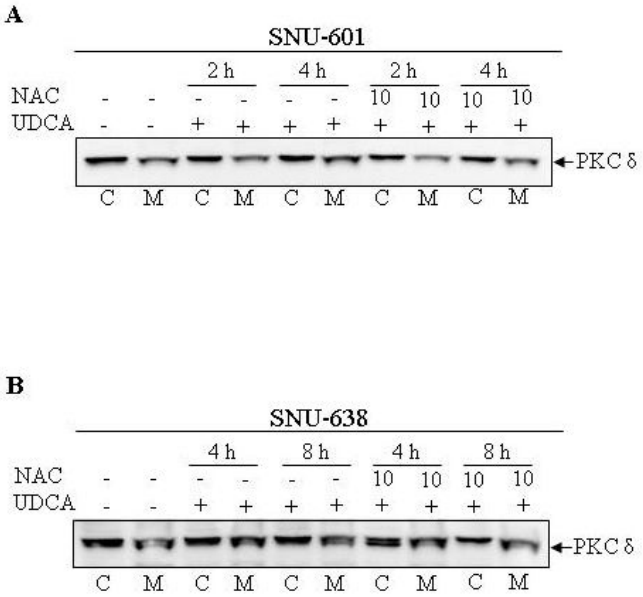


Fig. 13: UDCA-triggered PKC δ activation is mediated by ROS production. (**A**) Pretreated with 10 mM NAC for 1 h, and treated with 1000 μM UDCA for 2 or 4 h in SNU-601 cells. Cytosolic protein (fraction 1-C) and organelle/membrane protein (fraction 2-M) were then extracted by using ProteoExtract Subcellular Proteome Extraction kit. Protein level of PKC δ was measured by Western blot analysis. (**B**) Pretreated with 10 mM NAC for 1 h, and treated with 1000 μM UDCA for 4 or 8 h in SNU-638 cells. Cytosolic protein (fraction 1-C) and organelle/membrane protein (fraction 2-M) were then extracted by using ProteoExtract Subcellular Proteome Extraction kit. Protein level of PKC δ was measured by Western blot analysis.

3.8. UDCA-induced expression of DR5 to trigger apoptosis is partially regulated by MEK/ERK1 activation in SNU-601 cells but not in SNU-638 cells

It is well established that the ERK signaling pathway is activated in response to certain cellular stresses. To investigate the possible involvement of ERK pathway in UDCA-induced apoptosis in human gastric cancer cells, both cancer cell lines were treated with 1000 μM UDCA as a function of time (1, 3, 6, 9 or 12 h). By analysis of Western blot, we investigated that UDCA activated markedly phosphorylation status of ERK1/2 after 1 h, highly sustained until 6 h and recovered slowly to basal level following time treatment in SNU-601 cells (Fig. 14A). In contrast, we also investigated that UDCA decreased dramatically phosphorylation status of ERK1/2 after 1 h and continued decreasingly phosphorylation status of ERK1/2 following time treatment in SNU-638 cells (Fig. 14A). Moreover, we investigated that the phosphorylation status of MEK1/2 was similar to activation of ERK1/2 indicating that phosphorylation of ERK by MEK1/2 increased ERK activity in SNU-601 cells (Fig. 14A). In contrast, phosphorylation status of MEK1/2 reduced dramatically like phosphorylation status of ERK following time treatment in SNU-638 cells (Fig. 14A). The results suggest that UDCA markedly activates phosphorylation status of MEK/ERK in SNU-601 cells but not in SNU-638 cells.

In order to investigate the relation between UDCA-activated MEK/ERK pathway and UDCA-induced apoptosis in SNU-601 cells, cells were treated with 1000 μM UDCA in the presence or absence of 50 μM PD98059 (MEK1 inhibitor) or 20 μM UO126 (MEK1/2 inhibitor) for 24 h. By analysis of MTT assay, we investigated that UDCA-induced cell death was partially suppressed by PD98059 or UO126 in SNU-601 cells (Fig.

14B). Next, by analysis of Western blot, we investigated that UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage was partially suppressed by PD98059 or UO126 in SNU-601 cells (Fig. 14C). Furthermore, to confirm the relation between UDCA-activated MEK/ERK and UDCA-induced apoptosis, we used LICE/caspase-8 colorimetric assay kit to measure caspase-8 activation. As expected, we investigated that UDCA-induced caspase-8 activation was also partially suppressed by PD98059 or UO126 in SNU-601 cells (Fig. 14D).

In order to confirm involvement of MEK/ERK pathway to regulate UDCA-induced apoptosis in SNU-601 cells, we used siRNA specific for ERK1, and ERK2 to knock-down the phosphorylation and expression of ERK1 and ERK2, respectively. SNU-601 cells were transfected with siRNA ERK1, siRNA ERK2 or siRNA Control by using AMAXA method, incubated at 37^0 C in 5 % CO_2 contained humidified incubator for 42 h and then treated with 1000 μM UDCA for 24 h. By analysis of Western blot, the expression of ERK1 and ERK2 or phosphorylation of ERK1/2 were significantly reduced in SNU-601 cells treated with specific siRNA ERK1 or siRNA ERK2, respectively when compared with SNU-601 cells treated with siRNA Control (Fig. 15). Interestingly, we investigated that siRNA ERK1 but not siRNA ERK2 partially suppressed UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage SNU-601 cells (Fig. 15), indicating that ERK1 but not ERK2 have partial effect to regulate UDCA-induced apoptosis in SNU-601 cells.

To determine the partial role of MEK/ERK1 to link UDCA-induced expression of DR5, we used UO126 or PD98059 to examine its possible effect to regulate UDCA-

induced expression of DR5. SNU-601 were treated with 1000 μM UDCA in the presence or absence of 20 μM UO126 or 50 μM PD98059 for 24 h. we investigated that UDCA-induced expression of DR5 were blocked significantly by UO126 or partially by PD98059 in SNU-601 cells (Fig. 16), indicating that UDCA-induced expression of DR5 is regulated by MEK/ERK1 activation. Taken together, these results suggest that UDCA-induced expression of DR5 to trigger apoptosis is partially regulated by MEK/ERK1 activation in SNU-601 cells.

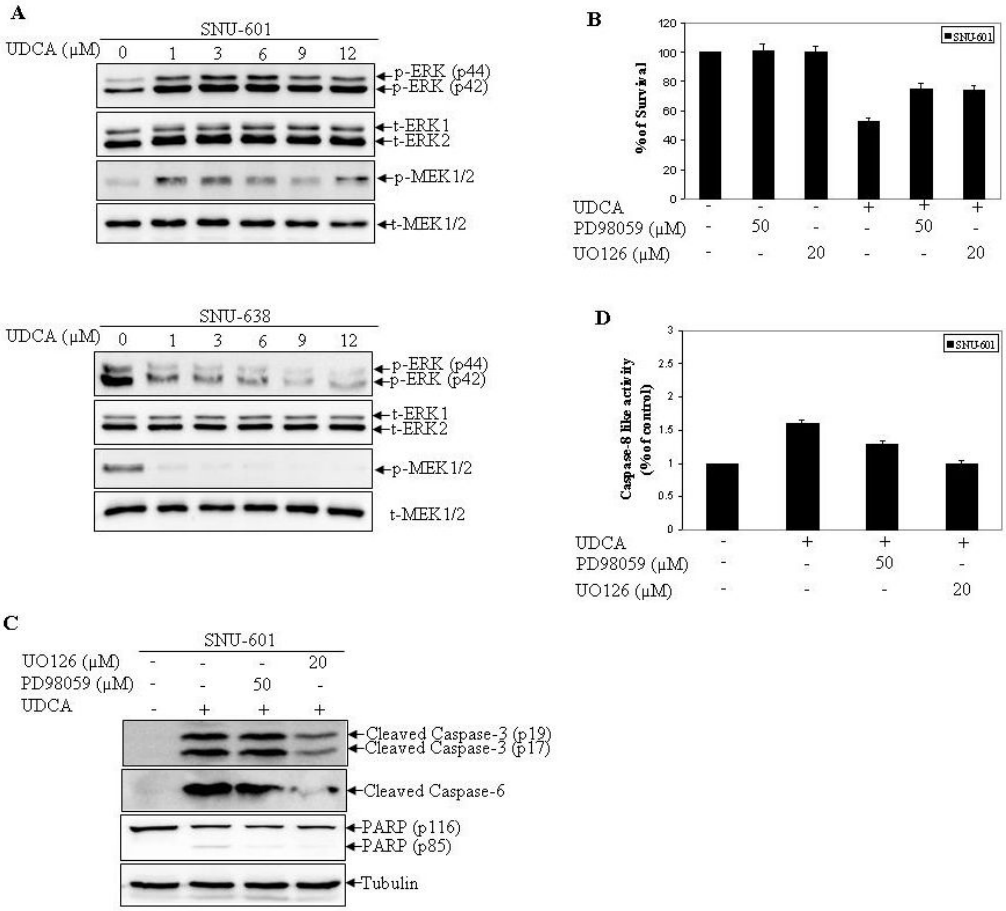

A
SNU-601
UDCA (μM) 0 1 3 6 9 12
p-ERK (p44)
p-ERK (p42)
t-ERK1
t-ERK2
p-MEK1/2
t-MEK1/2
SNU-638
UDCA (μM) 0 1 3 6 9 12
p-ERK (p44)
p-ERK (p42)
t-ERK1
t-ERK2
p-MEK1/2
t-MEK1/2
B
SNU-601
% of Survival
UDCA - - - + + +
PD98059 (μM) - 50 - - 50 -
UO126 (μM) - - 20 - - 20
C
SNU-601
UO126 (μM) - - - 20
PD98059 (μM) - - 50 -
UDCA - + + +
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PARP (p116)
PARP (p85)
Tubulin
D
SNU-601
Caspase-8 like activity (% of control)
UDCA - + + +
PD98059 (μM) - - 50 -
UO126 (μM) - - - 20

Fig. 14: Involvement of MEK/ERK pathway in UDCA-induced apoptosis in SNU-601 cells but not in SNU-638 cells. (**A**) SNU-601 and SNU-638 cells were treated with 1000 μM UDCA as a function of time (1, 3, 6, 9 or 12 h). Protein levels of p-ERK1/2, t-ERK1/2, p-MEK1/2 and t-MEK1/2 were then measured by Western blot analysis. (**B, C, D**) SNU-601 cells were pretreated with 50 μM PD98059 or 20 μM UO126 for 1 h, and treated with 1000 μM UDCA for 24 h. (**B**) Cell viability was then determined by a MTT assay. (**C**) Protein levels of cleaved caspase-3, cleaved caspase-6, cleaved PARP and tubulin were measured by Western blot analysis. (**D**) Caspase-8 activation was assessed by measuring IETD-pNA substrate cleavage by FLICE/Caspase-8 colorimetric assay kit.

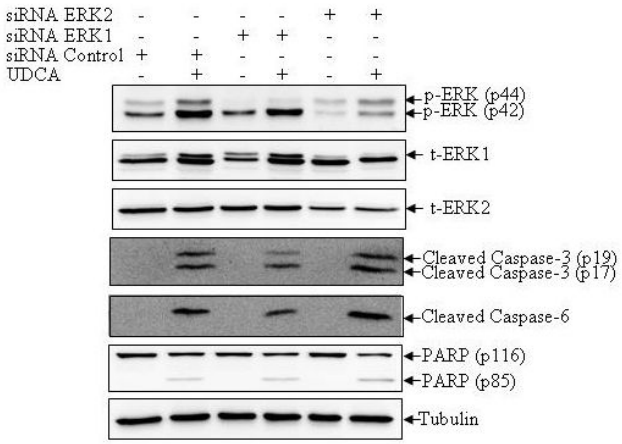


Fig. 15: Knock-down of ERK1 suppressed partially UDCA-induced apoptosis in SNU-601 cells. SNU-601 cells were transiently transfected with siRNA ERK1, siRNA ERK2 or siRNA Control by using AMAXA method. siRNA-transfected cells (siRNA ERK1, siRNA ERK2 or siRNA Control) were treated with 1000 μM UDCA for 24 h. Protein levels of cleaved caspase-3, cleaved caspase-6, cleaved PARP, p-ERK, t-ERK1, t-ERK2 and tubulin were then measured by Western blot analysis.

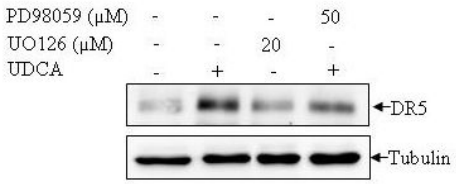


Fig. 16: UDCA-induced expression of DR5 was regulated by MEK/ERK1 activation in SNU-601 cells. SNU-601 cells were treated with 1000 μM UDCA in the presence or absence of 20 μM UO126 or 50 μM PD98059 for 24 h. Protein levels of DR5 and tubulin were then measured by Western blot analysis.

3.9. UDCA-induced expression of DR5 to trigger apoptosis is not related to c-Jun and NF-kB

It is well demonstrated that transcriptional factor including p53, c-Jun and NF-kB are known to play a crucial role in sensitivity and resistance to apoptosis death in many transformed cells, and also expression of DR5 can be regulated via stress-related mechanisms such as activation of p53, activation of JNK or ERK and activator protein as AP1 or NK-kB. We investigated UDCA-induced expression of DR5, which is responsible for trigger apoptosis, and both cancer cell lines are mutated p53, it has been shown that UDCA-induced apoptosis is independent of p53. To determine the possible association between c-Jun and NF-kB with UDCA-induced expression of DR5 to trigger apoptosis in human gastric cancer cells, we used siRNA specific to c-Jun and NF-kB to knock-down the expression of c-Jun and NF-kB, respectively. Both cancer cell lines were transfected with siRNA c-Jun, siRNA NF-kB or siRNA Control by using AMAXA method, incubated at 37^0 C in 5 % CO_2 contained humidified incubator for 42 h and then treated with 1000 μM UDCA. By using HO/PI double staining to identify condensed or fragmented nuclei like apoptotic cell death, we investigated that UDCA-induced apoptotic cell death was not blocked by siRNA c-Jun or siRNA NF-kB in both cancer cell lines, respectively (Fig. 17A). The expression of c-Jun or NF-kB were significantly reduced in both cancer cell lines treated with specific siRNA c-Jun or NF-kB when compared with both cancer cell lines treated with siRNA Control to determine these transfection ability, respectively. Furthermore, by analysis of Western blot, we investigated that UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage was not suppressed by siRNA c-Jun or siRNA NF-kB in both cancer cell lines (Fig. 17B), indicating that UDCA-induced apoptosis is not

related to c-Jun and NF-kB. On the other hand, we also investigated UDCA-induced expression of DR5 was not suppressed by siRNA c-Jun or NF-kB in both cancer cell lines (Fig. 17B). Taken together, these results suggest that UDCA-induced expression of DR5 to trigger apoptosis is not related to c-Jun and NF-kB in human gastric cancer cells.

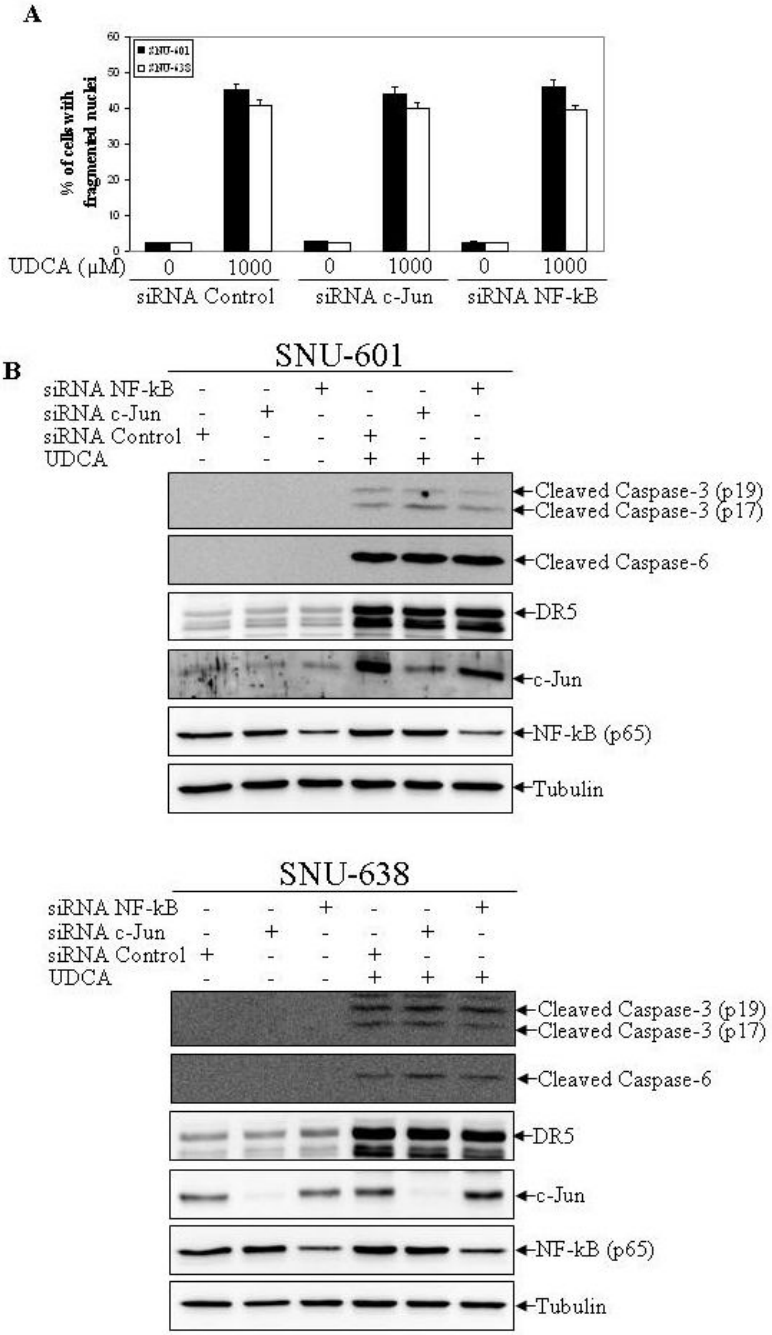
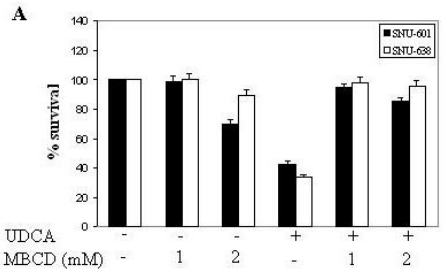

A
SNU-601
SNU-638
% of cells with fragmented nuclei
UDCA (μM)
0
1000
siRNA Control
siRNA c-Jun
siRNA NF-kB
B
SNU-601
siRNA NF-kB
siRNA c-Jun
siRNA Control
UDCA
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
DR5
c-Jun
NF-kB (p65)
Tubulin
SNU-638
siRNA NF-kB
siRNA c-Jun
siRNA Control
UDCA
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
DR5
c-Jun
NF-kB (p65)
Tubulin

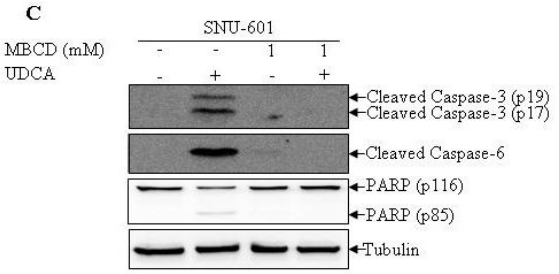
Fig. 17: UDCA-induced expression of DR5 and apoptosis were not related to c-Jun and NF-kB. (**A, B**) SNU-601 and SNU-638 cells were transiently transfected with siRNA NF-kB, siRNA c-Jun or siRNA Control by using AMAXA method. siRNA-transfected cells (siRNA NF-kB, siRNA c-Jun or siRNA Control) were treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638. (**A**) Cells were then stained with HO/PI double staining to identify apoptotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells under fluorescence microcopy. (**B**) Protein levels of cleaved caspase-3, cleaved caspase-6, DR5, c-Jun, NF-kB and tubulin were measured by Western blot analysis.

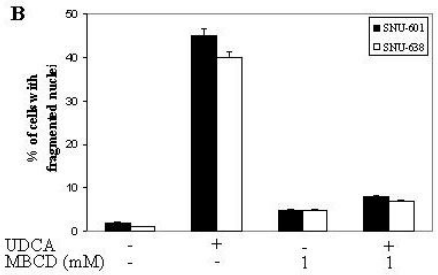
3.10. UDCA-induced expression of DR5 to trigger apoptosis is regulated by lipid raft

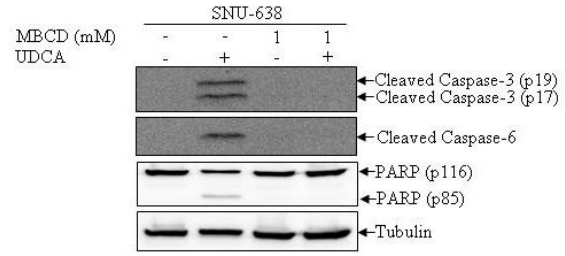
It is well established that cholesterol is an important constituent of cell membranes where it plays a crucial role in maintaining intergrity and fluidity. In addition, cholesterol-enriched microdomains, so called lipid rafts, are important signal transduction platforms which have been related to apoptosis and changes in plasma cholesterol levels have been associated with FAS-FADD, DR4 or DR5-FADD complex formation and caspase-8 activation. To investigate the possible association between lipid raft and UDCA-induce apoptosis in human gastric cancer cells, we used lipid raft depleting agent (MBCD) to examine its possible effect to regulate UDCA-induced apoptosis. Both cancer cell lines were treated with 1000 μM UDCA in the presence or absence of 1- or 2 mM MBCD. By analysis of MTT assay, we investigated that UDCA-induced cell death was completely suppressed by MBCD in both cancer cell lines (Fig. 18A). Next, by using HO/PI double staining to identify condensed or fragmented nuclei like apoptotic cell death, we investigated that UDCA-induced apoptotic cell death was completely suppressed by MBCD (Fig. 18B). Furthermore, by analysis of Western blot, we also investigated that UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage was completely suppressed by MBCD (Fig. 18C). To confirm the possibility of lipid raft to regulate UDCA-induced apoptosis, we used LICE/caspase-8 colorimetric assay kit to measure caspase-8 activation. As expected, we investigated that UDCA-induced activation of caspase-8 was completely suppressed by MBCD in both cancer cell lines (Fig. 18D), indicating that UDCA-induced apoptosis is crucially regulated by lipid raft in human gastric cancer cells.

In order to determine the significant role of lipid raft to link UDCA-induced expression of DR5, we used MBCD to examine its possible effect to regulate UDCA-induced expression of DR5. Both cancer cell lines were treated with 1000 μM UDCA in the presence or absence of 1 mM MBCD for 24 h. By analysis of Western blot, we investigated that UDCA-induced expression of DR5 was blocked completely by MBCD in both cell lines (Fig. 19), indicating that UDCA-induced expression of DR5 is crucially regulated by lipid raft. Taken together, these results suggest that UDCA-induced expression of DR5 to trigger apoptosis is crucially regulated by lipid raft in human gastric cancer cells.


A
% survival
SNU-601
SNU-638
UDCA
MBCD (mM)


C
SNU-601
MBCD (mM)
UDCA
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PARP (p116)
PARP (p85)
Tubulin


B
% of cells with fragmented nuclei
SNU-601
SNU-638
UDCA
MBCD (mM)


SNU-638
MBCD (mM)
UDCA
Cleaved Caspase-3 (p19)
Cleaved Caspase-3 (p17)
Cleaved Caspase-6
PARP (p116)
PARP (p85)
Tubulin

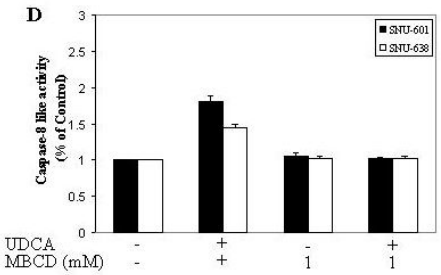

D
Caspase-8 like activity (% of Control)
SNU-601
SNU-638
UDCA
MBCD (mM)

Fig. 18: UDCA-induced apoptosis was regulated by lipid raft. (**A**) Pretreated with 1- or 2 mM MBCD for 1 h and treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Cells viability was then determined by a MTT assay. (**B, C, D**) Pretreated with 1 mM MBCD for 1 h and treated with 1000 μM for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. (**B**) Cells were then stained with HO/PI double staining to identify apoptotic cells. Cells with condensed or fragmented nuclei were counted as apoptotic cells under fluorescence microcopy. (**C**) Protein levels of cleaved caspase-3, cleaved caspase-6, cleaved PARP and tubulin were measured by Western blot analysis. (**D**) Caspase-8 activation was assessed by measuring IETD-pNA substrate cleavage by FLICE/Caspase-8 colorimetric assay kit.

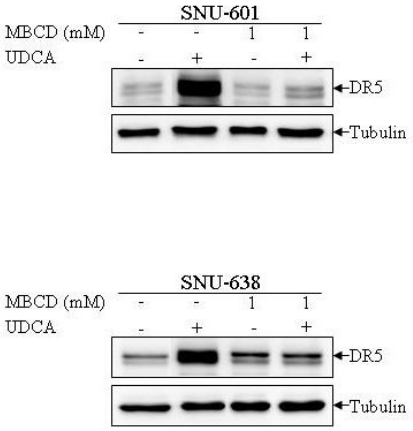


Fig. 19: UDCA-induced expression of DR5 was regulated by lipid raft. Pretreated with 1 mM MBCD for 1 h and treated with 1000 μM UDCA for 24 h in SNU-601 cells and for 36 h in SNU-638 cells. Protein levels of DR5 and tubulin were measured by Western blot analysis.

3.11. UDCA-activated ROS/PKC δ is regulated by lipid raft

We investigated that UDCA-induced apoptosis is regulated by PKC δ activation and lipid raft, and PKC δ is downstream event of ROS production in human gastric cancer cells. To determine the relation between lipid raft and PKC δ activation which are responsible for UDCA-induced expression to trigger apoptosis, both cancer cell lines were treated with 1000 μM UDCA in the presence or absence of 1 mM MBCD. Cytosolic protein (fraction 1) and organelle/membrane protein (fraction 2) were then extracted by using ProteoExtract Subcellular Proteome Extraction Kit. As expected, we investigated that UDCA-activated translocation of PKC δ from cytosol to membrane fraction was suppressed by MBCD in both cell lines (Fig. 20A), indicating that UDCA-activated the translocation of PKC δ from cytosol to membrane is regulated by lipid raft. So, PKC δ is a downstream event of lipid raft in human gastric cancer cells.

In order to determine the possible association between lipid raft and ROS, both cancer cell lines were treated with UDCA in the presence or absence of 1 or 2 mM MBCD for 3- or 5 h. As expected, by assay for ROS, we investigated that UDCA-stimulated ROS production was completely suppressed by MBCD (Fig. 20B), indicating that UDCA-stimulated ROS production was significantly regulated by lipid raft. So, ROS is downstream event of lipid raft in human gastric cancer cells. Thus, we clearly demonstrate that PKC δ is a downstream event of ROS. Collectively, these results suggest that UDCA-activated ROS/PKC δ is regulated by lipid raft. So, lipid raft is crucial regulator to regulate UDCA-stimulated ROS production to activate the translocation of PKC δ from cytosol to membrane leading to expression of DR5 to trigger apoptosis in human gastric cancer cells.

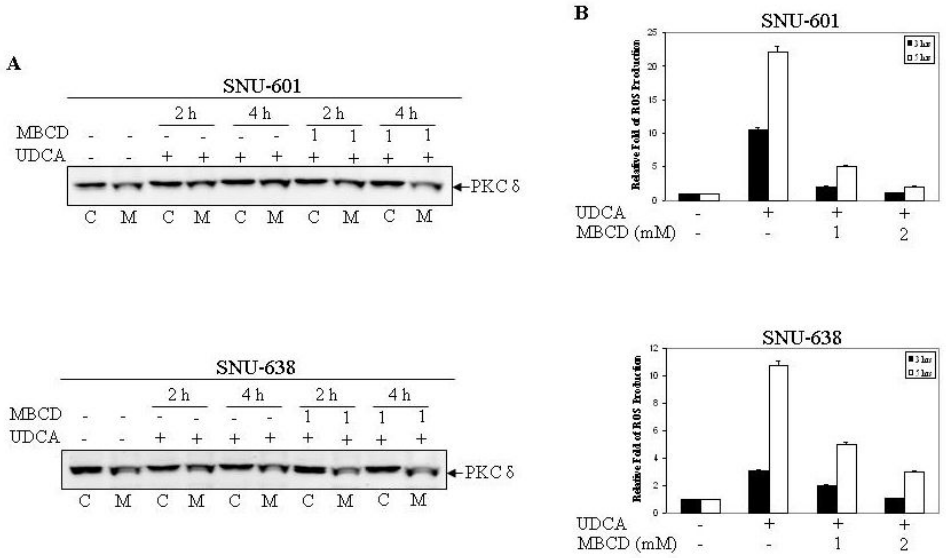


Fig. 20: UDCA-activated ROS/PKC δ was regulated by lipid raft. (**A**) Pretreated with 1 mM MBCD for 1 h and treated with 1000 μM UDCA for 2 or 4 h in SNU-601 cells and for 4 or 8 h in SNU-638 cells. Cytosolic protein (fraction 1-C) and organelle/membrane protein (fraction 2-M) were then extracted by using ProteoExtract Subcellular Proteome Extraction Kit. Protein levels of PKC δ were measured by Western blot analysis. (**B**) Pretreated with 1- or 2 mM MBCD for 1 h and treated with 1000 μM UDCA as a function of time (3 or 5 h) in both cancer cell line. ROS production was then measured after incubation for 1 h with 50 μM DCFH-DA and 100 μg HO. The values of ROS production were obtained by determining the ratio of DCFH-DA/HO signals per well.

3.12. UDCA-induced MEK/ERK activation is regulated by lipid raft in SNU-601 cells

Our results showed that UDCA-induced expression of DR5 to trigger apoptosis is partially regulated by MEK/ERK1 activation in SNU-601 cells and the crucial role of lipid raft to regulate apoptosis. To determine the possible involvement of lipid raft and MEK/ERK activation, SNU-601 cells were treated with UDCA in the presence or absence of 1 mM MBCD as a function of time. By analysis of Western blot, we investigated that UDCA-induced phosphorylation status of ERK was significantly suppressed by MBCD as a function of time (Fig. 21A), indicating that UDCA-induced MEK/ERK activation was regulated by lipid raft in SNU-601 cells.

It is well established that an important downstream mediator of ROS-induced signaling are the MAPK such as JNK, p38 and ERK activation. Therefore, to examine whether UDCA-stimulated ROS production is the upstream signal responsible for activation of MEK/ERK1, SNU-601 cells were treated with 1000 μM UDCA in the presence or absence of 10 mM NAC or 100 μM BHA for 1 or 3 h. Unexpectedly, we investigated that UDCA-activated phosphorylation status of ERK was not prevented by NAC or BHA as a function of time in SNU-601 cells (Fig. 21B), indicating that UDCA-activated MEK/ERK activation is independent of ROS. Taken together, the results suggest that UDCA-induced MEK/ERK activation is regulated by lipid raft. So, lipid raft is crucial regulator to regulate MEK/ERK1 activation leading to expression of DR5 to trigger apoptosis in SNU-601 cells.

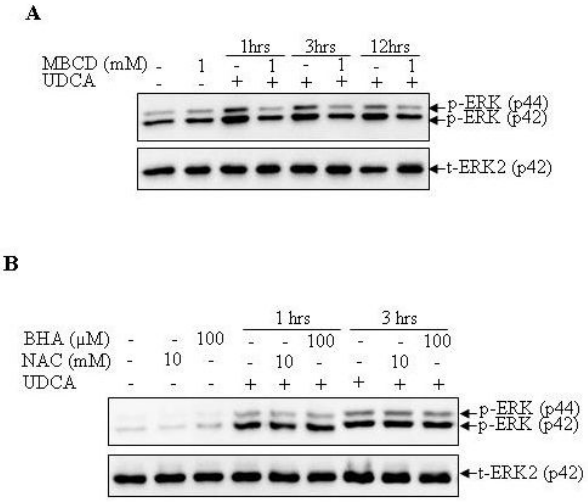


Fig. 21: UDCA-induced MEK/ERK1 activation was regulated by lipid raft in SNU-601 cells. (**A**) SNU-601 cells were treated with 1000 μM UDCA in the presence or absence of 1 mM MBCD as a function of time (1, 3 or 12 h). Protein levels of p-ERK and t-ERK2 were then measured by Western blot analysis. (**B**) SNU-601 cells were treated with 1000 μM UDCA in the presence or absence of 10 mM NAC or 100 μM BHA for 1 or 3 h. Protein levels p-ERK and t-ERK2 were then measured by Western blot analysis.

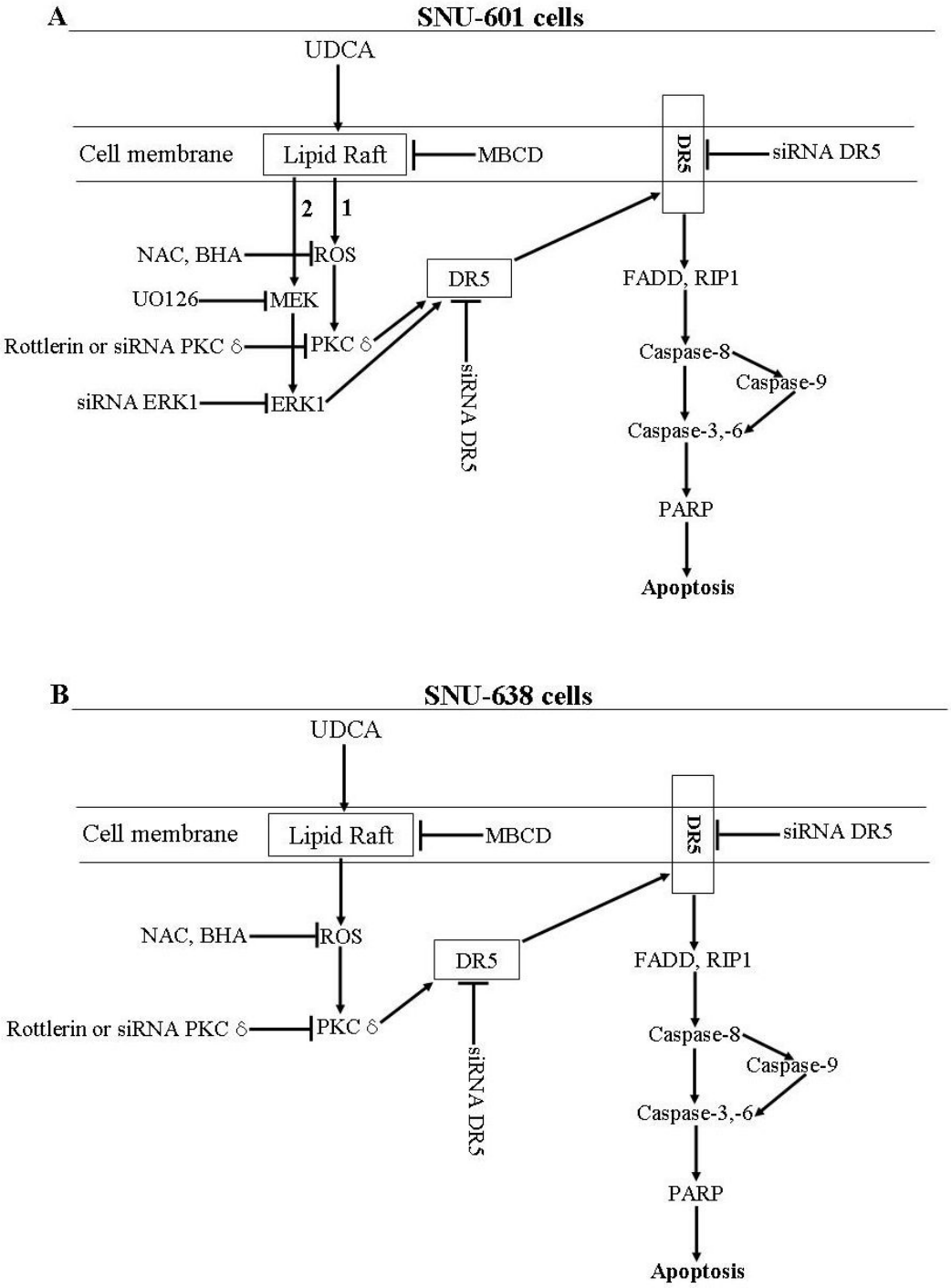

A
SNU-601 cells
UDCA
Cell membrane
Lipid Raft
MBCD
DR5
siRNA DR5
2
1
NAC, BHA
ROS
UO126
MEK
Rottlerin or siRNA PKC δ
PKC δ
siRNA ERK1
ERK1
DR5
siRNA DR5
FADD, RIP1
Caspase-8
Caspase-9
Caspase-3,-6
PARP
Apoptosis
B
SNU-638 cells
UDCA
Cell membrane
Lipid Raft
MBCD
DR5
siRNA DR5
NAC, BHA
ROS
Rottlerin or siRNA PKC δ
PKC δ
DR5
siRNA DR5
FADD, RIP1
Caspase-8
Caspase-9
Caspase-3,-6
PARP
Apoptosis

Fig. 22: Schematic model for the molecular mechanism of UDCA-induced apoptosis in human gastric cancer cells. (**A**) UDCA-induced expression of DR5 to trigger apoptosis is regulated by lipid raft/ROS/PKC δ and lipid raft/MEK/ERK1 pathway in SNU-601 cells, respectively. Briefly, (the first pathway) UDCA stimulated ROS production through lipid raft to activate the translocation of PKC δ from cytosol to membrane leading to induce expression of DR5 to trigger apoptosis, (the second pathway) UDCA-induced MEK/ERK1 activation through lipid raft to induce expression of DR5 to trigger apoptosis. UDCA-induced expression of DR5 to trigger apoptosis can be blocked by lipid raft depleting agent (MBCD), ROS scavengers (NAC and BHA), a specific inhibitor of PKC δ (Rottlerin) and a specific inhibitor of MEK1/2 (UO126). siRNA DR5, siRNA PKC δ and siRNA ERK1 can also be suppressed UDCA-induced expression of DR5 to trigger apoptosis. (**B**) UDCA-induced expression of DR5 to trigger apoptosis is regulated by lipid raft/ROS/PKC δ pathway in SNU-638. Briefly, UDCA stimulated ROS production through lipid raft to activate the translocation of PKC δ from cytosol to membrane leading to induce expression of DR5 to trigger apoptosis. UDCA-induced expression of DR5 to trigger apoptosis can be blocked by lipid raft depleting agent (MBCD), ROS scavengers (NAC and BHA) and a specific inhibitor of PKC δ (Rottlerin). siRNA DR5 and siRNA PKC δ can also be suppressed UDCA-induced expression of DR5 to trigger apoptosis in SNU-638 cells.

CHAPTER IV: DISCUSSION

Recently, beside of the important roles of UDCA such as anti-apoptotic, anti-inflammatory, immunodulation and cholesetic effect have been shown to exert tumor suppressing activity by pro-apoptotic mechanism [15, 16] and enhance CPT-11 induced-apoptosis in colon cancer cells, and promote an apoptotic response of tumor cells to photosensitizers and Bcl-2/Bcl-X_L antagonist HA14-1 [55, 56]. On the other hand, pro-apoptotic activity of UDCA was also demonstrated in hepatocytes [57]. The tumor suppressive activity of UDCA is explained by its potential to activate pro-apoptotic mechanisms, inhibit the cell cycle, or prevent oncogenic factors. In addition, UDCA inhibit selectively proliferation and induce apoptosis of HepG2 and BEL7402 cell lines by blocking cell cycle and regulating the expression of Bax/bcl-2 genes [23]. However, the molecular mechanisms responsible for UDCA-induced apoptosis have not been clearly established. In the present study, for the first time, we investigated that UDCA induces apoptosis through directly death receptor and indirectly mitochondrial pathway but not ER stress pathway in human gastric cancer cells. We demonstrated that UDCA stimulates ROS production through lipid raft to activate the translocation of PKC δ from cytosol to membrane leading to induce expression of DR5 to trigger apoptosis, suggesting UDCA-induced expression of DR5 to trigger apoptosis is significantly regulated by lipid raft/ROS/PKC δ pathway. On the other hand, we demonstrated that UDCA induced phosphorylation status of MEK/ERK through lipid raft to induce expression of DR5 to trigger apoptosis, suggesting UDCA-induced expression of DR5 to trigger apoptosis is partially regulated by lipid raft/MEK/ERK1 pathway in SNU-601 cells.

Activation of caspases plays an important role in apoptosis triggered by various pro-apoptotic signals [21]. Both major apoptotic pathways including death receptor and mitochondrial pathway, involved in an ordered activation of a set of caspases, which in turn cleave cellular substrates leading to the morphologic and biochemical changes of apoptosis. The activation of caspase-8 and caspase-9 has been documented to play central roles in mediating apoptosis signaled by death receptors and mitochondria, respectively [21]. However, caspase-8 can activate caspase-9-mediated apoptotic pathway via activating or cleaving Bid protein [24, 25]. It is reported that toxic bile acids have been shown to induce apoptosis in a Fas- and TRAIL R-dependent manner, involving recruitment of the Fas-associated death domain (FADD), activation of caspase-8 and Bid as well as downstream effector caspases [4, 5], and Lithocholic acid (LCA)-induced apoptosis in colon cancer cell lines proceeds through a mitochondrial/caspase-9-dependent pathway initiated by caspase-8 [6]. In this study, we investigated that UDCA-induced apoptosis is dependent on caspases-3, -6, -9 and -8 but not caspase-4 in human gastric cancer cells. We found that UDCA-induced apoptosis was completely suppressed by Z-VAD-FMK, Z-IETD-FMK, Z-VEID-FMK and Z-DEVD-FMK. In addition, UDCA increased an activation of caspase-8 in time-dependent manner and Z-IETD-FMK completely suppressed UDCA-induced activation of caspase-3, caspase-6 and PARP cleavage, indicating that caspase-8 activation is required for UDCA-induced apoptosis and caspase-8 is an upstream initiator caspase. Previous reports have shown that p53 is mutated in both human gastric cancer cells (SNU-601 and SNU-638 cells) [58]. Therefore, UDCA-induced apoptosis is independent on p53 in these cells. However, Z-LEHD-FMK significantly suppressed UDCA-induced apoptotic cell death, indicating that caspase-8

activation was an early upstream event of caspase-9 activation. It is also reported that caspase-4 has been identified as a mediator of ER stress to trigger apoptosis [59, 60, 61], and GCDCA-induced apoptosis is mediated through the ER stress pathway in liver cells [7, 8]. But, in this study, we found that UDCA-induced apoptosis was not suppressed by Ac-LEVD-CHO, indicating that UDCA-induced apoptosis was independent of caspase-4. These results suggest that UDCA induces apoptosis through directly death receptor and indirectly mitochondrial pathways but not ER stress pathway in human gastric cancer cells.

The death receptors include TNFR1, CD95/Fas, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). TRAIL binds to the cell surface death receptor DR4 and DR5 or Fas ligand binds to CD95/Fas, which in turn recruit intracellular FADD. Through its death effector domain, FADD recruits caspase-8 to the receptors for the assembly of a death-inducing signaling complex (DISC) [23]. It is reported that toxic bile acids have been shown to induce apoptosis in a Fas- and TRAIL-dependent manner [4, 5] and bile acids up-regulate DR5 expression thereby sensitizing hepatocytes to TRAIL-mediated apoptosis [53, 62]. In this study, we investigated that UDCA induced expression of DR5 in concentration- and time-dependent manner, especially UDCA induced expression of DR5 at cell membrane. Previous reports have shown that the DR5 plays a crucial role in sensitizing tumor cells to apoptosis induced by TRAIL and chemotherapeutic agents [50, 51]. In this study, our results showed that knock-down expression of siRNA DR5 significantly suppressed UDCA-induced apoptosis through inhibition of activation of caspase-3, caspase-6, PARP cleavage and caspase-8 activation. On the other hand, we also found siRNA Fas but not siRNA DR4 reduced UDCA-induced apoptosis in SNU-601 cells and siRNA DR4 but not siRNA Fas reduced UDCA-induced apoptosis in SNU-638 cells, indicating that DR5 is a

common death receptor regulating UDCA-induced apoptosis in both cancer cell lines. Previous reports have shown toxic bile acids induce apoptosis involving recruitment of FADD in hepatocyte and colon cancer cells [4, 5]. In this study, we found that knock-down of FADD or RIP1, adaptor of death receptor pathway, by specific siRNA FADD or RIP1, respectively reduced UDCA-induced apoptosis, indicating that UDCA-induced apoptosis was mediated through FADD and RIP1 in human gastric cancer cells.

PKC δ, a member of the novel PKC super-family, is actively involved in cell apoptosis in a stimulus and tissue specific manner. Depending on the cell types and apoptotic stimuli, PKC δ translocates to nearly all subcellular organelles, including the nucleus, mitochondria, the golgi complex, endoplamic reticulum and plama membrane [27]. It is reported that translocation of PKC δ to membrane is observed in response to UV radiation in mouse epidermal cell lines [63]. DCA activates the translocation of PKC δ from cytosol to membrane and evidence for tumor promoting in colon cancer cells. So, PKC plays an important role in colorectal carcinogenesis. However, UDCA inhibited DCA-activated the translocation of PKC δ in these cells [28]. Comparing with the effects of UDCA-inhibited DCA-induced the translocation of PKC δ in colon cancer cells, we observed that UDCA activated the translocation of PKC δ, but not PKC α, PKC θ and PKC ε from cytosol to membrane. Some reports have shown that PKC δ is essential for etoposide-induced apoptosis in salivary [64] and involvement of PKC δ in DNA damage-induced apoptosis [65]. In this study, we found that specific inhibitor of PKC δ (rottlerin) significantly suppressed UDCA-induced expression of DR5 and apoptosis, suggesting that rottlerin blocks downstream signaling events of PKC δ in response to UDCA-induced expression of DR5 to trigger apoptosis in both cancer cell lines. Recently, a variety of PKC

δ-independent actions of rottlerin has been recently reported [66, 67, 68]. Therefore, the specific effect of was confirmed by siRNA PKC δ to knock-down activation of PKC δ. We also observed that UDCA-induced expression of DR5 and apoptosis was blocked by siRNA PKC δ, suggesting that PKC δ was an important regulator to regulate UDCA-induced expression of DR5 to trigger apoptosis in human gastric cancer cells.

ROS is one of critical factors inducing apoptosis. It is reported that oxidative stress induced by chemo-preventive drugs reduces mitochondrial membrane potential and causes cytochrome C release from mitochondria, resulting in activation of caspase-9 [69, 70] and other studies have emphasized the role of ROS in the activation of the death receptor pathway and caspase-8 during apoptosis [70, 71]. Moreover, oxidative stress induced by bile acid can be generated through a multi-factorial mechanism involving NADPH oxidase activation and interaction of bile acids with mitochondria to directly generate ROS [32]. In addition, some chemotherapeutic agents such as sulforaphane, curcumin and rosiglitazone increase expression of DR5 through ROS production, and proteasome inhibitor (MG132) induces expression of DR5 through ROS-dependent of p53 activation [50, 51, 54]. In this study, we observed that UDCA activated ROS production for 3- or 5 h and ROS scavengers including NAC and BHA significantly suppressed UDCA-induced expression of DR5 and apoptosis, indicating that ROS was crucial regulator to regulate UDCA-induced expression of DR5 to trigger apoptosis. Interestingly, we also observed that NAC blocked UDCA-activated the translocation of PKC δ from cytosol to membrane, indicating that UDCA stimulated ROS production to activate the translocation of PKC δ from cytosol to membrane and PKC δ was downstream event of ROS.

Lipid raft plays an important role in cellular signaling, functioning as physical platforms to concentrate and assemble the signal transduction machinery [48], and have been shown to regulate signal transduction by activating or suppressing the phosphorylation caspases related to cell growth, survival and death and other physiological processes [49]. Lipid raft disruption by cholesterol depletion inhibits loss of mitochondrial transmembrane potential, caspase activation and apoptosis, whereas cholesterol replenishment restored these responses. It is reported that depletion of membrane cholesterol (lipid raft) by MBCD suppresses DCA-induced apoptosis [72]. In this study, we observed that depletion of lipid raft by MBCD completely suppressed UDCA-induced expression of DR5 and apoptosis. In addition, we also showed that UDCD-activated ROS production and the translocation of PKC δ from cytosol to membrane were significantly suppressed by MBCD, indicating that UDCA-stimulated ROS production through lipid raft to activate the translocation of PKC δ from cytosol to membrane, which lead to expression of DR5 to trigger apoptosis. These results suggest that UDCA-induced apoptosis was significantly regulated by lipid raft/ROS/PKC δ/DR5 pathway in human gastric cancer cells.

MAPK cascades are involved in the signaling of various cellular responses such as cell death, proliferation and inflammation [35, 36]. The ERK1/2 cascade is activated through receptor-mediated signaling stimuli and is associated with cell proliferation, differentiation and survival [37, 38]. However, in some cases, ERK activation contributes to cell death [39, 40]. It is reported that the p38/ERK/PI3K pathways are involved in protection of TUDCA against GCDCA-induced apoptosis in rat hepatocytes [41] and other reports shown that bile acid regulates transcriptional factors via ERK and JNK pathways

modulates the apoptotic response of hepatocyte [73]. However, we observed that UDCA activated phosphorylation of MEK/ERK1/2 in SNU-601 cells but not in SNU-638 cells. In addition, inhibition of MEK1/2 phosphorylation by UO126 or knock-down of ERK1 by specific siRNA for ERK1 partially suppressed UDCA-induced expression of DR5 and apoptosis, indicating that UDCA-induced expression of DR5 and apoptosis was partially regulated by MEK/ERK1 pathways in SNU-601 cells. Previous reports have shown that ROS stimulates activation of ERK1/2 in various cells [74, 75]. However, we investigated that NAC or BHA did not have effect to block UDCA-activated phosphorylation of ERK1/2, indicating that ROS did not regulate UDCA-activated ERK in SNU-601 cells. It is reported that depletion of plasma membrane cholesterol with MBCD induces ERK activation via a PI3K-dependent pathway [76]. On the other hand, depletion of cholesterol in lipid raft membranes inhibited EGFR/ERK signal transduction [77]. In this study, we observed that depletion of lipid raft by MBCD completely suppressed UDCA-activated phosphorylation status of ERK, indicating that UDCA-activated phosphorylation status of ERK was regulated by lipid raft in SNU-601 cells. Based on these results, we suggested that UDCA-induced expression of DR5 to trigger apoptosis was also partially regulated by lipid raft/MEK/ERK1 pathway in SNU-601 cells.

Both NF-kB and AP-1 is heterodimeric transcription factor. NF-kB consists of p50 and RelA, RelB and cRel subunits, and the most commonly encountered NF-kB dimer in mammalian cells is p65/p50 [78, 79]. AP-1 consists of various subunits including Fos and Jun family of proteins. Both NF-kB and AP-1 activates transcription of proinflamatory and other genes involved in cell proliferation and transcription [80, 81]. Previous reports have shown that DCA activates NF-kB and AP-1, whereas UDCA inhibits those induced by

interleukin-β1 and DCA in colon cancer cells [82]. In this study, we observed that UDCA did not activate both NF-kB and AP-1, and knock-down of NF-kB or AP-1 by specific siRNA for NF-kB or AP-1, respectively did not prevent UDCA-induced expression of DR5 and apoptosis, indicating that both transcription factors (NF-kB and AP-1) was not related to UDCA-induced expression of DR5 and apoptosis. Collectively, UDCA-induced expression of DR5 to trigger apoptosis was significantly regulated by lipid raft/ROS/PKC β pathway in human gastric cancer cells and partially regulated by lipid raft/MEK/ERK1 pathway in SNU-601 cells.

CHAPTER V: CONCLUSION

In conclusion, UDCA has a novel effect to induce apoptosis in human gastric cancer cells. UDCA induces apoptosis through directly the death receptor and indirectly mitochondrial pathway but not ER stress pathway. UDCA stimulates ROS production through lipid raft to activate the translocation of PKC δ leading to induce expression of DR5 to trigger apoptosis, suggesting that UDCA-induced expression of DR5 to trigger apoptosis is significantly regulated by lipid raft/ROS/PKC δ pathway in both cancer cell lines. On the other hand, UDCA induces phosphorylation status of MEK/ERK1 through lipid raft to induce expression of DR5 to trigger apoptosis, suggesting that UDCA-induced expression of DR5 to trigger apoptosis is partially regulated by lipid raft/MEK/ERK1 pathway in SNU-601 cells. From this study, UDCA can be suggested as a novel chemotherapeutic agent to develop for promising strategy to treat gastric cancer at clinical in future.

CHAPTER VI: REFERENCES

1. Joana D. Amaral, Ricardo J.S Viana, Rita M. Ramalho, Clifford J. Steer and Cecilia M.P Rodrigues. Bile acids: Regulation of apoptosis by Ursodeoxycholic acid. *J Lipid Res*. **50**: 1721-1734, 2009.

2. Debruyne PR, Bruyneel EA, Li X, Zimber A, Gespach C and Mareel MM. The role of bile acids in carcinogenesis. *Mutat Res*. **480-481**: 359-369, 2001.

3. Greim H, Trulzsch D, Czygan P, Rudick J, Hutterer F, Schaffner F, Popper H. Mechanism of cholestasis. 6. Bile acids in human livers with or without biliary obstruction. *Gastroenterology*. **63**: 846-850, 1972.

4. Faubion WA, Guicciardi ME, Miyoshi H, Bronk SF, Roberts PJ, Svingen PA, Kaufmann SH and Gorces GJ. Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas. *J Clin Invest*. **103**: 137-145, 1999.

5. Higuchi H, Yoon JH, Grambihler A, Werneburg N, Bronk SF and Gorces GJ. Bile acids stimulate cFLIP phosphorylation enhancing TRAIL-mediated apoptosis. *J Biol Chem*. **278**: 454-461, 2003.

6. Katona BW, Anant S, Covey DF and Stenson WF. Characterization of enantiomeric bile acid-induced apoptosis in colon cancer cell lines. *J Biol Chem*. **284**: 3354-3364, 2009.

7. Tsuchiya S, Tsuji M, Morio Y and Oguchi K. Involvement of endoplasmic reticulum in glycochenodeoxycholic acid-induced apoptosis in rat hepatocytes. *Toxicol Lett*. **166**: 140-149, 2006.

8. Lizaka T, Tsuji M, Oyamada, Morio Y and Oguchi K. interaction between caspase-8 activation and endoplasmic reticulum stress in glycochenodeoxycholic acid-induced apoptotic HepG2 cells. *Toxicology*. **241**: 146-156, 2007.

9. Angulo P. Use of ursodeoxycholic acid in patients with liver disease. *Curr Gastroenterol Rep*. **4**: 37-44, 2002.

10. Marzioni M, Francis H, Benedetti A, Ueno Y, Fava G, Venter J, Reichenbach R, Mancino MG, Summers R, Alpini G and Glaser S. Ca^{2+}-dependent cytoprotective effects of ursodeoxycholic and tauroursodeoxycholic acid on the biliary epithelium in a rat model of cholestasis and loss of bile ducts. *Am J Pathol*. **168**: 398-409, 2006.

11. Rodrigues CM, Fan G, Ma X, Kren BT and Steer CJ. A novel role for ursodeoxycholic acid inhibiting apoptosis by modulating mitochondrial membrane perturbation. *J Clin Invest*. **101**: 2790-2799, 1998.

12. Rodrigues CM, Ma X, Linehan-Stieers C, Fan G, Kren BT and Steer CJ. Ursodeoxycholic acid prevents cytochrome C release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. *Cell Death Differ*. **6**: 842-854, 1999.

13. Rodrigues CM and Steer CJ. The neuroprotective characteristic of ursodeoxycholic acid and its conjugates. In biology of Bile acids in health and disease. Van Berge Henegouwen G, Keppler D, Leuschner U, Paumgartner G and Stiehl A. *Kluwer Academic ublishers*. The Netherlands. 255-270, 2000.

14. Khare S, Cerda S, Wali RK, Von Lintig FC, Tretiakova M, Joseph L, Stoiber D, Cohen G, Nimmagada K, Hart J, Sitrin MD, Boss GR and Bissonnette M. Ursodeoxycholic acid inhibits Ras mutations, wild-type Ras activation, and cyclooxygenase-2 expression in colon cancer. *Cancer Res*. **63**: 3517-3523, 2003.

15. Khare S, Mustafi R, Cerda S, Yuan W, Jagadeeswaran S, Dougherty U, Tretiakova M, Samarel A, Cohen G, Wang J, Moore C, Wali R, Holgren C, Joseph L, Fichera A, Li YC and Bissonnette M. Ursodeoxycholic acid suppresses Cox-2 expression in colon cancer: role of Ras, p38, and CCAAT/enhancer-binding protein. *Nutr Cancer*. **60**: 349-400, 2008.

16. Liu H, Qin CY, Han GQ, Xu HW, Meng M, Yang Z. Mechanism of apoptotic effects induced selectively by ursodeoxycholic acid on human hepatoma cell lines. *World J Gastroenterol*. **13**: 1652-1658, 2007.

17. Parkin DM. Global cancer statistics in the year 2000. *Lancet Oncol*. **2**:533–43, 2001.

18. Sakamoto J, Matsui T, and Kodera Y. Paclitaxel chemotherapy for the treatment of gastric cancer. *Gastric Cancer*. **12**: 69–78, 2009.

19. Sutter AP, Fechner H. Gene therapy for gastric cancer: Is it promising? *World J Gastroenterol*. **21**: 380-387, 2006.

20. Daniel PT. Dissecting the pathway to death. *Leukemia*. **14**: 2035-2044, 2000.

21. Kroemer G, Galluzzi L and Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev*. **87**: 99-163, 2007.

22. Krammer PH. CD95(APO-1/Fas)-mediated apoptosis: Live and let die. *Adv Immunol*. **71**: 163-210, 1999.

23. Hao C, Song JH, Vilimanovich U and Kneteman NM. Modulation of TRAIL signaling complex. In: Litwack G. Editor. *Vitamins and hormones*. Academic Press. 81-99, 2004.

24. Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH and Peter ME. FLICE is activated by association with CD95 death-inducing signaling complex (DISC). *EMBO J.* **16**: 2794-2804, 1997.

25. Li H, Zhu H, Xu CJ and Yuan J. Cleavage of BID by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*. **94**:491-501, 1998.

26. Nishizuka Y. The molecular heterogeneity of protein kinase C and its implication for cellular regulation. *Nature*. **334**: 661-665, 1998.

27. Brodie C and Blumberg PM. Regulation of cell apoptosis by protein kinase c δ. *Apoptosis*. **8**:19-27, 2003.

28. Shah SE, Looby E, Volkov Y, Long A and Kelleher D. Ursodeoxycholic acid inhibits translocation of protein kinase C in human colonic cancer cell lines. *Eur J Cancer*. **41**: 2160-2169, 2005.

29. Nordberg J and Arner ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med*. **31**: 1287-1312, 2001.

30. Cadenas E and Davies KJ. Mitochondrial free radical generation, oxidative stress and, aging. *Free Radic Biol Med*. **29**: 220-230, 2000.

31. McCord JM. Human disease, free radicals and the oxidant/antioxidant balance. *Clin Biochem*. **26**: 351-357, 1993.

32. Reinehr R, Becker S, Eberle A, Grether-Beck S, and Haussinger D. Involvement of NADPH oxidase isoforms and Src family kinase in CD95-dependent hypatocyte apoptosis. *J Biol Chem*. **280**: 27179-27194, 2005.

33. Sommerfeld A, Reinehr R, Häussinger D. Bile acid-induced epidermal growth factor receptor activation in quiescent rat hepatic stellate cells can trigger both proliferation and apoptosis. *J Biol Chem*. **284**: 22173-22183, 2009.

34. Rodrigues CM, Fan G, Wong PY, Kren BT and Steer CJ. Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and reactive oxygen species production. *Mol Med*. **4**: 165-178, 1998.

35. Matsukawa J, Matsuzawa A, Takeda K and Ichijo H. The ASK1-MAP kinase cascades in mammalian stress response. *J Biochem*. **136**: 261-265, 2004.

36. Dong C, Davis RJ and Flavell RA. MAP kinases in the immune response. *Annu Rev Immunol*. **20**: 55-72, 2002.

37. Xia Z, Dickens M, Raingeaud J, Davis RJ and Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*. **270**: 1326-1331, 1995.

38. Cobb MH. MAP kinase pathways. *Prog Biophys Mol Biol*. **71**: 479-500, 1999.

39. Stanciu M, Wang Y, Kentor R, Burke N, Watkins S, Kress G, Reynolds I, Klann E, Angiolieri MJ, Johnson JW and DeFranco DB. Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *J Biol Chem*. **275**: 12200-12206, 2000.

40. Wang X, Martindale JL and Holbrook NJ. Requirement for ERK activation in cisplatin- induced apoptosis. *J Biol Chem*. **275**: 39435-39443, 2000.

41. Rao YP, Studer EJ, Stravitz RT, Gupta S, Qiao L, Dent P and Hylemon PB. Activation of the Raf-1/MEK/ERK caspade by bile acids occurs via epidermal growth factor receptor in primary rat hepatocytes. *Hepatology*. **35**: 307-314, 2002.

42. Qiao D, Chen W, Stratagoules ED and Martinez JD. Bile acid-induced activation of activator protein-1 requires both extracellular signal-regulated kinase and protein kinase C signaling. *J Biol Chem*. **275**: 15090-15098, 2000.

43. Brown DA and London E. Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol*. **14**: 111-136, 1998.

44. Burger K, Gimpl G and Fahrenholz F. Regulation of receptor function by cholesterol. *Cell Mol Life Sci*. **57**: 1577-1592, 2000.

45. Simons K and Toomre D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol*. **1**: 31-39, 2000.

46. Edidin M. The state of lipid rafts: From model membranes to cells. *Annu Rev Biophys Biomol Struct*. **32**: 257-283, 2003.

47. Dykstra M, Cherukuri A, Sohn HW, Tzeng SJ and Pierce SK. Location is everything: lipid rafts and immune cell signaling. *Annu Rev Immunol*. **21**: 457-481, 2003.

48. Simons K and Toomre D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol*. **1**: 31-9, 2000.

49. Dimanche-Boitrel MT, Meurette O, Rebillard A and Lacour S. Role of early plasma membrane events in chemotherapy-induced cell death. *Drug Resist Updat.* **8**: 5-14, 2005.

50. Kim YH, Jung EM, Lee TJ, Kim SH, Choi YH, Park JW, Park JW, Choi KS and Kwon TK. Rosiglitazone promotes tumor necrosis factor related apoptosis-inducing ligand induced apoptosis by reactive oxygen species-mediated up-regulation of death receptor 5 and down-regulation of C-FLIP. *Free Radia Biol Med*. **44**: 1055-1068, 2008.

51. Jung EM, Lim JH, Lee TJ, Park JW, Choi KS and Kwon TK. Curcumin sensitizes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through reactive oxygen species-mediated upregulation of death receptor 5 (DR5). *Carcinogenesis*. **26**: 1905-1913, 2005.

52. Shenoy K, Wu Y and Pervaiz S. Ly303511 enhances TRAIL sensitivity of SHEP-1 neuroblastoma cells via hydrogen-mediated mitogen-activated protein kinase activation and up-regulation of death receptors. *Cancer Res*. **69**: 1941-1950, 2009.

53. Higuchi H, Grambihler A, Canbay A, Bronk SF and Gores GJ. Bile acids up-regulate death receptor 5/TRAIL-receptor 2 expression via a c-Jun N-terminal kinase-dependent pathway involving Sp1. *J Biol Chem*. **1**: 51-60, 2004.

54. Chen JJ, Chou CW, Chang YF and Chen CC. Proteasome inhibitors enhance TRAIL-induced apoptosis through the intronic regulation of DR5: Involvement of NF-kB and reactive oxygen species-mediated p53 activation. *J Immunol*. **180**: 8080-8089, 2008.

55. Castelli M, Reiners JJ and Kessel D. A mechanism for the proapoptotic activity of ursodeoxycholic acid: effect on Bcl-2 conformation. *Cell Death Differ*. **11**: 906-914, 2004.

56. Ikegami T, Matsuzaki Y, Al Rashid M, Ceryak S, Zhang Y and Bouscarel B. Enhancement of DNA topoisomerase I inhibitor-induced apoptosis by ursodeoxycholic acid. *Mol Cancer Ther*. **5**: 68-79, 2006.

57. Qiao L, Yacoub A, Studer E, Gupta S, Pei XY, Grant S, Hylemon PB, Dent P. Inhibition of the MAPK and PI3K pathways enhances UDCA-induced apoptosis in primary rodent hepatocytes. *Hepatology*. **35**: 779-789, 2002.

58. Ku JL and Park JG. Biology of SNU cell lines. *Cancer Res Treat*. **1**: 1-19, 2005

59. Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, Manabe T, Yamagichi S, Bando Y, Imaizumi K, Tsujimoto Y, Tohyama M. Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and abeta-induced cell death. *J Cell Biol*. **165**: 347-356, 2004.

60. Liao PC, Tan SK, Lieu CH and Jung HK. Involvement of endoplasmic reticulum in paclitaxel-induced apoptosis. *J Cell Biochem*. **104**: 1509-1523, 2008.

61. Yukioka F, Matsuzaki S, Kawamoto K, Koyama Y, Hitomi J, Katayama T and Toyama M. Presenilin-1 mutation activates the signaling pathway of caspase-4 in endoplasmic reticulum stress-induced apoptosis. *Neurochem Internat.* **52**: 683-687, 2008.

62. Higuchi H, Bronk SF, Takikawa Y, Wernrburg N, Takimoto R, El-Deiry W and Gores GJ. The bile acid glucochenodeoxycholate induces TRAIL-receptor 2/DR5 expression and apoptosis. *J Biol Chem*. **42**: 38610-38618, 2001.

63. Chen N, Ma WY, Huang C, and Dong Z. Translocation of protein kinase Cε and protein kinase Cδ to membrane is required for ultraviolet B-induced activation of mitogen-activated protein kinases and apoptosis. *J Biol Chem*. **22**: 15389-15394, 1999.

64. Reyland ME, Anderson SM, Matassa AA, Barzen KA and Quissell DO. Protein kinase C δ is essential for etoposide-induced apoptosis in salivary gland acinar cells. *J Biol Chem*. **274**: 19115-19123, 1999.

65. Basu A. Involvement of protein kinase C delta in DNA damage-induced apoptosis. *J Cell Mol Med*. **7**: 341-350, 2003.

66. Soltoff SP. Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase Cdelta tyrosine phosphorylation. *J Biol Chem*. **276**: 37986–37992, 2001

67. Tapia JA, Jensen RT, Garcia-Marin LJ. Rottlerin inhibits stimulated enzymatic secretion and several intracellular signaling transduction pathways in pancreatic acinarcells by a non-PKC-delta-dependent mechanism. *Biochim Biophys Acta*, **1763**: 25-38, 2006

68. Lim JH, Park JW, Choi KS, Park YB and Kwon TK. Rottlerin induces apoptosis via death receptor 5 (DR5) upregulation through CHOP-dependent and PKC δ-independent mechanism in human malignant tumor cells. *Carcinogenesis*. **5**: 729-736, 2009.

69. Bagriacik EU, Uslu K, Yurtcu E, Stefek M and Karasu C. Stobadine inhibits doxorubicin-induced apoptosis through a caspase-9 dependent pathway in P815 mastocytoma cells. *Cell Biol Int*. **31**: 979-984, 2007.

70. Mates JM, Segura JA, Alonso FJ and Marquez J. Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis and carcinogenesis. *Arch Toxicol*. **82**: 273-299, 2008.

71. Kim BM and Chung HW. Hypoxia/reoxygenation induces apoptosis through a ROS-mediated caspase-8/Bid/Bax pathway in human lymphocytes. *Biochem Biophys Res Commun*. **363**: 745-750, 2007.

72. Jean-Louis S, Akare S, Ali MA, Mash EA and Meuillet E. Deoxycholic acid induces intracellular signaling through membrane perturbations. *J Biol Chem*. **21**: 14948-14960, 2006.

73. Bile acid regulation of C/EBPβ, CREB and c-Jun function, via the extracellular signal-regulated kinase and c-Jun NH_2-terminal kinase pathways, modulates the apoptotic response of hepatocytes. *Mol Cell Biol*. **23**: 3052-3066, 2003.

74. Wang SF, Yen JC, Yin PH, Chi CW and Lee HC. Involvement of oxidative stress-activated JNK signaling in the methamphetamine-induced cell death of human SH-SY5Y cells. *Toxicology*. **2-3**: 234-241, 2008.

75. Ruffels J, Griffin M and Dickenson JM. Activation of ERK1/2, JNK and PKB by hydrogen peroxide in human SH-SY5Y neuroblastoma cells: role of ERK1/2 in H2O2-induced cell death. *Eur J Pharmacol*. **483**: 163-173, 2004.

76. Chen X and Resh MD. Activation of mitogen-activated protein kinase by membrane-targeted Raf chimeras is independent of raft localization. *J Bio Chem.* **276:** 34617-34623, 2001.

77. Oh HY, Lee EJ, Yoon S, Chung BH, Cho KS and Hong SJ. Cholesterol level of Lipid raft microdomains regulates apoptotic cell death in prostate cancer cells through EGFR-mediated Akt and ERK signal transduction. *Prostate*. **67**: 1061-1069, 2007.

78. Ghosh S, May MJ and Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol*. **16**: 225-260, 1998.

79. Miyamoto S and Verma IM. Rel/NF-kB/Ikb story. *Adv Cancer Res*. **66**: 255-292, 1995.

80. Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappa B. *J Clin Invest*. **107**: 241-246, 2001.

81. Angel P and Karin M. The role of Jun, Fos and the AP-1 complex in proliferation and transformation. *Biochem Biophys Acta*. **1072**: 129-157, 1991.

82. Shah SA, Volkov Y, Arfin Q, Abdel-Latif MM and Kelleher D. Ursodeoxycholic acid inhibits interleukin beta 1 and deoxycholic acid-induced activation of NF-kB and AP-1 in human colon cancer cells. *Int J Cancer*. **118**: 232-239, 2006.

저작물 이용 허락서

학 과	바이오신약개발학과	학 번	20077735	과 정	석사, 박사
성 명	한글: 권동홍 한문 : 영문 : Duong Hong Quan				
주 소	광주광역시 동구 서석동 426-4번지				
연락처	E-MAIL : quanvspt@yahoo.com or quanvspt@gmail.com				
논문제목	한글 : Ursodeoxycholic acid에 의한 위암세포 고사유도 영어 : Ursodeoxycholic acid induces apoptosis in human gastric cancer cells				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의여부 : 동의(√) 반대( )

2009 년 12 월 29 일

저작자: Duong Hong Quan (서명 또는 인)

조선대학교 총장 귀하