의학박사 학위논문

Expression and Regulation of Gap Junction Proteins in Human Cholangiocytes by Excretory-Secretory Products of *Clonorchis sinensis* and N-Nitrosodimethylamine

사람 담관상피세포주에서 간흡충 분비항원과 **N-Nitrosodimethylamine**의 자극에 의한 세포연접 단백질의 발현조절

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서울대학교 대학원
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김 은 민

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사람 담관상피세포주에서 간흡충 분비항원과 N-Nitrosodimethylamine 의 자극에 의한 세포연접 단백질의 발현조절

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The Department of Medicine, Seoul National University College of Medicine

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by

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(Directed by Prof. Sung-Tae Hong)

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ABSTRACT

Clonorchis sinensis, the most prevalent parasite in Korea, has been reclassified as Group I bio-carcinogen for cholangiocarcinoma (CCA) in humans by IARC in 2009. In order to understand the mechanism of carcinogenesis of *C. sinensis*, the present study investigated cell proliferation and expression and regulation of gap junction proteins by stimulation with N-nitrosodimethylamine (NDMA) and/or excretory-secretory product of *C. sinensis* (ESP) in a human cholangiocyte line, H69. In cells exposed to NDMA and ESP, cellular proliferation and the proportion of cells in the G2/M phase increased to 37%. Moreover, the expression of the cell cycle protein E2F1 and the cell proliferation related proteins, ki67, and cytokeratin 19 were more than 30-fold increased when NDMA and ESP were added together. Especially, expression of gap-junction proteins (connexin 43 and connexin 26) and Cox-2 was increased while that of connexin 32 was reduced in the NDMA and ESP group. Connexin 43 silencing significantly suppressed expression of connexin 26 and Cox-2 in the same group. In conclusion, it is suggested that the connexin 43 plays a key role in regulation of inflammatory responses, which may cause cholangicarcinoma under stimulation by ESP of *C. sinensis* and NDMA.

Keywords: Cell cycle, *Clonorchis sinensis*, excretory-secretory products,

carcinogenesis, N-dimethylnitrosamine, Gap junction, Cox-2, human cholangiocyte cell line (H69)

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CONTENTS

LIST OF FIGURES

The ratio of Cx32/GAPDH in H69 cells transfected with Cx43-specific siRNA. Analysis showed that the expression of Cx32 no meaningful change in transfected H69 cells transfected with Cx43-specific siRNA. *P < 0.001 versus control siRNA

Figure 11. Amplification curves of real-time PCR for detection for Cox-2 gene. (A) Uptake of Cx43siRNA reduces Cox-2 expression, as confirmed using real-time PCR. (B) The ratio of Cox-2 /GAPDH in H69 cells transfected with Cx43-specific siRNA. Analysis showed that the expression of Cox-2 is remarkably reduced in transfected H69 cells transfected with Cx43-specific siRNA. *P < 0.001 versus control siRNA

LIST OF TABLES

LIST OF ABBREVIATIONS

Cs	*Clonorchis sinensis*
CCA	Cholangiocarcinoma
ESP	Excretory-secretory products of *C. sinensis*
H69	Human cholangiocyte cell line
Cox-2	Cyclooxygenase-2
Cx43	Connexin 43
Cx32	Connexin 32
Cx26	Connexin 26
CK19	cytokeratin-19
Ki67	MK167

INTRODUCTION

Clonorchis sinensis is a human liver fluke that induces cholangiocarcinoma (CCA) in human (IARC, 2009). Clonorchiasis has been endemic in Korea for a long time, especially among fish-eating residents along rivers. Still several species of freshwater fish are briskly transmitting *C. sinensis* infection in many riverside areas of southern Korea (Kim et al., 2008). In Korea, the last national survey on the status of intestinal helminthiasis in 2004 recorded a 2.9% egg positive rate of *C. sinensis* in the general population. The data estimated 1.3 million people of clonorchiasis in Korea (Kim et al., 2009). In the endemic areas of the liver fluke disease, CCA is more highly prevalent in Korea (Lim et al., 2005; Shin et al., 2011) and in Thailand (Sripa et al., 2010).

Humans get infection of *C. sinensis* by eating raw or undercooked-fish which carry the metacercariae. After human infection, the adult stage fluke inhabits the intrahepatic bile duct to induce irregular dilatation of the bile ducts and glandular hyperplasia, inflammation, periductal fibrosis, and/or cellular responses to antigenic stimulation of the infecting fluke (Hong, 2003). These conditions predispose to CCA, possibly through an enhanced susceptibility of DNA to damage by a chemical carcinogen (Lee et al., 1994). Chronic hepatobiliary damage is reported to be multi-factorial and considered to arise from a contributed mechanical irritation of the epithelium by flukes, particularly via their suckers, metabolites, and ESP antigens (Kim et al., 2008)

as well as host immune response (Hong et al., 2003). This inflammatory reaction also leads to excessive production of reactive oxygen species (ROS) and reactive nitrogen species which damage DNA. In turn, the damaged DNA may be related in the initiation and promotion of experimental CCA (Lee et al., 1994; Prakobwong et al., 2010).

The carcinogenesis mechanism of CCA by liver flukes is not well understood in detail. However, bile duct injury resulting directly by fluke activities and immunopathologic processes due to host inflammatory responses to the fluke and the ESP are suspected to be major factors in the liver fluke associated CCA (Hong and Fang, 2012).

A few studies have investigated mechanism of oncogenesis in liver fluke infections including *C. sinensis* and *O. viverrini* (Sripa et al., 2012; Hong and Fang, 2012). Increased levels of proinflammatory cytokines and nuclear factor kappa B (NFκB) that control cyclooxygenase-2 (Cox-2) and inducible nitric oxide synthase activities, disturb the homeostasis of oxidants/anti-oxidants and DNA repair enzymes, all of which appear to be involved in *O. viverrini*-associated inflammatory processes and progression to CCA (Yongvanit et al., 2012; Ninlawan et al., 2010). Consequently oxidative and nitrative stress-related cellular damage occurs due to the overproduction of reactive oxygen and nitrogen species in the inflamed target cells (Yongvanit et al., 2012).

Nitrosamines can be formed endogenously from nitrate and nitrite and secondary amines under certain conditions such as strongly pHs of the human

stomach (Erkekoglu et al., 2010). Humans are exposed to a wide range of nitrosamines from diet such as cured meat products, fried food, smoked preserved foods, and salty preserved foods. Among them, N-nitrosodimethylamine (NDMA) is a potent hepatotoxin that can cause fibrosis and tumors in the liver of rats through an activation of CYP450 enzymes (George et al., 2001).

Oncogenesis is a multi-step process in which alteration of cell cycle, apoptosis, oncogene expression, tumor suppressor and angiogenesis related genes are involved. Normal cells divide in a controlled cell cycle, and there are numerous components which control timing and frequency of the cell cycles. Previously, Kim et al. (2008) reported that HEK293T cells, which were exposed to the carcinogen NDMA and the ESP of *C. sinensis*, increased of their proliferation and the proportion of cells in the G2/M phase. The increased proliferation was induced by over-expression of E2F1 and down-regulation of pRb, indicating the involvement of the Rb-E2F1 pathway (Dimova et al., 2003). Sripa et al. (2012) reported that liver fluke ESP promote tumor cell growth, suppress apoptosis, and induce potent IL-6 production *in vitro* and *in vivo*.

In hamsters, which are infected with liver flukes such as *C. sinensis* or *O. viverrini*, are at greater risk of developing cholangiocarcinoma through NDMA-induced or inflammation-mediated carcinogenesis (Kim et al., 2005). The bile duct epithelial cells of an infected animal with liver fluke and

exposed *N*-nitroso compounds, may result neoplastic transformation (Zhang et al., 2007). Using this phenomenon, NDMA is commonly used as an initiator of carcinogenesis in a rodent model. DNA damage and reparative cellular replication were examined to confirm the effects of chemicals such as NDMA on hepatocytes (Trosko et al., 1993).

Kantima et al. (2010) showed that excretory-secretory antigens of *O. viverrini* induced pro-inflammatory response in vitro, which was through upregulation of TLR4, and their downstream signal transduction including MyD88-dependent IκB-α degradation, NFκB activation, and increased expression of IL-6 and IL-8. NFκB may also influence production of connexin 43 (Cx43), which is one of the gap-junction proteins, in liver cirrhosis (Vairappan et al., 2013).

Cell-to-cell communication through the gap junction channels is a critical factor in the life and death balance of cells, because gap junction intercellular communication (GJIC) has an important function in maintaining tissue homeostasis through the regulation of cell growth, differentiation, apoptosis, and adaptive functions of differentiated cells (Avanzo et al., 2007). Gap junctions are clusters of transmembrane channels that permit the direct intercellular exchange of ions, secondary messenger, and small signaling molecules influencing cell growth, differentiation and cancerous change (Balsubramaniyan et al., 2013). Among them, Cx43 is known to be critically involved in various immune inflammatory situations and participated in the

regulation of almost all steps of the inflammatory response, including antigen presentation, cytokine production, and inflammatory cell migration (Cicirata et al., 2004; Li et al., 2011). Alteration of Cx expression has been recorded in development of cancers (Trosko and Ruch, 1998). In HCC for instance, reduction of Cx32 expression is accompanied by the appearance of Cx43. The latter is spread around the plasma membrane and cytoplasm of cancerous hepatocytes. It has been suggested that the extent of intracellular Cx43 localization is related with the malignant potential of the liver tumor (Vinken et al., 2012).

In order to evaluate interactions of the intracellular molecules, the present study investigated changes of cellular proliferation, proinflammatory molelcules and connexin production in cholangiocytes H69 which were exposed to the ESP of *C. sinensis* and the carcinogen NDMA.

MATERIALS AND METHODS

1. Preparation of ESP

(1) Animals

Male Sprague–Dawley rats at 6 weeks of age were purchased from the Koatech Co. (Seoul, Korea), and housed in an ABL-2 animal facility in Seoul National University College of Medicine (SNU-091019-2). All rats were bred in filter cages under positive pressure according to institutional-approved guidelines.

(2) Recovery of metacercariae of *C. sinensis*

Pseudorasbora parva, the second intermediate hosts of *C. sinensis*, which were naturally infected with *C. sinensis*, were purchased at Sancheong-gun, Gyeongsangnam-do,─Republic of Korea, which is an endemic area of clonorchiasis. Metacercariae of *C. sinensis* were collected after digesting fish with pepsin-HCl (0.6%) artificial gastric juice for 1 h at 37 °C.

(3) Infection of *C. sinensis* in Experimental and Collection of ESP

Sprague–Dawley rats were individually infected orally with 50 metacercariae of *C. sinensis*. Eight weeks post-infection, adult worms were collected from bile ducts and washed several times with phosphate-buffered saline (PBS). The freshly isolated worms were then incubated in sterile PBS

containing antibiotics for 24 h in an atmosphere of 5% CO_2 at 37℃. After incubation, the medium was centrifuged for 10 min at 800 rpm to remove the worms and debris. The supernatant was then further centrifuged for 10 min at 3000 rpm and filtered with a syringe-driven 0.45-μm filter unit. The amount of protein in each extract was measured using the Bradford assay (Thermo, Rockford, IL, USA).

2. Cell culture and experimental design

(1) Cell culture

In this experiment, the human cholangiocyte cell line H69 cells were cultured for more than 60 days, the median change every 72 hours, then were treated with NDMA and ESP. H69 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, LA, USA) and DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, LA, USA), 2 mM l-glutamine, 100 μg/ml penicillin, 0.243 mg/ml adenine (Sigma A68626), insulin 5 μg/ml (Sigma I6634, SL, USA), 10 μg/ml epinephrine(Sigma E4250, SL, USA), 2 nM+ 5 μg/ml Triiodonine-transferrin (Sigma T8158, SL, USA), 30.000 ng/ml epidermal growth factor (R&D 236-EG, CA, USA), 1.1 μm hydrocortisone and 100 U/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

(2) Experimental design in H69 cells

Based on the hypotheses to be tested, H69 cells were divided into four

groups and cultured for more than 60 days, the median change every 72 hours, then were treated with NDMA and ESP: control, cultured in plain medium; 100 ng/ml NDMA, cultured in medium containing 100 ng/ml NDMA; ESP, cultured in medium containing 10 μg/ml ESP; and NDMA + ESP, cultured in medium containing 10 μg/ml and 100 ng/ml each NDMA and ESP.

3. Cellular proliferation assay

I used the PrestoBlue cell viability reagent method to evaluate the degree of cellular proliferation. For each assay, cells were seeded at a density of 5 x 10^3 cells/well on 96-well plates. After 24 h of incubation, the medium was replaced with 2% FBS-DMEM without phenol red. The cells were then incubated in the presence of PBS (vehicle) or with 100 ng/ml NDMN or without 10 μg/ml ESP for another 72 h. the PrestoBlue cell viability (1 mg/ml) was dissolved in warm medium and 1.25 mM phenazine methosulfate (PMS) was prepared in PBS. Following the incubation of the cells for the indicated periods, 50 μl of the PrestoBlue cell viability reagent was added to each well. The plates were then incubated for 1 h. The conversion of PrestoBlue cell viability was quantified by measuring the absorbance at 570 and 600 nm using a microtiter plate reader.

4. Cell cycle analysis

For cell cycle analysis, H69 cells were plated in six-well culture plates at

2×10^5 cells/well in 2 ml of DMEM containing 10% FBS. They were then treated with 100 ng/ml NDMA with or without 10 μg/ml ESP for 72 h and stained with propidium iodide (PI). The PI-stained cells were analyzed using a FACSCalibur multicolor flow cytometer (Becton-Dickinson, NJ, USA), and the data were analyzed using CellQuest software (Becton-Dickinson, NJ, USA).

5. Western blotting

For Western blots, cells were lysed using 1% Nonidet P-40 in a buffer containing 150 mM NaCl, 10 mM NaF, 1 mM PMSF, 200 μM Na_3VO_4, and 50 mM HEPES, pH 7.4. Equal amounts of protein were separated by 8 and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon; Millipore, MA, USA). The membranes were then probed with antibodies against E2F1, Ki-67, Ck-19, Cox-2, connexin 43, connexin 32, connexin 26, and calnexin. The primary antibodies were detected using goat anti-rabbit or rabbit anti-mouse secondary antibodies conjugated with HRP and visualized using an enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech, NY, USA).

6. Antibody

Polyclonal or monoclonal antibodies were used to detect the expression of several cell-cycle-related proteins, anti-E2F1 (sc-193, Santacruz, CA, USA), and anti-ki67 (SP6, Abcam, MA, USA), were used to detect the expression of cell proliferation proteins and anti-Cox-2 (c-9897, Cayman, MI, USA), anti-cytokeratin-19 (Ab53119, Abcam, MA, USA) as a cancer related maker and connexin 26 (138100, Invitrogen, NY, USA), connexin 32 (358900, Invitrogen, NY, USA), and connexin 43 (138300, Invitrogen, NY, USA). Antibodies against calnexin (BD 610523, NJ, USA) as control was purchased from Transduction Laboratories (BD Biosciences, NJ, USA) and used at 1:1,000 dilution. Anti-mouse, anti-rabbit, and anti-goat IgG antisera conjugated with horseradish peroxidase (HRP) were purchased from DAKO (Glostrup, Denmark).

7. Confocal microscopic observation

The cells were washed with cold PBS three times and fixed with 2% paraformaldehyde in PBS for 30 min. Permeabilization was done by treating cells with 0.2% (w/v) Triton X-100 in PBS for 5 min and then blocked with 0.5% BSA in PBS for 1 h. After blocking, the cells were incubated with primary antibodies (connexin 26, 32, and 43; Invitrogen) diluted in BSA-PBS at room temperature for 2 h and incubated in secondary antibodies diluted in BSA-PBS at room temperature for 30 min. After washing with 1×PBS DAPI

stain was done. The cells were observed under a confocal laser scanning microscope (LSM PASCAL, Carl Zeiss, Germany).

8. Silencing of Cx43 with siRNA

Three selected sequences of human Cx43-siRNA (TriFECTa^{TM}kit disRNA Duplex, IDT, CA, USA) which contained negative and positive control and three specific siRNA for connexin 43-1, 2, and 3 (NM-00165; duplex 1, NM-00165; duplex 2, NM-00165; duplex 3) were purchased from IDT. The transfection experiments were performed using using TransIT-TKO^{TM} kit (Mirus, CA, USA) and following the manufacturer's instructions. Briefly, final of 25 nM siRNA was mixed with 10 ul TransIT-TKO for 6well plate containing $2x10^5$ H69 cells for 72h and then treated with NDMA or/and ESP of *C. sinensis* for 72h. Medium and cells (rinsed 2 times with ice-cold PBS) were harvested 3 days later. The efficiency of transfection was checked of gap junction proteins (connexin 26, connexin 32, and connexin 43) by real-time PCR.

9. Real-time PCR

RNA samples from each cell line were column purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription and real-time PCR (RT-PCR) was performed to detect the mRNA expression of Cx26, Cx32, Cx43, and Cox-2 or GAPDH as control using a transcription kit (Applied

Biosystems, CA, USA). The thermal cycling parameters of the reverse transcription were modified according to the Applied Biosystems manual. Hexamer incubation at 25℃ for 10 min and reverse transcription at 42℃ for 30 min was followed by reverse transcriptase inactivation at 95℃ for 5 min. Total 20 ng of cDNA from the previous step was subjected to RT-PCR using specific sets of primers in a total reaction volume of 25 (Applied Biosystems, CA, USA). RT-PCR was performed in an optical 96-well plate with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, CA, USA), using Taqman probe detection chemistry. The running protocol was as follows: initial denaturation stage at 95℃ for 10 min, 40 cycles of amplification at 95℃ for 15 s and 60℃ for 1 min. After PCR, a dissociation curve was constructed by increasing temperature from 65℃ to 95℃ for detection of PCR product specificity. A cycle threshold (Ct) value was recorded for each sample.

10. Statistics analysis

Statistical significance was analyzed by the Student's *t*-test. A *P* value of less than 0.05 was considered to be statistically significant. And data were represented by the mean ± standard deviation.

RESULTS

1. ESP and NDMA synergistically affect the proliferation of H69

To investigate the role of NDMA and ESP in the proliferation of H69 cells cell viability assay was applied. Cellular proliferation in H69 cells treated with NDMA or/and ESP of *C. sinensis* was increased compared to that in the controls. Although each compound increased proliferation when used alone, co-stimulation with NDMA or/and ESP of *C. sinensis* had the greatest effect on cell growth. The average increase compared to the control was: NDMA, 112%; ESP, 120%; and NDMA+ESP, 146% (P=0.0002). NDMA+ESP synergistically affected cellular proliferation (Fig. 1).

2. Cell cycle distribution upon NDMA and/or ESP treatment in H69 cells

Cellular proliferation is tightly connected with cell cycle progression; thus, we monitored cell cycle progression using PI staining (Fig. 2). In H69 cells cells treated with NDMA, ESP, or NDMA + ESP for 72 h, the number of cells in the G0/G1 phase decreased. In contrast, the number of cells in the G2/M phase, which included cells undergoing rapid proliferation, was increased compared to the control (Fig. 2). Fewer S-phase cells were identified in the ESP and NDMA + ESP groups. The percentage of G2/M-phase cells in each group was: control, 17%; NDMA, 20%; ESP, 26%; NDMA and ESP, 37%

(P=0.007).

3. Gap junction proteins in H69 cells

Expression of connexin 26 and connexin 43 was increased in NDMA+ESP treated cell group. The expression of Cx26 and Cx43 was analyzed by immunoblotting. Under confocal microscopy, the intracellular concentration of Cx 26 and Cx 43 in each group was observed. In Western blotting, Cx26 and Cx43 showed increased expression in Group 3 and Group 4 cell line (Fig. 3). The expression of Cx32 was markedly decreased in group 4. Under confocal immunofluorescence microscopy, intracellular concentration of Cx32 and Cx 43 was up-regulated in NDMA+ESP (Fig. 5-7).

4. Modulation of inflammation related or transformation proteins by the addition of NDMA and ESP in H69 cells

I used immunoblotting to detect the regulation of cell-cycle-related proteins in each group with calnexin as a loading control. The expression of several proteins, including E2F1, Ki67, Cy-19, and Cox-2 was upregulated, especially in the NDMA + ESP group (Fig. 4).

5. Down-regulation of Cx43 by Cx43-specific small interfering RNA reduced the expression of Cx26 and Cox-2 in H69 cells

To evaluate the expression of the other gap junction proteins and Cox-2 by Cx43 down-regulation, H69 cells were harvested after treated with NDMA or/and ESP of *C. sinensis* for 72 hours. The Cx43siRNA reduction was more than 70%, compared with liposome-only control (Fig. 8). Real-time PCR showed that Cx43 silencing was markedly resulted in down-regulation of Cx26 and Cox-2 genes (Fig. 9 and Fig. 11 respectively). The level of Cx32, however had no significant changes (Fig. 10).

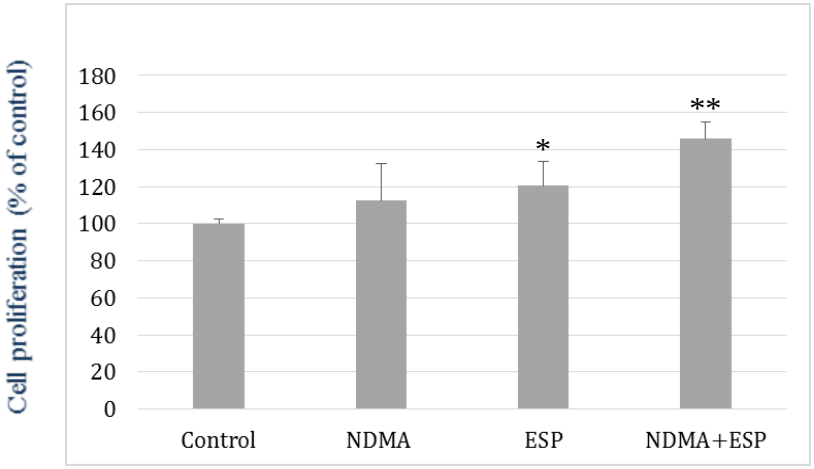


Fig. 1. Effect of NDMA and ESP of *C. sinensis* on cell proliferation of human cholangiocyte cell line (H69 cells). Cells were plated in 96-well plates (5×10^3 cells/well). Cellular proliferation in each treatment was determined using the PrestoBlue cell viability assay. Bars and whiskers indicate cell growth as the percentage of the control ± standard deviation (n = 4). **P* < 0.05 and ***P* < 0.01 versus Control

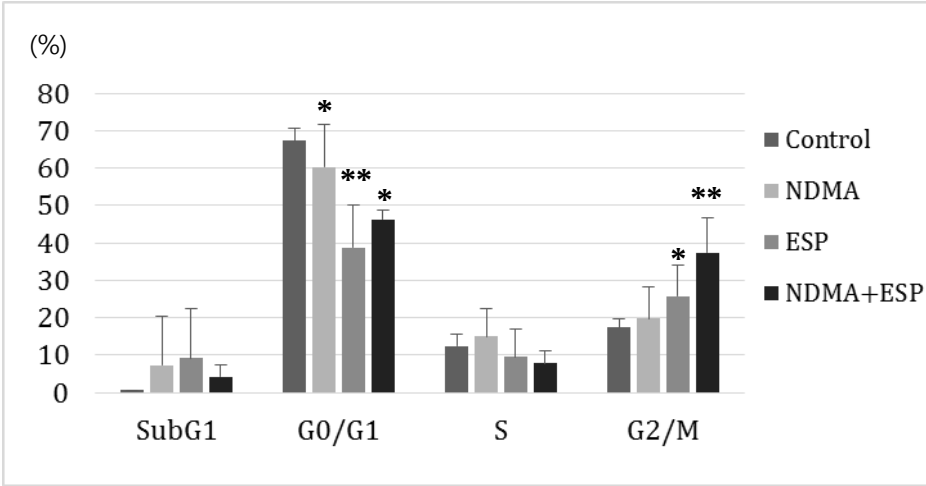


Fig. 2. Effects of the excretory/secretory products of *C. sinensis* (ESP) and NDMA on the cell numbers by cell cycle phases in of human cholangiocyte cell line (H69 cells). After H69 cells were treated with NDMA and/ or ESP for 72h and PI staining was performed to determine the percentages of cells in each phase. **P* < 0.05 and ***P* < 0.01 versus Control

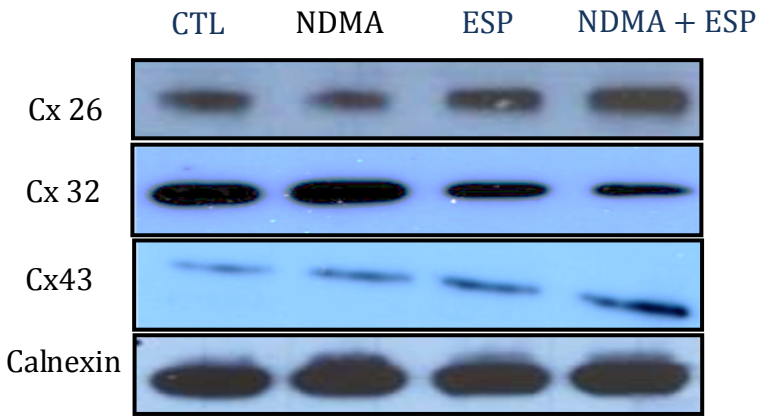


Fig. 3. Expression of gap-junction proteins, connexin 26, connexin 32, and 43 proteins in H69 cells after treatment with NDMA and/ or ESP by western blotting. H69 cells were incubated with either PBS (vehicle) or NDMA and/ or ESP for 72h, the cells were collected for protein extraction.

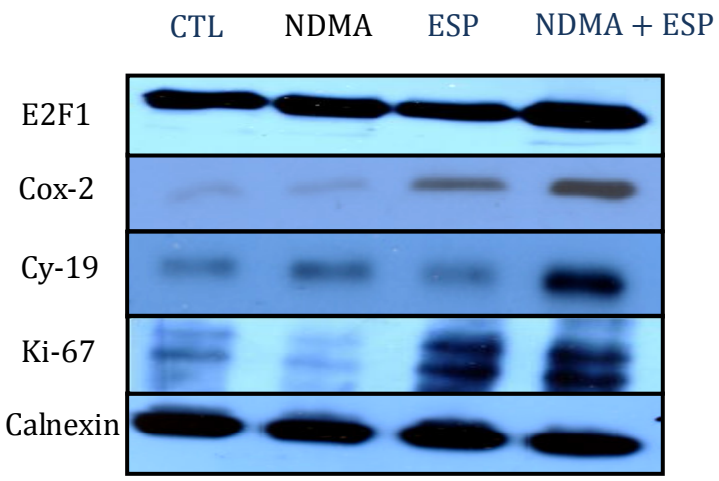


Fig. 4. Expression of inflammation related or transforming proteins in H69 cells after treatment with NDMA and/ or ESP by western blotting. H69 cells were incubated with either PBS (vehicle) or NDMA and/ or ESP for 72 h, the cells were collected for protein extraction.

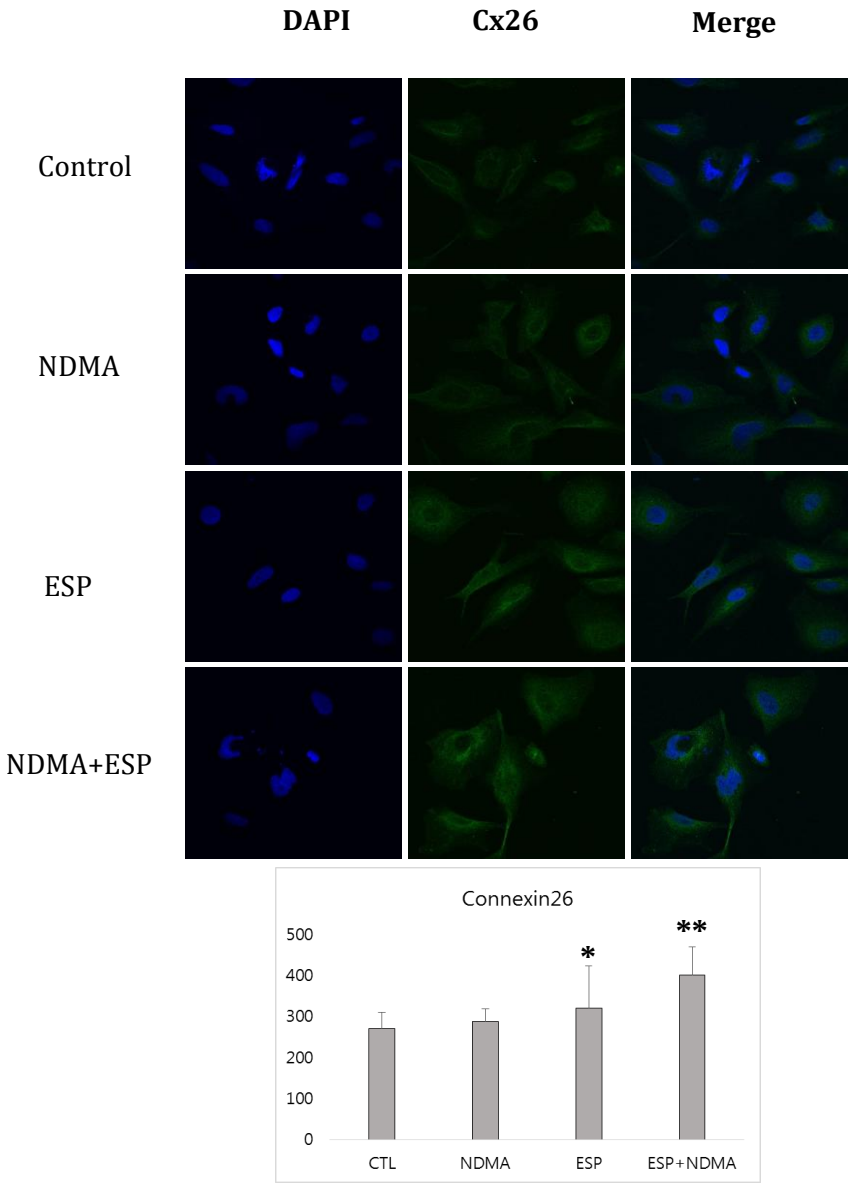


Fig. 5. Concentration of intracellular connexin 26 in human cholangiocyte cell line (H69 cells). After H69 cells were treated with NDMA and/or ESP for 72h measured by laser scanning microscopy (X 4,000). *$P < 0.05$ and **$P < 0.01$ versus Control

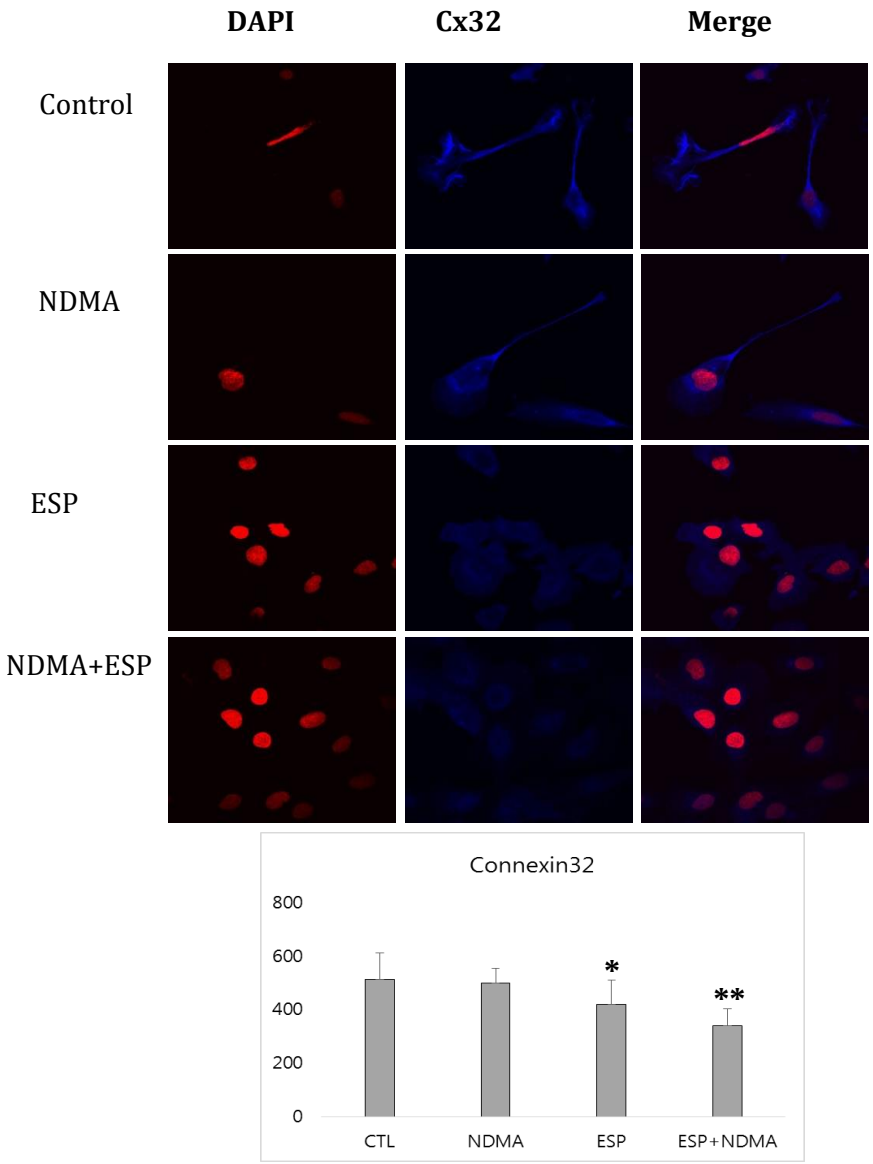


Fig. 6. Concentration of intracellular connexin 32 in human cholangiocyte cell line (H69 cells). After H69 cells were treated with NDMA and/or ESP for 72h measured by laser scanning microscopy (X 4,000). *$P < 0.05$ and **$P < 0.01$ versus Control

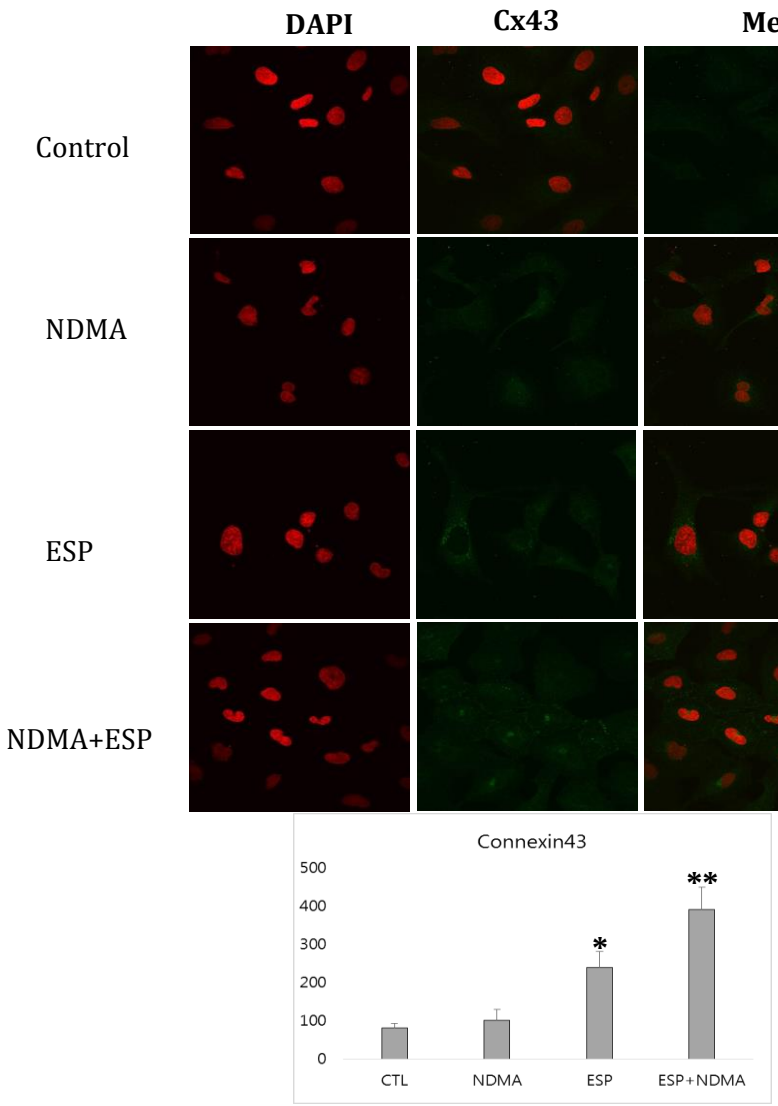
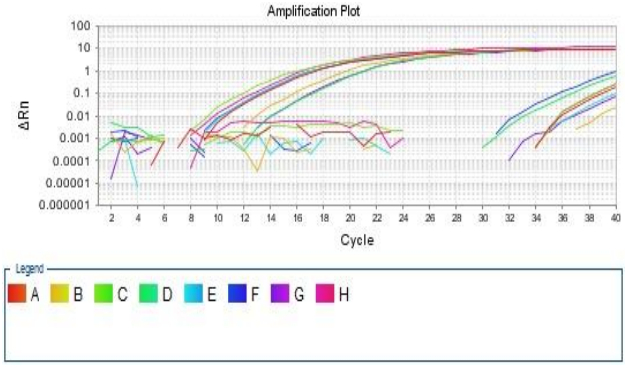


Fig. 7. Concentration of intracellular connexin 43 in human cholangiocyte cell line (H69 cells). After H69 cells were treated with NDMA and/or ESP for 72h measured by laser scanning microscopy (X 4,000). *$P < 0.05$ and **$P < 0.01$ versus Control

A



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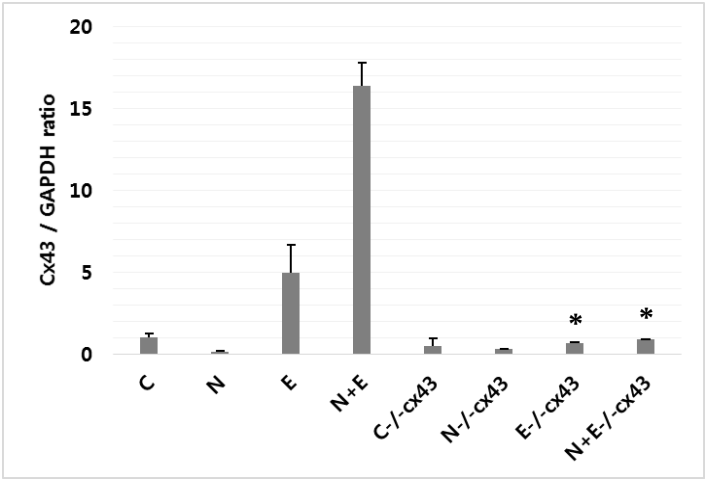
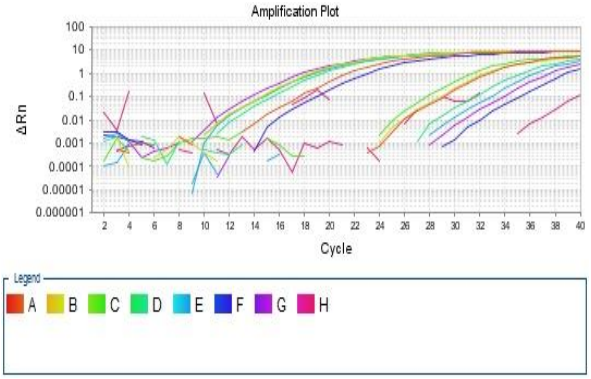


Fig. 8. Amplification curves of real-time PCR for detection for Cx43 gene. (A) Uptake of Cx43siRNA reduces Cx43 expression, as confirmed using real-time PCR. (B) The ratio of Cx43/GAPDH in H69 cells transfected with Cx43-specific siRNA. Analysis showed that the expression of Cx43 was remarkably reduced in transfected H69 cells transfected with Cx43-specific siRNA. *P < 0.001 versus control siRNA

A



B

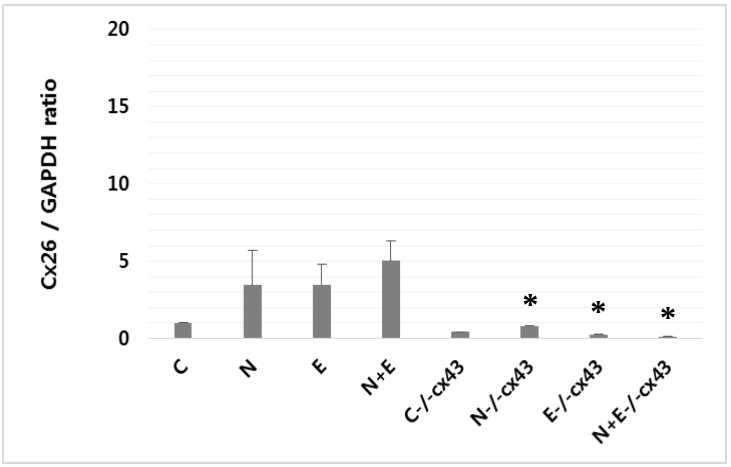
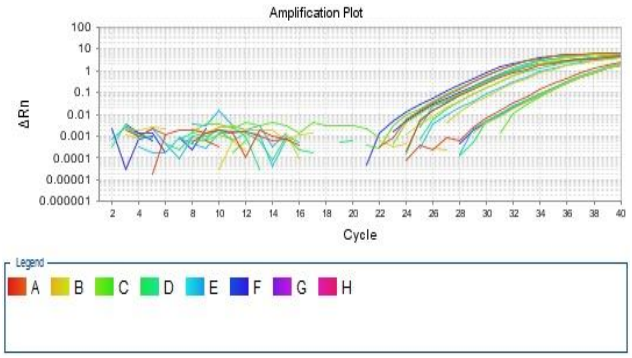


Fig. 9. Amplification curves of real-time PCR for detection for Cx26 gene. (A) Uptake of Cx43siRNA reduces Cx26 expression, as confirmed using real-time PCR. (B) The ratio of Cx26/GAPDH in H69 cells transfected with Cx43-specific siRNA. Analysis showed that the expression of Cx26 is remarkably reduced in transfected H69 cells transfected with Cx43-specific siRNA. *$P < 0.001$ versus control siRNA

A



B

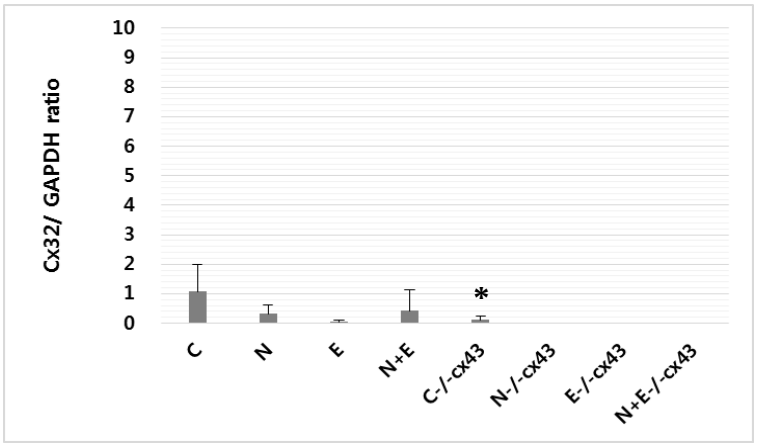
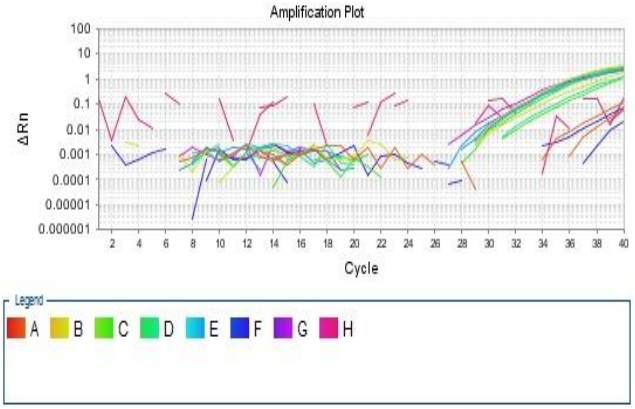


Fig. 10. Amplification curves of real-time PCR for detection for Cx32 gene. (A) Uptake of Cx43siRNA did not change Cx32 expression, as confirmed using real-time PCR. (B) The ratio of Cx32/GAPDH in H69 cells transfected with Cx43-specific siRNA. Analysis showed that the expression of Cx32 no meaningful change in transfected H69 cells transfected with Cx43-specific siRNA. *$P < 0.001$ versus control siRNA

A



B

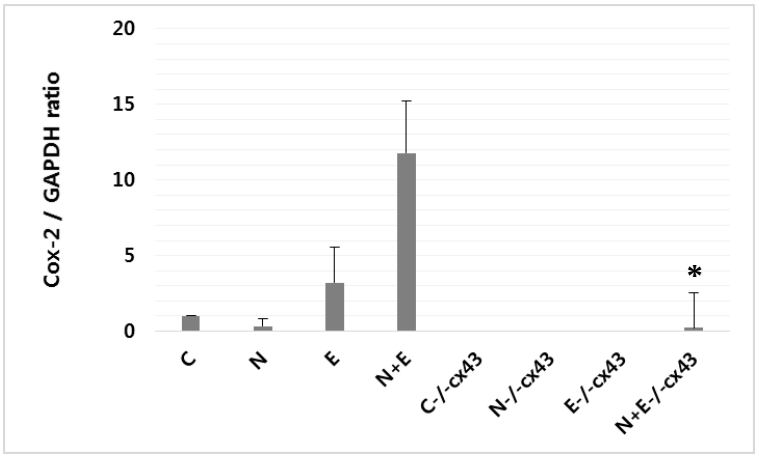


Fig. 11. Amplification curves of real-time PCR for detection for Cox-2 gene. (A) Uptake of Cx43siRNA reduces Cox-2 expression, as confirmed using real-time PCR. (B) The ratio of Cox-2 /GAPDH in H69 cells transfected with Cx43-specific siRNA. Analysis showed that the expression of Cox-2 is remarkably reduced in transfected H69 cells transfected with Cx43-specific siRNA. *$P < 0.001$ versus control siRNA

Table 1. Full spectrum of connexins expressed in rodent and human liver

Connexins	Localisation	References
Cx26	HP, KC, SC, SEC	Nicholson et al. (1987)
		Zhang and Nicholson (1989)
Cx31.9/Cx30.2	NS	Belluardo et al. (2001)
		Nielsen and Kumar (2003)
Cx32	HP, BEC, SEC	Bode et al. (2002)
		Kumar and Gilula (1986)
		Nicholson et al. (1987)
		Paul (1986)
Cx37	AEC, PEC	Chaytor et al. (2001)
		Saito et al. (2000)
		Shiojiri et al. (2006)
		Willecke et al. (1991)
Cx39	NS	Cicirata et al. (2004)
Cx40	AEC, PEC	Chaytor et al. (2001)
		Shiojiri et al. (2006)
Cx43	AEC, BEC, GC, KC, PEC, SC, SEC	Berthoud et al. (1992)
		Bode et al. (2002)
		Chaytor et al. (2001)
		Shiojiri et al. (2006)

AEC, hepatic artery endothelial cell; BEC, biliary epithelial cell; GC, Glisson's capsule; HP, hepatocyte; KC, Kupffer cell; NS, not specified; PEC, portal vein endothelial cell; SC, stellate cell; SEC, sinusoidal endothelial cell.

Table 2. Oligonucleotide sequences for connexin 43 specific SiRNA

Oligonucleotide name	Oligonucleotide sequences
Connexin 43 Duplex Seqeunces	
	5'-AGCGUUUGCUAUGACCAAUUCUUCC-3' **3'-UGUCGCAAACGAUACUGGUUAAGAAGG-5'**
Endogenous Gene Positive Control	
	5'-GCCAGACUUUGUUGGAUUUGAAATT **3'-AAUUUCAAAUCCAACAAAGUCUGGCUU-5'**
Negative Cotnrol	
	5'-CGUUAAUCGCGUAUAAUACGCGUAT-3' **3'-AUACGCGUAUUAUACGCGAUUAACGAC-5'**

Table 3. Oligonucleotide primers and detection probe for real-time PCR for the detection of connexin 26, connexin 32, connexin 43, and GAPDH as control

Oligonucleotide name	Oligonucleotide sequences
Connexin 26	
GJB2 F	**CCC CTA AAG CCT CAA AAC AAA G**
GJB2 R	**GAA ACA AAT GCC GAT ATC CTC TG**
GJB2 probe	**56-FAM/CCT TAC ACC /ZEN/AAT AAC CCC TAA CAG CCT /3IABkFQ**
Connexin 32	
GJB1 F	**GCA CAG ACA TGA GAC CAT AGG**
GJB1 R	**CAA ACC TGT CCA GTT CAT CCT**
GJB1 probe	**56-FAM/CCT ATC CCT /ZEN/GAG GCC ACC CAG /3IABkFQ**
Connexin 43	
GJA1 F	**ACT TGG CGT GAC TTC ACT AC**
GJA1 R	**AGC AGT TGA GTA GGC TTG AAC**
GJA1 probe	**56-FAM/AGG CAA CAT /ZEN/GGG TGA CTG GAG C/3IABkFQ**
Cox-2	
PTGS2 F	**ACT TGG CGT GAC TTC ACT AC**
PTGS2 R	**AGC AGT TGA GTA GGC TTG AAC**
PTGS2 probe	**/56-FAM/AGG CAA CAT /ZEN/GGG TGA CTG GAG C/3IABkFQ/**
GAPDH	
GAPDH F	**ACA TCG CTC AGA CAC CAT G**
GAPDH R	**TGT AGT TGA GGT CAA TGA AGG G**
GAPDH probe	**5HEX/AAG GTC GGA /ZEN/GTC AAC GGA TTT GGT C/3IABkFQ**

DISCUSSION

The present study demonstrated for the first time that NDMA or/and ESP of *C. sinensis* potently increased Cx43 expression in a human cholangiocyte line, H69. In addition, despite ESP or/and NDMA stimulation, Cx43 knockdown induced inhibition of Cox-2 in H69. Pinlaor et al. (2005) reported that *O. viverrini* somatic extracts can induce up-regulation of TLR2 in Raw 264.7 macrophage cells and trigged NFκB signalling, stimulate inducible nitric oxide synthase (iNOS) and Cox-2 expression. The induction of iNOS under inflammatory situations was implicated of NO in the up-regulation Cx43 (Li et al., 2011). Therefore, an involvement of iNOS in elevating Cx43 expression under inflammatory situation is suspected in this study either. This should be demonstrated further.

In the present study, ESP of *C. sinensis* and NDMA have a synergistic effect on the proliferation of human cholangiocytes (Fig. 1). In addition to the cell proliferation and switched cell division cycles, expression of gap-junction proteins Cx43 and Cx26 were increased in H69 cells by NDMA or/and ESP (Fig. 3, Fig. 5, Fig. 7). Most normal cells have functional GJIC, whereas most cancer cells have dysfunctional GJIC (Yamasaki and Naus, 1996). Zhang et al. (2007) demonstrated that Cx43 expression promoted hepatocellular carcinoma via cell to cell communication. Fujimoto et al. (2005) showed that Cx32 had a suppressive effect in a metastatic renal cell carcinoma cell line. However, there is no study on the influence of cell communication through gap

junctions regarding to cholangiocarcinoma.

ESP of *C. sinensis* induced proliferation of cells by increased expression of E2F1, Ck19, and Ki67 when NDMA costimulated the cells. The increased proliferation of cells and increased proportion of G2/M phase cells were same as previously reported in HEK 293T cells (Kim et al., 2008 a). Kim et al. (2008 b) observed that treatment with ESP increased the proliferation of the cells compared with the control level and was more effective than treatment with NDMA, and indicated that NDMA and ESP synergistically affected the regulation of cell cycle-related proteins of HEK 293T cells. When H69 cells were co-stimulated with NDMA and ESP, the degree of proliferation was increased more (Fig. 1).

I also investigated the cell cycle distribution of the cells (Fig. 2). Treatment with NDMA+ESP maximized the proportion of G2/M-phase cells, implying that NDMA and ESP synergistically affect cell cycle progression (Fig. 2). I, therefore, analyzed the expression of a number of cell proliferation and inflammation related proteins (Fig. 4), including E2F1, Ki67, and Ck19, which is an essential regulator of the G2/M transition (Sowersss, 2003). Furthermore, E2F1 may induce cell cycle progression, resulting in cellular proliferation, by acting as a transcription factor. An increase in Ki67 positive biliary epithelial cells was seen in primary biliary cirrhosis (PBC), is characterized by progressive, immune-mediated destruction of bile ducts (< 75 micron diameter) and secondary changes related to cholestasis and

indicating cell cycle activity (Tan et al., 2012). C-Met is involved in early events of carcinogenesis, and Ki67 is involved during the formation of invasive carcinoma (Sowersss, 2003). Biliary epithelial cells retain Ck7, 17, and 19 after neoplastic transformation in almost all cases (Pua et al., 2009). Increased expression of E2F1 was repeatedly confirmed as a stimulatory molecule of cell proliferation in this study. Two other molecules of cytokeratin, Ck19, and a transformation protein, Ki67, were also noticed stimulating and modifying the proliferation of H69 cells.

I also investigated prostaglandin (Cox-2) as major inducer of inflammation. Cox-2 was over-expressed when stimulated with NDMA and ESP. The over-expression of Cox-2 has been observed in various inflammatory diseases and bile duct carcinoma cells, manly in the cytoplasm (Nobuyasu et al., 2001). Importantly, bile duct epithelial cells in primary sclerosing cholangitis showed very strong expression of Cox-2 protein that is comparable with carcinoma cells. On the other hand, primary biliary cirrhosis epithelial cells showed moderate levels of Cox-2 expression (Nobuyasu et al., 2001). In this context, the over expression of Ck19, Ki67, and Cox-2 in H69 cells may transform the H69 cells which are stimulated by NDMA and ESP of *C. sinensis*.

In the present study, the expression of Cx43 and Cx26 were increased in H69 cells by stimulating with NDMA and ESP of *C. sinensis*. On the other hand, Cx32, which has a suppressive effect in a metastatic renal cell carcinoma cell line, was significantly decreased. Increased expression of hepatic Cx43 was

noted in cirrhosis and acute-on-chronic liver failure mouse model by LPS, which was related to the severity of inflammation. This increased Cx43 expression was likely to be an adaptive protective response to the liver to allow better cell-to-cell communication (Balasubramaniyan et al., 2013). The expression of Cx26 and Cx32, major connexins in the liver, was known to be extremely low in several hepatocellular carcinoma cell lines (HSU), but Cx43, a minor connexin in the liver, was highly expressed in metastatic cancer (Vinken et al., 2012). Connexin proteins were recognized to be involved in cell modification by NDMA and ESP of *C. sinensis* stimulation.

The Cx43 knock down using siRNA significantly suppressed expression of Cx26 and Cox-2 in H69 cells when they were stimulated with NDMA and ESP. Indeed, early hepatic progenitor cells are found to switch from Cx43 to Cx26 production, but especially to a Cx32 modus, upon differentiation into hepatocytes (Neveu et al., 1995; Naves et al., 2001; Paku et al., 2004). In general, there are several known connexins in cells according to the intracellular locations (Table 1) (Vinken et al., 2012). Intercellular communication through the gap junction is inhibited by increased expression of Cox-2 as frequently observed in several forms of human malignancies (Lee et al., 2004). Recently, several reports have suggested that the carcinogenic mechanisms of hydrogen peroxide, TPA, and quinones may be involved in the inhibition of GJIC through Cx43 phosphorylation via ERK1/2 activation in rat liver epithelial cells (Surh et al., 2002; Lee et al., 2004). Furthermore, the

increased expression of Cx43 is positively correlated with NFκB activation in human muscular arteries of patients undergoing coronary artery bypass graft surgery (Li et al., 2009). NFκB plays a central role in general inflammatory as well as immune responses. The 5'-flanking region of the Cox-2 promotor contains NFκB binding site (Lee et al., 2004). In agreement with this notion, NFκB has been shown to be a critical regulator of Cox-2 expression in many cell lines (Surh et al., 2002). These findings, taken together, suggested that Cx43 expression induced over-expression of Cox-2 through the NFκB activation. In conclusion, it is suggested that the connexin 43 plays a key role in regulation of inflammatory responses, which may cause cholangicarcinoma under stimulation by ESP of *C. sinensis* and NDMA.

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국문 초록

간흡충의 만성 감염시 담관암이 발생할 수 있다. 간흡충에 의한 암발생에 가장 중요한 것은 숙주의 염증반응인데, 이에 대한 기전이 잘 알려져 있지 않다. 이 연구에서는 인접한 세포의 연결통로서 세포 사이의 이온이나 정보를 주고받을 수 있는 연결통로인 세포연접 (Gap junction)이 염증반응을 조절하는지를 사람 담관상피세포주 (H69)에서 관찰하였다. 세포연접 중 Connexin 43 (Cx43)은 염증반응 조절에 관여하여, 특히 암 발생 이나 암세포증식 관련한다. 이와 함께 대표적인 염증반응 유도 물질인 prostaglandin 의 발현 정도를 확인하기 위하여 Cox-2 도 함께 관찰 하였다. 실험군으로 정상 대조군과, 100 ng/ml 의 NDMA 처리한 군, 10 μg/ml 의 간흡충 분비항원 처리한 군, 그리고 NDMA 와 간흡충 분비항원을 동시에 처리한 군으로 나누어 실험하였고, H69 세포는 NDMA 와 분비항원을 72 시간 마다 처리하여 60 일 이상 배양된 것을 이용했다. 간흡충항원을 단독으로 처리하거나 NDMA 와 간흡충 분비항원을 동시에 처리한 H69 세포의 증식이 현저하게 증가하였고 이와 더불어 Cx43 과 Cox-2 의 발현량도 유의하게 증가하였다. 반면 암억제에 관여하는 Cx32 는 감소하는 양상을 볼 수 있었다. Cx43 이 염증반응을

조절한다는 것을 확인하기 위해, Cx43 유전자의 발현을 억제 시켰더니 H69 세포에서 간흡충 분비항원과 NDMA 의 자극에도 Cx26 과 Cox-2 의 발현량이 현저히 억제 되었다.

본 연구 결과는 간흡충분비항원과 극소량의 발암물질에 의해서도 염증반응이 유도 되며, Cx43 가 Cox-2 의 발현을 조절하는 보아서, 담관암을 유도 할 수 있는 염증반응 조절에 Cx43 가 중요한 통로임을 알 수 있다.

주요어: 간흡충, 담관암, 세포연접, N-dimethylnitrosamine, 간흡충 분비항원

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감사의 글

이 논문이 완성되기까지 도와주신 모든 분들께 감사 드립니다. 지도교수님이신 홍성태 교수님께 깊은 감사 드립니다. 많이 부족한 제게 좋은 기회를 많이 주셨고 그 때 마다 믿어 주셨기에 값진 여러 경험을 많이 하였습니다. 그 분에게서 받은 여러 부분의 지도와 편달은 과학자로 이제 걸음마를 시작하는 저에게 크나큰 밑거름이 되었습니다. 졸업이 끝이 아니라 이제부터는 선생님의 제자로서 선생님께서 흐뭇하게 웃으시도록 많은 노력 하겠습니다. 또한 채종일 교수님의 가르침과, 아낌없는 격려 덕분에 제가 형태학의 관심에 끈을 놓지 않았던 것 같습니다. 홍성태, 채종일 교수님의 가르침 덕분에 무언가를 내 눈으로 발견하여 다른 사람에게 도움을 줄 수 있었던 여러 번의 값진 순간들을 평생 잊지 못할 것 입니다. 배영미 교수님은 제가 실험실 생활을 하는 동안 실험을 과학적으로 접근하는 방법을 알게 해 주셨고, 많은 지식과 실험기법을 섭렵하게 도와주셨고, 힘든 시기를 보낼 때 심적으로 많은 위로를 주셨습니다 정말 감사합니다. 심사위원으로 고생해주신 김우호 교수님 최민호 교수님 배영안 교수님께도 감사 드립니다. 제게 많은 위로와 격려를 주셨던 신은희 교수님께도 진심으로 감사 드립니다. 짧은 감사의 글로 모두 표현할 수 없지만 제가 한

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