Connexin 43 plays an important role in the transformation of human cholangiocytes upon stimulation with *Clonochis sinensis* excretory-secretory protein and *N*-nitrosodimethylamine

Short title: Connexin 43 is an important target in human cholangiocarcinoma

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29 **Abstract**

30 **Background**

31 *Clonorchis sinensis* is a group I bio-carcinogen responsible for
32 cholangiocarcinoma (CHCA) in humans. However, the mechanism by which
33 *C. sinensis* promotes carcinogenesis is unclear.

34 **Methodology**

35 Using the human cholangiocyte line H69, we investigated cell proliferation
36 and gap junction protein expression after stimulation with the hepatotoxin *N*-
37 nitrosodimethylamine (NDMA) and/or excretory-secretory products (ESP) of
38 *C. sinensis*, which induce inflammation. NDMA and ESP treatment increased
39 proliferation by 146% and the proportion of cells in the G2/M phase by 37%.
40 Moreover, the expression of the cell cycle protein E2F1 and the cell
41 proliferation-related proteins Ki-67 and cytokeratin 19 increased in response
42 to combined treatment with NDMA and ESP. The gap-junction proteins
43 connexin (Cx) 43 and Cx26 also increased. In contrast, Cx32 expression
44 decreased in cells treated with NDMA and ESP. Cox-2 was also upregulated.
45 Silencing of Cx43 reduced cell proliferation and significantly suppressed
46 Cx26 and Cox-2 expression.

47 **Conclusions**

48 These results suggest that Cx43 is an important factor in CHCA induced by *C.*
49 *sinensis* ESP and NDMA and further investigations targeting this pathway
50 may allow prevention of this deadly disease.

51 **Author summary**

52 *Clonorchis sinensis*, a human fluke, resides in the liver of humans and is
 53 commonly found in the common bile duct and gall bladder. This parasite is
 54 the main cause of cholangiocarcinoma, also called bile duct cancer, in
 55 humans. Of note, the excretory-secretory products (ESP) of *C. sinensis* are
 56 known to cause inflammation in the biliary epithelium, which may ultimately
 57 result in neoplasms via production of reactive oxygen species and subsequent
 58 DNA damage. Together with *N*-nitrosodimethylamine (NDMA), a potent
 59 hepatotoxin that can cause fibrosis and tumors in the liver, ESP led to an
 60 increase in the growth and proliferation of cholangiocytes. Our results showed
 61 that the ESPs of *C. sinensis* induced pro-inflammatory responses by
 62 increasing the levels of proinflammatory cytokines and nuclear factor kappa B
 63 (NFκB), which in turn, enhanced the production of connexin 43 (Cx43), a
 64 gap-junction protein. Therefore, Cx 43 can serve as a potential target for
 65 developing a therapeutic strategy for the treatment of cholangiocarcinoma in
 66 humans.

68 **Introduction**

69 *Clonorchis sinensis* is a human liver fluke that induces cholangiocarcinoma
 70 (CHCA) in humans [1]. Clonorchiasis has been endemic in Asia for a long
 71 time, especially among residents who live along rivers and consume raw

72 freshwater fish [2].

73 The mechanism by which *C. sinensis* induces CHCA is not well
74 understood, but chronic hepatobiliary damage, a precursor to CHCA, in
75 clonorchiasis is a multi-factorial outcome of the mechanical and biochemical
76 irritation of the biliary epithelium by flukes via their suckers, metabolites, and
77 excretory-secretory products (ESP) [3]. Local inflammation and the systemic
78 immune response in the host [4-7] produce reactive oxygen species and
79 reactive nitrogen compounds, which may cause DNA damage, leading to
80 neoplasms [8, 9].

81 *N*-nitrosodimethylamine (NDMA) is a potent hepatotoxin that can cause
82 fibrosis and tumors in the liver of rats via the activation of CYP450 enzymes
83 [10] and hamsters infected with *C. sinensis* are at a greater risk of developing
84 NDMA-induced or inflammation-mediated CHCA than uninfected hamsters
85 [11, 12]. Previously, we reported that exposure to NDMA and the ESP of *C.*
86 *sinensis* increases HEK293T cell proliferation and the proportion of cells in
87 the G2/M phase [3].

88 CHCA is potentially caused by increased levels of proinflammatory
89 cytokines and nuclear factor kappa B (NFκB), which regulate the activities of
90 cyclooxygenase-2 (Cox-2) and inducible nitric oxide synthase, and disturb the
91 homeostasis of oxidants/anti-oxidants and DNA repair enzymes [13]. ESPs of
92 *C. sinensis* induce pro-inflammatory responses *in vitro* by the upregulation of
93 TLR4 and its downstream transduction signals, including MyD88-dependent

94 I κ B- α degradation and NF κ B activation [14, 15]. NF κ B may also influence
95 the production of Cx43, a gap-junction protein, in liver cirrhosis [16, 17].

96 Gap junctions are clusters of transmembrane channels on the cell
97 membrane that permit direct intercellular exchange of ions, secondary
98 messengers, and small signaling molecules influencing cell growth,
99 differentiation, and cancerous changes [17-21]. Among gap junction proteins,
100 Cx43 is involved in almost all steps of the inflammatory response of cells,
101 cytokine production, and inflammatory cell migration [17, 20, 22]. The
102 substantial role of Cx43 in carcinogenesis is highlighted by the fact that high
103 levels of Cx-43 expression increase the invasion of breast tumor cells and
104 promote tumors in melanoma [22].

105 Alterations of Cx expression have been reported in cancer [21, 23]. In
106 hepatocellular carcinoma (HCC), for instance, reduced Cx32 expression is
107 accompanied by increased expression of Cx43, which promotes HCC via cell-
108 to-cell communication [16, 20, 23]. Fujimoto et al. [23] have shown that Cx32
109 has a suppressive effect in metastatic renal cell carcinoma. However, the role
110 of connexins in cancer is still controversial [23], and the influence of gap
111 junctions in CHCA caused by *C. sinensis* has not yet been examined.

112 In this study, to determine the mechanisms underlying the carcinogenic
113 effects of ESP from *C. sinensis*, we investigated changes in cell proliferation,
114 proinflammatory molecules, and connexin production in cholangiocytes (H69
115 cell line) exposed to ESP from *C. sinensis* and the carcinogen NDMA. We

found that the silencing of Cx43 reduced ESP- and NDMA-induced cell proliferation and the expression of Cox-2.

Methods

Preparation of ESP

Animals

The animal experimental protocol was approved and reviewed by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Health System, Seoul, Korea (approval no. SNU-060511-1) and followed the National Institutes of Health (NIH) guideline for the care and use of laboratory animals (NIH publication no. 85–23, 1985, revised 1996). The facility is accredited by the Ministry of Food and Drug Administration and by the Ministry of Education, Science and Technology (LNL08-402) as an animal experiment facility. Male Sprague–Dawley rats at 6 weeks of age were purchased from Koatech Co. (Seoul, Korea) and housed in an ABL-2 animal facility at Seoul National University College of Medicine. All rats were bred in filter cages under positive pressure according to institutionally approved guidelines.

Recovery of metacercariae of *C. sinensis*

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

Infection of experimental animals with *C. sinensis* 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% CO₂ at 37°C. 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). The concentration of endotoxin (LPS) was

measured using an LAL QCL-1000 Kit (LONZA, Switzerland), and as a result, the LPS contained in 10 µg/mL of ESP was measured to be less than 0.001 (EU). Therefore, there is no effect of LPS on the results of this paper.

Cell culture and experimental design

Cell culture

SV40-transformed human cholangiocytes (H69) from Dr. Dae-Gon Kim of Chonbuk National University for providing were divided into four treatment conditions and cultured for more than 180 days; the medium was replaced every 72 h. The cells were then treated as follows: 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 NDMA and 100 ng/mL ESP. H69 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) and DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 2 mM L-glutamine, 100 µg/mL penicillin, 0.243 mg/mL adenine (Sigma, St. Louis, MO, USA; A68626), 5 µg/mL insulin (Sigma; I6634), 10 µg/mL epinephrine (Sigma; E4250), 5 µg/mL Triiodonine-transferrin (Sigma; T8158), 30 ng/mL epidermal growth factor (R&D Systems, Minneapolis, MN, USA; 236-EG), 1.1 µM

hydrocortisone, and 100 U/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

Cell proliferation assay

The PrestoBlue cell viability reagent was utilized to evaluate cell proliferation. For each assay, cells were seeded at a density of 5×10^3 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 NDMA with or without 10 µg/mL ESP for another 72 h. The PrestoBlue cell viability reagent (1 mg/mL) was dissolved in warm medium, and 1.25 mM phenazine methosulfate (PMS) was prepared in PBS. Following incubation 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 reagent was quantified by measuring the absorbance at 570 and 600 nm using a microtiter plate reader.

Cell cycle analysis

For the 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, Franklin lakes, NJ, USA), and data were analyzed using CellQuest software (Becton-Dickinson).

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 μ M Na₃VO₄, 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, Billerica, MA, USA). The membranes were then probed with antibodies against E2F1, Ki-67, Ck19, 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, North Massapequa, NY, USA). The western blotting results were obtained by a densitometric analysis using ImageJ (NIH, Bethesda, MD, USA).

Antibodies

Polyclonal or monoclonal antibodies were used to detect the expression of cell-cycle-related proteins, including anti-E2F1 (sc-193; Santa Cruz

Biotechnology, Santa Cruz, CA, USA) and anti-Ki-67 (SP6; Abcam, Cambridge, MA, USA). The other antibodies included anti-Cox-2 (c-9897; Cayman Chemicals, Ann Arbor, MI, USA) and anti-cytokeratin-19 (Ab53119; Abcam) as cancer-related makers, and anti-connexin 26 (138100; Invitrogen, Carlsbad, CA, USA), anti-connexin 32 (358900; Invitrogen), and anti-connexin 43 (138300; Invitrogen). An antibody against calnexin (BD 610523) used as a control was purchased from Transduction Laboratories (BD Biosciences, San Jose, CA, USA) and used at a 1:1,000 dilution. Anti-mouse, anti-rabbit, and anti-goat IgG antisera conjugated with horseradish peroxidase (HRP) were purchased from DAKO (Glostrup, Denmark).

Confocal microscopy

Cells were washed with cold PBS three times and fixed with 2% paraformaldehyde in PBS for 30 min. Permeabilization was performed by treating the cells with 0.2% (w/v) Triton X-100 in PBS for 5 min and then blocking with 0.5% BSA in PBS for 1 h. After blocking, the cells were incubated with primary antibodies against connexin 26, 32, and 43 (Invitrogen) diluted in BSA-PBS at 25°C for 2 h and then incubated in secondary antibodies diluted in BSA-PBS at room temperature for 30 min. After washing with 1× PBS, the cells were stained with DAPI and observed under a confocal laser scanning microscope (LSM PASCAL; Carl Zeiss, Jena, Germany).

241

242 **Cx43 silencing with siRNA**

243 Three selected human Cx43-siRNAs (TriFECTa Kit disRNA Duplex,
244 IDT, San Jose, CA, USA), with negative and positive controls, and specific
245 siRNA targeting connexin 43 (NM-00165) (Table 1) were purchased from
246 IDT. The transfection experiments were performed using the TransIT-TKO
247 Kit (Mirus, Madison, WI, USA) following the manufacturer's instructions.
248 Briefly, a 25 nM siRNA solution was mixed with 10 μ L of TransIT-TKO and
249 added to the wells of a 6-well plate containing 2×10^5 H69 cells for 72 h.
250 Cells were then treated with NDMA, ESP of *C. sinensis*, or the combination
251 for 72 h. Medium and cells (rinsed 2 times with ice-cold PBS) were harvested
252 3 days later. The efficiency of transfection was assessed by measuring the
253 expression of gap junction proteins (connexin 26, connexin 32, and connexin
254 43) by real-time PCR.

255 Table 1. Connexin43-specific siRNA oligonucleotide sequences

Oligonucleotide name	Oligonucleotide sequences
Connexin 43 Duplex	5'-AGCGUUUGCUAUGACCAAUUCUUC-3'
Sequences	3'-UGUCGCAAACGAUACUGGUUAAGAAGG-5'
Endogenous Gene	5'-GCCAGACUUUGUUGGAUUUGAAATT-3'
Positive Control	3'-AAUUUCAAUCCAACAAAGUCUGGCUU-5'
Negative Control	5'-CGUUAUUCGCGUAUAAUACGCGUAT-3'

3'-AUACGCGUAUUAUACGCGAUUAACGAC-5'

256

257 Real-time PCR

258 RNA samples from each cell line were column-purified using the
 259 RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription and real-
 260 time PCR (RT-PCR) were performed to determine the mRNA expression
 261 levels of *Cx26*, *Cx32*, *Cx43*, and *Cox-2*, using *GAPDH* as a control (Applied
 262 Biosystems, Santa Clara, CA, USA). The thermal cycling parameters for
 263 reverse transcription were modified according to the Applied Biosystems
 264 manual. Hexamer incubation at 25°C for 10 min and reverse transcription at
 265 42°C for 30 min was followed by reverse transcriptase inactivation at 95°C
 266 for 5 min. cDNA (20 ng) from the previous step was subjected to RT-PCR
 267 using specific sets of primers (Table 2 in a total reaction volume of 25 µL
 268 (Applied Biosystems). RT-PCR was performed in an optical 96-well plate
 269 using an ABI PRISM 7900 HT Sequence Detection System (Applied
 270 Biosystems) and TaqMan probe detection chemistry. The running protocol
 271 was as follows: initial denaturation at 95°C for 10 min, and 40 cycles of
 272 amplification at 95°C for 15 s and 60°C for 1 min. After PCR, a dissociation
 273 curve was constructed by increasing the temperature from 65°C to 95°C to
 274 evaluate the PCR amplification specificity. The cycle threshold (Ct) value was
 275 recorded for each sample.

276 Table 2. Oligonucleotide primers and detection probe for real-time PCR

Oligonucleotide	Oligonucleotide sequences
name	
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

277

278 **Statistical analysis**

279 Statistical significance was analyzed using Student's *t*-tests. Differences were
 280 considered statistically significant at a $*P < 0.05$, $**P < 0.01$ and $***P <$
 281 0.001 versus control. Data are presented as the mean $\pm$ standard error of the
 282 mean (SEM) from at least three independent experiments.

283

284 **Results**

285

286 **ESP and NDMA synergistically increase H69 cell proliferation**

287 The roles of NDMA and ESP in the proliferation of H69 cells, as
 288 determined by cell viability, were investigated. Proliferation was higher in
 289 H69 cells treated with NDMA and ESP of *C. sinensis* than in control cells.
 290 Compared to control cells, the average increase for cells treated with NDMA
 291 was 112%, for cells treated with ESP was 120% ($P < 0.05$), and for cells
 292 treated with NDMA + ESP was 146% ($P < 0.001$). NDMA and ESP had
 293 synergistic effects on cell proliferation (Fig 1A).

294

Fig 1: Effects of NDMA and ESP of *C. sinensis* on cell proliferation and

cell cycle progression in human cholangiocytes (H69 cells). A. Cells were

plated in 96-well plates (5×10^3 cells/well). Cell proliferation for each

treatment was determined using the PrestoBlue cell viability assay. **B.** After

H69 cells were treated with NDMA and ESP for 72 h, PI staining was

performed to determine the percentage of cells in each phase. Data represent

the mean $\pm$ SE of five independent experiments. $*P < 0.05$, $**P < 0.01$ and

$***P < 0.001$ versus control.

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

cells

Cell cycle progression was monitored using propidium iodide (PI)

staining (Fig 1B). In H69 cells treated with NDMA, ESP, or NDMA + ESP

for 72 h, the number of cells in the G0/G1 phase significantly decreased, but

cells in the G2/M phase increased significantly compared to cell counts in the

control group (Fig 1B). Fewer S-phase cells were identified in the ESP- and

NDMA + ESP-treated cells than in control cells. There is no significant

increase in G2/M phase in cells treated with NDMA compared to control

group (Fig 1B). The proportions of G2/M-phase cells in each condition were

as follows: control, 17%; NDMA, 20%; ESP, 26%; NDMA + ESP, 37% ($P =$

0.007).

Upregulation of inflammation- or transformation-related proteins by NDMA and ESP in H69 cells

Immunoblotting was utilized to detect the regulation of cell-cycle-related proteins in each group using calnexin as a loading control. The expression levels of several cell proliferation- and inflammation-related proteins, including E2F1, Ki-67, Cy-19, and Cox-2 (an essential regulator of the G2/M transition), were upregulated, especially in NDMA + ESP-treated cells (Fig 2).

Fig 2: Expression of inflammation- or transformation-related proteins in H69 cells after treatment with NDMA and ESP, assessed by western blotting. H69 cells were incubated with PBS (vehicle), NDMA, ESP, or both for 72 h. The cells were collected for protein extraction. The blots of each groups were run under same experimental conditions and the images were cropped from different parts of the same gels, DATA represent the mean $\pm$ SE of five independent experiments. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ versus control.

Gap junction proteins in H69 cells

The expression levels of Cx26 and Cx43 were high in NDMA + ESP-treated cells, as confirmed by immunoblotting (Fig 3). The intracellular concentrations of Cx26 and Cx43 in each condition were observed under a confocal microscope. Western blotting indicated increases in Cx26 and Cx43

expression in cells treated with ESP and NDMA + ESP (Figs 3). The expression of Cx32 (blue) was markedly lower in cells treated with NDMA + ESP than in other cells. Immunofluorescence confocal microscopy indicated that the intracellular concentrations of Cx26 (green) and Cx43 (green) increased in cells treated with NDMA + ESP (Fig 4).

Fig 3: Expression of the gap-junction proteins connexin 26, connexin 32, and connexin 43 in H69 cells after treatment with NDMA and/or ESP, as determined by western blotting. H69 cells were incubated with either PBS (vehicle) or NDMA and/or ESP for 72 h, and the cells were collected for protein extraction. The blots of each groups were run under same experimental conditions and the images were cropped from different parts of the same gels, DATA represent the mean $\pm$ SE of five independent experiments. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ versus control.

Fig 4: Concentration of intracellular connexin 26, connexin 32, and connexin 43 in human cholangiocytes (H69 cells). After treatment with NDMA and ESP for 72 h, the concentration of intracellular connexin 26 in H69 cells was measured by laser scanning microscopy ($\times 4,000$). Scale bar = 25 μ m. Data represent the mean $\pm$ SE of five independent experiments. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ versus control.

Reduced cell proliferation upon NDMA and ESP treatment in H69 cells by Cx43 silencing compare to silencing negative control.

Downregulation of Cx43 by Cx43-specific small interfering RNA resulted in all groups were not significant, even though NDMA and ESP stimulated cells. The average increases compared to the control were as follows: NDMA, 104.5%; ESP, 105.1%; and NDMA + ESP, 107.6% (Fig 5A).

Fig 5: Effect of connexin 43 silencing in H69 cells. **A.** Reduced cell proliferation upon NDMA and ESP treatment in H69 cells by connexin 43 silencing. **B.** Uptake of Cx43 siRNA reduces Cx43 expression, as confirmed by real-time PCR. Cx43 expression was remarkably reduced in H69 cells transfected with Cx43-specific siRNA. **C.** Ratio of Cx26/GAPDH in H69 cells transfected with Cx43-specific siRNA. Cx26 expression was remarkably reduced in H69 cells transfected with Cx43-specific siRNA. **D.** The ratio of Cox-2/GAPDH in H69 cells transfected with Cx43-specific siRNA. Cox-2 expression was remarkably reduced in H69 cells transfected with Cx43-specific siRNA. Data represent the mean $\pm$ SE of five independent experiments. * $P < 0.001$ versus control siRNA.

Downregulation of Cx43 by Cx43-specific small interfering RNA reduced the expression of Cx26 and Cox-2 in H69 cells

To evaluate the effect of Cx43 downregulation on the expression of other

gap junction proteins and Cox-2, H69 cells were harvested after treatment with NDMA and ESP of *C. sinensis* for 72 h. Transfection with Cx43 siRNA resulted in a reduction in Cx43 expression of greater than 70% when compared with that of the liposome-only control (Fig 5A). Real-time PCR showed that Cx43 silencing resulted in the downregulation of Cx26 and Cox-2 (Figs 5C and 5D, respectively). However, Cx32 expression was not affected (not shown).

Discussion

Our results provided the first evidence for the involvement of gap junction proteins in the pathogenesis of CHCA by *C. sinensis*. NDMA and ESP of *C. sinensis* increased Cx43 expression substantially in H69 human cholangiocytes. In addition, in the presence of ESP and NDMA stimulation, Cx43 knockdown inhibited Cox-2 expression in H69 cells. Yan et al. [15] reported that iNOS is highly expressed in Kupffer cells, sinusoidal endothelial cells, and biliary epithelial cells in BALB/c mice after *C. sinensis* infection. Nitric oxide (NO) formation and nitrosation may contribute to the development of *C. sinensis*-associated carcinogenesis [11, 12, 14, 15]. The induction of iNOS under inflammatory conditions suggests that NO is involved in the upregulation of Cx43 [16, 19]. Therefore, we also expect the involvement of iNOS in the elevation of Cx43 expression under inflammatory conditions in the present *C. sinensis* model. Further studies are warranted to

405 test this hypothesis.

406 Our results indicated that ESP of *C. sinensis* and NDMA had a synergistic
 407 effect on the proliferation of human cholangiocytes (Fig 1). In addition to the
 408 observed increase in cell proliferation and alteration of the cell cycle, the
 409 expression of the gap junction proteins Cx43 and Cx26 increased in H69 cells
 410 treated with NDMA and ESP. Most normal cells have functional gap
 411 junctional intercellular communication, in contrast to the dysfunctional
 412 communication of cancer cells [17-22]. When used in combination with
 413 NDMA, ESP of *C. sinensis* induced cell proliferation and increased the
 414 expression of E2F1, Ck19, and Ki-67. When H69 cells were co-stimulated
 415 with NDMA and ESP, cell proliferation increased. Additionally, as shown in
 416 Fig 1B, treatment with NDMA + ESP maximized the proportion of cells in
 417 the G2/M phase, implying that NDMA and ESP synergistically stimulate cell
 418 cycle progression. We analyzed the expression of a number of cell
 419 proliferation-, cell cycle-, and inflammation-related proteins (Fig 2), including
 420 E2F1, Ki-67, and Cy19 [24-26]. E2F1 is able to induce cell cycle progression,
 421 resulting in cell proliferation [3, 24]. Consistent with these previous findings,
 422 we observed that increased expression of E2F1 stimulated cell proliferation.
 423 C-Met is involved in early events of carcinogenesis, and Ki-67 is involved in
 424 the formation of invasive carcinoma [25-28]. Biliary epithelial cells retain
 425 Cy19 expression after neoplastic transformation in almost all cases [26, 28].
 426 In our study, Cy19 and Ki-67 were upregulated in response to the stimulation

of H69 cell proliferation.

Cyclooxygenase 2 (Cox-2), an enzyme involved in the production of prostaglandins, was over-expressed when cells were stimulated by NDMA and ESP. Cox-2 over-expression has been observed in various inflammatory diseases and in bile duct carcinoma cells, mainly in the cytoplasm [29-30]. Importantly, bile duct epithelial cells in primary sclerosing cholangitis show very strong Cox-2 expression, comparable to that in carcinoma cells. In contrast, epithelial cells in primary biliary cirrhosis show moderate levels of Cox-2 expression [29, 30]. In this context, the over-expression of Cx19, Ki-67, and Cox-2 may result in the transformation of normal H69 cells to cancer-like cells by stimulation with NDMA and ESP of *C. sinensis*. In the present study, Cx43 and Cx26 expression levels were increased in H69 cells upon stimulation with NDMA and ESP of *C. sinensis*. In contrast, Cx32 was significantly downregulated. Increased expression of hepatic Cx43 was noted in cirrhosis and in a mouse model of acute-on-chronic liver failure in response to LPS, and this effect was related to the severity of inflammation [19]. This increased Cx43 expression was likely an adaptive protective response of the liver to enable better cell-to-cell communication [16, 20, 21]. The expression levels of Cx26 and Cx32, major connexins in the liver, are extremely low in several HCC lines, but Cx43, a minor connexin in the liver, is highly expressed in metastatic cancer [17, 20-22]. Cx43 knockdown using siRNA reduced cell proliferation and significantly suppressed the expression of Cx26

449 and Cox-2 in H69 cells stimulated with NDMA and ESP compare to silencing
450 negative control stimulated with NDMA and ESP. The connexin proteins
451 Cx43 and Cx26 are involved in cell modification upon stimulation by NDMA
452 and ESP of *C. sinensis*.

453 In general, cells contain several known connexins, classified according to
454 their intracellular location (Table 3).

455 Table 3. Full spectrum of connexins expressed in rodent and human livers

Connexins	Localization	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)

456 AEC, hepatic artery endothelial cell; BEC, biliary epithelial cell;
457 GC, Glisson's capsule; HP, hepatocyte; KC, Kupffer cell; NS, not specified;
458 PEC, portal vein endothelial cell; SC, stellate cell; SEC, sinusoidal endothelial cell.
459 Intercellular communication via gap junctions is inhibited by increased Cox-
460 2 expression, as is frequently observed in several forms of human
461 malignancies [29, 30]. Recently, several reports have suggested that the
462 carcinogenic mechanisms of hydrogen peroxide, TPA, and quinones may be
463 involved in the inhibition of GJIC by Cx43 phosphorylation via ERK1/2
464 activation in rat liver epithelial cells [13, 17]. Furthermore, increased
465 expression of Cx43 is positively correlated with NFκB activation in muscular
466 arteries of patients undergoing coronary artery bypass graft surgery [31].
467 NFκB plays a central role in general inflammatory and immune responses.
468 The 5'-flanking region of the *Cox-2* promoter contains an NFκB binding site,
469 and NFκB is a critical regulator of Cox-2 expression in many cell lines [13,
470 31]. Taken together, the present findings suggest that Cx43 expression

induces Cox2 over-expression via NFκB activation. However, until now, the link between NFκB activation, Cx43 expression, and Cox2 over-expression has not been clearly established. In the future, it will be interesting to examine the relationship between the GJIC and NFκB activation, Cox-2 by ESP of *C. sinensis* and NDMA.

In conclusion, our results suggest that Cx43 plays a key role in cell proliferation, potentially leading to CHCA development upon stimulation by ESP of *C. sinensis* and NDMA.

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