Lipocalin 2 (LCN2) is a promising target for cholangiocarcinoma treatment and bile LCN2 level is a potential cholangiocarcinoma diagnostic marker

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Cholangiocarcinoma (CCA) is a devastating disease due to resistance to traditional chemotherapies and radiotherapies. New therapeutic strategies against CCA are urgently needed. This study investigated the role of lipocalin-2 (LCN2) in human cholangiocarcinoma as a potential therapeutic target and diagnostic marker. So far, the role of LCN2 in cancer is still controversial and studies regarding the role of LCN2 in CCA are limited. LCN2 knockdown inhibited CCA cell growth in vitro and in vivo through induction of cell cycle arrest at G0/G1 phases and decreased metastatic potential due to repression of epithelial-mesenchymal transition (EMT). Overexpression of LCN2 in CCA cells increased cell metastatic potential. We showed for the first time that the N-myc downstream regulated gene 1 (NDRG1) and NDRG2, known as tumor suppressor genes, are negatively regulated by LCN2 in CCA cells. LCN2 concentration in bile was higher in patients with CCA than that in patients with gallstones, with a cutoff value of 20.08 ng/ml making this a potential diagnostic marker. Higher LCN2 expression was associated with worse survival in patients with CCA. LCN2 is a promising target for CCA treatment and bile LCN2 level is a potential diagnostic marker for CCA.

Cholangiocarcinoma (CCA) is an epithelial malignancy arising from the bile ducts, and ranks as the second most common liver malignancy, after hepatocellular carcinoma. Recently, due to increased recognition and incidence, the interest in treatments for this cancer has increased¹. Most CCA develops de novo without obvious risk factors. The 5 year survival rate of CCA is very low due to late diagnosis and resistance to traditional anti-cancer regimens². Curative radical surgery remains the standard and most effective treatment for CCA; however, most patients with CCA are not good candidates for operation due to advanced disease at the time of diagnosis. Thus, the development of new therapeutic targets for CCA should be prioritized.

Lipocalin-2 (LCN2), also known as NGAL, uterocalin, or 24p3, belongs to the lipocalin superfamily. LCN2 is a secreted protein with the ability to interact with other ligands and has been found to be a transporter of some hydrophobic substances³. Originally, the main function of LCN2 was believed to be the capture and transport into the cytoplasm of iron ions, contributing to its bactericidal properties, among others. LCN2 is also categorized as a stress protein due to activation of iron-dependent defense systems following exposure to stress stimuli⁴. Recently, the oncogenic role of LCN2 has been described in severe cancers, with higher LCN2 expression in cancerous cells.

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compared to non-cancerous cells\textsuperscript{5}. Many studies have also identified a pro-neoplastic role for \textit{LCN2} and related mechanisms\textsuperscript{6,7}. However, controversies over its function remain. Some studies have shown that \textit{LCN2} acts as a tumor suppressor gene in ovarian cancer, pancreatic cancer and colon cancer\textsuperscript{8–10}. Studies investigating the role of \textit{LCN2} in CCA are still very limited.

The N-myc downstream regulated gene (NDRG) protein family comprises 4 members, NDRG1, NDRG2, NDRG3, and NDRG4\textsuperscript{11}. NDRG proteins are widely expressed in human tissues, with \textit{NDRG4} mainly expressed in the heart and brain\textsuperscript{12}. \textit{NDRG1} and \textit{NDRG2} have been widely studied and identified as tumor suppressor genes in a variety of cancers\textsuperscript{13–17}.

EMT is a process during which epithelial cells change towards a mesenchymal cell phenotype, playing a vital role in cancer cell metastasis. After EMT, cancer cells have increased motility and become more invasive. EMT also renders cancer cells more resistant to chemotherapy and surveillance of immune cells due to increased stem cell-like characteristics\textsuperscript{18–20}.

MMPs are proteases that digest collagen, which is one of the main components of the extracellular matrix. Cancers with higher MMP expression tend to have higher invasiveness\textsuperscript{21,22}.

Previously, our group has shown high \textit{LCN2} expression in human CCA samples\textsuperscript{23}. In the current study, we investigated the role of \textit{LCN2} in human CCA, including the effect of \textit{LCN2} on CCA cell growth and metastatic potential \textit{in vitro} and \textit{in vivo} xenografted tumor growth. The relation between NDRGs and LCN2 in CCA cells were also studied for the first time. Furthermore, \textit{LCN2} expression in human samples was studied to relate \textit{LCN2} levels to clinical characteristics and survival of patients with CCA. The level of \textit{LCN2} in bile in patients with CCA was measured for comparison with levels in gall stone patients. Overall, we aimed to provide a new therapeutic target and diagnostic marker for CCA.

**Results**

**Characterization of \textit{LCN2} mRNA expression in CCA cells.** \textit{LCN2} expression was evaluated in 8 CCA cell lines: RBE, SSP-25, TFK-1, SNU308, SNU1079, TGBC-24, HUCCT1, and YSCCC. \textit{LCN2} mRNA expression in each cell line was determined by RTqPCR. As shown in Fig. 1A, SNU308 cells had the highest level of \textit{LCN2} mRNA expression, and expression was lowest in RBE cells.

**Effect of \textit{LCN2} knockdown on CCA cell cycle progression and expressions of cell-cycle control related proteins.** Previously we have shown that the doubling time of SNU308-LCN2si cells is increased as compared to SNU308-COLsi cells\textsuperscript{23}, suggesting an oncogenic role for \textit{LCN2} in human CCA. Therefore, we further evaluated the effect of \textit{LCN2} on SNU308 cell cycle progression. As shown in Fig. 1B, SNU308-LCN2si cells had an increased number of cells in G0/G1 phase compared to SNU308-COLsi cells (56.26% and 41.44%, respectively), suggesting that knockdown of \textit{LCN2} in SNU308 cells induces G0/G1 arrest, thus inhibiting cell
growth. Further, to understand how LCN2 influences SNU308 cell cycle progression, the expression of cyclin dependent kinase inhibitors (CKIs) p21 and p27, cyclin dependent kinase (CDK) 4 and CDK6, and cyclin D3 were investigated. As shown in Fig. 1C,D, LCN2 knockdown induced higher p21 expression and lower CDK4 and CDK6 expression in SNU308 cells with no obvious impact on p27 and cyclin D3 expression, initiating the G0/G1 arrest noted in Fig. 1B.

Effect of LCN2 knockdown on SNU308 metastatic potential. For evaluation of the role of LCN2 in SNU308 cell metastatic potential, we then conducted invasion and migration assays. As shown in Fig. 2A, the population of SNU308-LCN2si cells had much fewer invading and migrating cells as compared to the population of SNU308-COLsi cells. This shows that LCN2 knockdown attenuates metastatic potential. Since epithelial-mesenchymal transition (EMT) is a key step in cancer cell metastasis, we then investigated the effect of LCN2 on EMT-related transcription factors. Figure 2B,C demonstrate that Snail, Slug, Twist, Zeb-1, and Zeb-2 expression levels decreased in SNU308-LCN2si cells compared to SNU308-COLsi cells, implying that LCN2 knockdown inhibited EMT. We further evaluated expression of E-cadherin and P-cadherin in SNU308 cells after LCN2 knockdown. Figure 2D shows that E-cadherin was upregulated while P-cadherin was downregulated in SNU308-LCN2si cells. Intracellular and extracellular MMP-2 and MMP-9 expression were also decreased (Fig. 2E,F) after LCN2 knockdown.

Effect of LCN2 overexpression on cell proliferation and metastatic potential in RBE cells. LCN2 overexpression was induced in RBE cells (Fig. 3A) to evaluate its effect on RBE cell proliferation, invasion, and migration. RBE-LCN2 cells exhibited higher LCN2 mRNA expression and LCN2 secretion than RBE-CMV2 cells (Mock transfection of LCN2) (Fig. 3A,B). Figure 3C shows that RBE-LCN2 cells had a similar doubling time to RBE-CMV2 cells (The calculation of double time was described previously ref. 23). Figure 3D demonstrate that migration and invasion were increased to 155 ± 5% and 165 ± 7% respectively in RBE-LCN2 cells as compared to RBE-CMV2 cells.
Effect of LCN2 knockdown or overexpression on NDRG1 and NDRG2 expression in CCA cells. The NDRG gene family is widely regarded as comprising several tumor suppressor genes. NDRG1 and NDRG2 expression was evaluated following LCN2 overexpression or knockdown. Figure 4A reveals that the expression of NDRG1 and NDRG2 was increased after LCN2 knockdown in SNU308 cells but decreased after LCN2 overexpression in RBE cells, as determined by western blot. This result is supported by the higher expression of NDRG1 and NDRG2 mRNA in SNU308-LCN2si cells as compared to SNU308-COLsi cells (Fig. 4B,C). The reporter assay confirms this, as transiently transfecting SNU308 cells with an NDRG1 reporter using an LCN2 expression vector decreased reporter activity (Fig. 4D). Similar results were observed in the NDRG2 reporter assay (Fig. 4E). Overall, we concluded that NDRG1 and NDRG2 are downstream of LCN2 in CCA cells.

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Effect of LCN2 knockdown on SNU308 cell growth in vivo. To investigate the effect of LCN2 knockdown on SNU308 cell growth in vivo, tumors comprised of either SNU308-LCN2si cells or SNU308-COLsi cells were xenografted into nude mice. 4 weeks later, the xenografted tumors were harvested and weighed. Figure 5 shows that tumors from the SNU308-LCN2si cell group had a much lower tumor weight and smaller tumor volume than that in the SNU308-COLsi cell group, indicating that LCN2 knockdown was able to inhibit CCA cell growth in vivo.

Evaluation of LCN2 concentration in bile from CCA or gallstone patients. The median LCN2 levels in the bile for 30 patients with CCA and 36 gallstone patients were 59.26 ng/ml and 10.19 ng/ml, respectively. The LCN2 level in patients with CCA was statistically higher than that in gallstone patients (P < 0.001, Fig. 6A). The area under the ROC curve was 0.81 with an optimal cutoff value, which had the largest Youden index, of 20.08 ng/ml. The test had a sensitivity of 0.87 and a false positive rate of 0.25 (Fig. 6B).

Relationship between LCN2 expression and clinicopathological characteristics of patients with CCA. LCN2 was diffusely expressed in the cytoplasm of CCA cells with 42 (53.8%) patients graded as exhibiting high expression (based on H-scores ≥ 40) (Fig. 6C,D). Higher LCN2 expression was associated with lower albumin level and a higher rate of positive surgical margin in patients with CCA (Supplemental Table 1). After univariate and subsequent multivariate analysis, only LCN2 expression was found to be negatively associated with overall survival (OS) of patients with CCA (p < 0.001, relative risk, 3.615 (1.721–7.592)) (Supplemental Table 2 and Fig. 6E).

Discussion
LCN2 is widely deemed as an oncogene in a variety of cancers despite some enduring controversy, but studies regarding LCN2 anti-cancer mechanisms remain limited. Moreover, the fact that the findings of these studies are sometimes contradictory indicates that LCN2 may work in a highly cell- or tissue- specific manner. Our results demonstrate that LCN2 acts as an oncogene in human CCA, as LCN2 expression was positively correlated with
CCA cell growth and metastatic potential and negatively associated with CCA patient survival. We investigated the mechanisms whereby LCN2 influences CCA cell growth and metastasis. Furthermore, the bile of patients

Figure 4. Evaluation LCN2 effect on NDRG1 and NDRG2 expressions in CCA cells. (A) The western blot to show the downregulation or upregulation of NDRG1 and NDRG2 as LCN2 overexpression in RBE cells or knockdown in SNU308 cells. (B) Relative NDRG1 mRNA expression of SNU308-LCN2si cells to SNU308-COLsi cells. Each value was a mean ± SD of three independent determinations. Experiment was done at least three times (**P < 0.01). (C) Relative NDRG2 mRNA expression of SNU308-LCN2si cells to SNU308-COLsi cells. Each value was a mean ± SD of three independent determinations. Experiment was done at least three times (**P < 0.01). (D) The activity of NDRG1 reporter in SNU308 cells as treated with indicated concentrations of LCN2 expression vectors. Each value was a mean ± SD of three independent determinations. Experiment was done at least three times (**P < 0.01). (E) The activity of NDRG2 reporter in SNU308 cells as treated with indicated concentrations of LCN2 expression vectors. Each value was a mean ± SD of three independent determinations. Experiment was done at least three times (**P < 0.01).

Figure 5. LCN2 knockdown repressed SNU308 cell growth in vivo. SNU308-COLsi and SNU308-LCN2si cells were xenografted into the nude mice (= 8 for each group). Four weeks later, the tumors were harvested and weighted. Our result clearly shows that LCN2 knockdown significantly inhibited SNU308 cell growth in vivo (**P < 0.01).
with CCA exhibited a much higher LCN2 concentration compared to individuals without CCA. Taken together, our data suggest that LCN2 could be a promising therapeutic target and diagnostic marker for human CCA.

Cell cycle progression in normal cells is under strict control to maintain homeostasis. However, due to constitutively high mitogenic signaling in cancer, the cell cycle of cancer cells is usually dysregulated, leading to uncontrolled proliferation. Thus, targeting cell cycle progression is an effective way to curb cancer cell growth24.

To evaluate the effect of LCN2 on CCA cell cycle progression, 8 CCA cell lines were analyzed for LCN2 mRNA expression, with SNU308 showing the highest LCN2 expression (Fig. 1A). We therefore knocked down LCN2 in SNU308 cells and analyzed the distribution of cells at each stage of the cell cycle within the population. Figure 1B reveals that more SNU308-LCN2si cells were in G0/G1 phase compared to SNU308-COlsi cells, indicating that LCN2 knockdown can induce cell cycle arrest at G0/G1.

To initiate cell division, cells must pass a restriction point located in mid to late G1 phase, for which release of E2F-1 from its inhibitor, retinoblastoma protein (RB) is necessary 25. This requires RB to be phosphorylated, involving specific CDKs and cyclins, and is negatively controlled by specific CKIs. Among others, CDK4, CDK6, and cyclin D3 are responsible for G1/S transition. Figure 1C,D demonstrate that CDK4 and CDK6 expression was decreased in SNU308-LCN2si cells, while no obvious change in expression of cyclin D3 occurred. Two CKI initiators of G1/S transition, p21 and p27, were also examined. As shown in Fig. 1C,D, LCN2 knockdown increased p21 expression with no significant impact on p27. Taken together, we concluded that LCN2 knockdown in SNU308 cells can retard cell cycle progression at G0/G1 phase by downregulation of CDK4 and CDK6 and upregulation of p21, leading to the inhibition of cell proliferation noted in our previous study 23. Following xenograft of SNU308-LCNsi or SNU308-COLsi tumors into nude mice, tumor growth from SNU308-LCNsi cells was much slower than from SNU308-COLsi cells (Fig. 5). Collectively, our results suggest that LCN2 knockdown could repress SNU308 cell growth in vitro and in vivo.

It has been reported that EMT is positively associated with cancer progression, resulting in poor prognosis 26. The regulation of EMT process is well orchestrated. Among others, Snail, Slug, Twist, Zeb1, and Zeb2 are transcriptional factors responsible for EMT induction 27. Figure 2B,C demonstrate that all five transcriptional factors were downregulated following LCN2 knockdown in SNU308 cells, indicating that LCN2 knockdown could inhibit EMT progress in CCA cells, attenuating the metastatic potential noted in Fig. 2A. Another hallmark of EMT is the downregulation of E-cadherin 28. To initiate metastasis, cancer cells must detach from the primary tumor first. E-cadherin, a transmembrane glycoprotein, plays a vital role in calcium-dependent cell-cell adhesion 29,30 and its expression has been negatively associated with cancer invasiveness and prognosis 31,32. Figure 2D...
shows that SNU308-LCNsi cells expressed E-cadherin more highly than SNU308-COLsi cells, further supporting the finding that the EMT process is attenuated by LCN2 knockdown in SNU308 cells. The downregulation of P-cadherin, which is another marker of mesenchymal cells and linked with CCA cell migration, was observed in LCN2 knockdown SNU308 cells also demonstrated EMT process was repressed in SNU308-LCNsi cells (Fig. 2D).

Previously, LCN2 has been shown to form an LCN2/MMP complex that protects MMP-9 from degradation, thus increasing MMP-9 activity. In CCA, LCN2 knockdown in CCA cells had been shown to repress invasion through reduction of LCN2/MMP-9 complex formation. Our result indicates that knockdown of LCN2 in CCA cells could decrease both intracellular and extracellular MMP-2 and MMP-9 expression, reducing cell invasiveness (Fig. 2D,E).

The effect of LCN2 on CCA cells was further demonstrated through overexpression in RBE cells (Fig. 3). It is obvious that RBE-LCN2 cells exhibited higher migration and invasion abilities than RBE-CMV2 cells, although cell proliferation was similar in both cell types (Fig. 3B–D).

NDRG1 has been reported to induce differentiation, cell cycle arrest, and cell growth inhibition in a variety of cancers. Similar to NDRG1, many studies have implied an anti-cancer role for NDRG2. Since NDRG1 and NDRG2 belong to the N-Myc downstream-regulated gene family, their expression is modulated by N-myc. Besides N-myc, NDRG1 and NDRG2 have also been reported to be regulated by p53, HIF-1α, and PTEN, among others. As shown in Fig. 4, we knocked down or overexpressed LCN2 in CCA cells, NDRG1 and NDRG2 protein expression was increased or decreased, respectively (Fig. 4A). The RTqPCR and reporter assay also confirmed that both NDRG1 and NDRG2 expression was regulated by LCN2 (Fig. 4B–E). We showed for the first time that NDRG1 and NDRG2 are downstream targets of LCN2 in CCA cells.

LCN2 has been applied as a tumor marker in bladder cancer and has been found to be higher in urine of patients diagnosed with non-papillary bladder cancer as compared to papillary bladder cancer. To evaluate the clinical relevance of LCN2 expression in CCA, bile from CCA and gall stone patients was collected for measurement of LCN2 concentration. Medium concentration of 59.26 ng/ml or 10.19 ng/ml were obtained for CCA or gall stone patients, respectively, with the optimal cutoff value to differentiate patients with CCA from those without CCA being 20.08 ng/ml (sensitivity of 0.87 and false positive rate of 0.25). Our result suggests that LCN2 measurement of LCN2 concentration. Medium concentration of 59.26 ng/ml or 10.19 ng/ml were obtained for CCA or gall stone patients, respectively, with the optimal cutoff value to differentiate patients with CCA from those without CCA being 20.08 ng/ml (sensitivity of 0.87 and false positive rate of 0.25). Our result suggests that LCN2 concentration in bile could be a valuable diagnostic marker for human CCA. Other report also demonstrated the similar result although the cutoff value was much higher than our current finding, which may be due to they recruited both malignant or benign biliary obstruction patients for comparison.

LCN2 has been shown to be able to render cancer cells more resistant to chemotherapy and target therapy and has been adversely associated with cancer patients’ survival. In our current study, higher LCN2 expression was linked with poor OS of patients with CCA (Fig. 6C–E), further justifying the development of LCN2-targeting therapies in CCA treatment.

Our current study indicates that LCN2 could be a promising target for CCA treatment, since it acts as an oncogene in CCA cells in vitro and in vivo, and higher LCN2 expression is linked to poor OS of patients with CCA. In conjunction with the finding that bile LCN2 concentration was higher in patients with CCA, we conclude that in addition to being a potential therapeutic target, bile LCN2 concentration is a good diagnostic marker for CCA.

Materials and Methods
Cell culture. SNU308 and RBE cells, human CCA cell lines, were purchased from the Korean Cell Line Bank (KCLB: 28 Yongon-dong, Chongno-gu, Seoul 110–744, Korea). The cells were tested using the PromegaGenePrint 10 System and mRNA was analyzed using ABI PRISM 3730 GENETIC ANALYZER and GeneMapper Software V3.7.

Knockdown of LCN2 in SNU308 cells. SNU308 cells were transducted with control small hairpin RNA lentiviral particles (Sc-10808-V, Santa Cruz Biotechnology) according to the manufacturer’s instructions. Two days after transduction, the cells (SNU-COLsi and SNU-NGLAsi) were selected by incubation with 10 μg/ml puromycin dihydrochloride for another 3 generations. The detailed procedures were described previously.

LCN2 expression vector constructs and stable transfection. The expression vector containing coding region of LCN2 (HG10222-M-Y) was purchased from Sino Biological Inc (Beijing, China). The expression vector was introduced into REB cells by electroporation. The LCN2-transfected cells (REB-LCN2) were selected with hygromycin B (50 μg/ml; Invitrogen, Carlsbad, CA) for at least 4 generations. The mock-transfection (REB-CMV2) cells were transfected with pCMV2HA expression vector (Sino Biological Inc) and were selected as REB-LCN2 cells.

Cell cycle analysis. Cells were serum starved for the 24 hours preceding analysis as previously described. The cells were fixed in ice-cold 75% ethanol. The fixed cells were stained by propidium iodide buffer containing 100 mM sodium citrate, 0.1% Triton X-100, 0.2 mg/ml RNase, and 50 μg/ml PI at 4 °C for 1 h. Cell cycle analysis was performed using a FACSCalibur cytometer and CellQuest Pro software (BD Biosciences, San Jose, CA).

Matrigel invasion assay. The matrigel invasion assay was conducted as previously described. 48 hours afterwards, the invading cells were fixed with 4% paraformaldehyde in 1 × PBS, stained, digitally photographed and counted under the microscope (IX71, Olympus, Tokyo, Japan). Experiments were performed in triplicate and repeated at least three times.

Trans-well filter migration assay. The migration assay was conducted as previously described. 24 hours afterwards, the migrating cells were stained and counted under four random high-power microscopic fields (HPF; 100X) per filter. The experiments were performed in triplicate and repeated at least three times.
Real-time reverse transcription-polymerase chain reaction (RT-qPCR). Total RNA Isolation kit (Promega, Madison WI, USA) was used to extract total RNA. For each sample, cDNA was generated from 2 μg of RNA using Superscript RNAase H− (Invitrogen, Carlsbad, CA, USA) with random hexamer primers. qPCR was performed using an ABI StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). FAM dye-labeled TaqMan MGB probes for human NDRG1 (Hs00608387_m1), NDRG2 (Hs01045115_m1), LCN2 (Hs00194353_m1) and β-actin (Hs01060665_g1) were purchased from Applied Biosystems.

Western blotting. Western blots were performed as described previously47. Cells were washed once with PBS and lyzed in the lysis buffer containing 50 mM Tris-Cl, 50 mM β-glycerophosphate, 50 mM NaCl, 1 mM Na3Vo4, 1 mM EDTA, 1 mM EGTA, 1% NP40, and freshly adding 1 mM DTT, 1 mM PMSF, 2 μg/ml Aprotinin, 2 μg/ml Leupetin, and 2 μg/ml Pepstatin right before lysis. The cell lysates were then rotated for 30 minutes and centrifuged at 13,000 g for 30 minutes at 4 °C and the supernatant was collected. The protein concentration of the cell lysate was determined by using BCA reagent (Thermo Scientific Pierce) with BSA as standards. The antibodies used in this experiment are listed in the Supplementary data.

Reporter assay for NDRG1 and NDRG2. The NDRG1 (−4714 to +46) reporter vectors were constructed as described previously51. A 5.2 kb DNA fragment was subtracted from a BAC clone (PR11-998D10; Invitrogen) and cloned into the pGEM-3 vector (Promega BioScience) with the Kpn I cutting site. The DNA fragment containing the enhancer/promoter of the NDRG2 gene (−4253 to −1) was synthesized with primers (Sp6 and 5′-AAGCTTCTATAATAGGCGGATCCGC-3′) by PCR using pGEM-3NDRG2 as target DNA. This DNA fragment was cloned into the pGL3-Basic vector (Promega BioScience) vector at the Hind III cutting sites. Cells were then transiently transfected and luciferase activity was determined in relative light units (RLU) as described previously17.

Statistical analyses. All data were presented as the mean ± standard deviation (SD). Differences between the experimental and control groups were calculated using the student’s t-test. Differences in tumor weights between the experimental and control animals, and LCN2 levels in bile between the two groups of patients were compared by the Mann-Whitney U test. The cut-off value of LCN2 level was determined by receiver operating characteristic (ROC) curve analysis52. Overall survival rate was evaluated using the Kaplan–Meier method. Several clinicopathological variables were considered for the initial single-variable analysis, which was performed using an ABI StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).
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Author Contributions
K.-C.C. and T.-S.Y. wrote the manuscript and designed this experiment. R.-C.W., J.-H.S., C.-T.C. and S.-Y.W. helped conduct the experiment. H.-H.J. and C.-N.Y. are in charge of the whole experiment conduction and paper writing.

Additional Information
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