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ORIGINAL RESEARCH

# RETRACTED ARTICLE: iNOS is associated with tumorigenicity as an independent prognosticator in human intrahepatic cholangiocarcinoma

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**Background:** Inducible nitric oxide synthase (iNOS) has supposed to duplicate in inflammation, infection, liver cirrhosis, and neoplastic diseases. This study was designed to explore the biological and clinical function of iNOS in intrahepatic cholangiocarcinoma (ICC).

**Methods:** RT-PCR (Real-time quantitative PCR) and immunohistochemical staining were used to analyze the expression of iNOS in ICC and adjacent tissues. CCK8, transwell assays, flow cytometry were conducted to detect the proliferation, apoptosis, cell cycle. Western blotting was performed to detect the expression of target proteins. Multivariate analyses were conducted to analysis associates between clinicopathological values and survival.

**Results:** We found that levels of iNOS mRNA and protein were dramatically increased in ICC samples and positively correlated with complicated bile duct stone, differentiation, pathology T, pathology M, Wip1, MMP-2, and MMP-9. iNOS expression was significantly correlated with the poor survival of ICC patients. Furthermore, iNOS was high expression in ICC cell lines (QBC939, ICC-9810, SSP-25) compare with human normal biliary epithelium cell line (HIBEpiC); both iNOS knockdown and iNOS inhibitor (1400 W) suppressed cell proliferation, invasion, and migration though nitric oxide production in ICC cells. Down-regulation of iNOS also induced G0/G1 cell cycle arrest and ICC cell apoptosis. Moreover, iNOS knockdown treatment significantly decreased Wip1, MMP-9, and MMP-2 gene expression.

**Conclusion:** Lowly expressed iNOS-inhibited proliferation yet promoted apoptosis of ICC cells. Our data show targeted inhibition of iNOS in ICC may have therapeutic value.

**Keywords:** intrahepatic cholangiocarcinoma, iNOS, prognosis

## Background

Intrahepatic cholangiocarcinoma (ICC) is an aggressive and poorly understood biliary malignancy that is frequently diagnosed at advanced stages, which limits its treatment options.[1] In recent years, the incidence and mortality of ICC have drastically increased with geographic variation.[2,3] Furthermore, the long-term survival of patients with unresectable ICC is dismal, with less than 5–10% of the patients surviving at 5 years after the diagnosis.[2,4] Surgical resection remains the only approach with curative intent, achieving a 5-year survival rate of approximately 20% at early T1–T2 stages; however, patients with an unresectable tumor have a dismal median survival of approximately 9 months.[5] Still worse, in sharp contrast to the use of standard therapies for patients with advanced lung/breast/colorectal cancers and hepatocellular carcinoma, systemic chemotherapy and molecular-targeted



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therapies had limited success in treating ICC;[6] furthermore, most patients have advanced disease at the time of diagnosis and are not suitable candidates for surgery.[7,8] Therefore, molecular markers serving as predictive factors are needed to provide effective therapy as well as to understand mechanisms underlying ICC progression.

ICC were often observed in primary sclerosing cholangitis, chronic hepatitis C or B virus infection, hepatolithiasis, and chronic alcohol abuse involving chronic inflammation of the large bile duct may be major risk factors for cholangiocarcinoma.[9] Obviously, the common feature of these chronic inflammatory conditions was highly reactive nitrogen species (RNS) and reactive oxygen species (ROS), which may play key roles in cholangiocarcinoma.[9]

Inducible nitric oxide synthase (iNOS) is a pro-inflammatory enzyme associated with a wide array of malignant disease.[10,11] In fact, the effective of iNOS during cancer progression is totally complex and incompletely understood. Both inhibiting and inhibiting actions have been reported, presumably depending on the local concentration of iNOS within the tumor microenvironment.[12] iNOS exerts multiple effects in carcinoma, such as angiogenesis, malignant transformation, and metastasis. Nitric oxide (NO) activated by iNOS has a potentially cytotoxic/cytostatic effect upon inflammation-associated cancer.[13] On the other hand, a common feature of ICC etiologies is high RNS and ROS.[9,14] Previous studies showed that iNOS expression was highly expressed in reactive epithelial and biliary intraepithelial neoplasia.[14]

However, the roles of biological significance of iNOS in ICC or its precursors or metastatic lesions remain elusive. In the present study, we aimed to examine the frequency of iNOS expression and determine whether this aberration correlates with ICC disease progression.

## Methods

### Patient and tissue samples

Forty-five ICC and distant normal tissue samples were obtained from ICC patients who underwent radical resection between April 2011 and October 2013 at the Department of Hepatobiliary Surgery, Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University, Changsha, Hunan, China. None of the patients received any preoperative chemo- and radiotherapy or other medical interventions. Follow-up data were obtained by phone interview, postal letter communication, and the outpatient clinical database. All patients were monitored from the date of initial surgery until death or the closing date of the present study (November 30, 2016). The mean follow-up period was 43 months (Range between 26 and 60 months). This study was reviewed and approved by the Hunan Normal University (NO.HNSRMYY201511), and informed consent was obtained from each patient following institutional review board protocols. The patient consent (clinical samples) was written informed consent, and all informed consent forms are available if required.

### Immunohistochemical staining

Formalin-fixed and paraffin-embedded specimens were used for immunohistochemistry. Specimens were cut at a 4-mm thickness and subsequently deparaffinized and rehydrated. Antigen retrieval was performed by microwaving in 0.01 M citrate buffer for 10 mins. After washing in PBS, the slides were blocked for endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 mins at room temperature. The sections were then blocked with 10% goat serum for 30 mins, followed by incubation with a primary anti-iNOS antibody (Abcam, USA) at a 1:200 dilution overnight at 4°C. After washing, the sections were incubated with a biotin-labelled secondary antibody for 10 mins at room temperature. Next, a streptavidin peroxidase reagent was applied for 10 mins. Coloration was developed via a 4–5 min incubation with a 3,3′-diaminobenzidine solution, and the sections were counterstained with hematoxylin. The slides were washed with distilled water, dried, and coverslipped using a permanent mount. As a negative control, PBS was used instead of the primary antibody. Immunohistochemical staining of iNOS on ICC tissue was evaluated in accordance with our previous studies.[16–18] Briefly, all tissue sections were reviewed under a light microscope, and at least five fields were scored at ×400 magnification independently by two pathologists who were unaware of any clinical or outcome data. The staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), or 3 (strong). The extent of stained cells was scored as 0 (0–5%), 1 (6–25%), 2 (26–50%), or 3 (51–100%). The immunoreactive score was determined by the sum of the intensity and extent of staining. Summed scores ≥3 points were considered to be significant overexpression and were considered to be positive to simplify data analysis.

### Cell lines and cell culture

Three ICC cell lines (QBC-939, ICC-9810, and SSP-25) were obtained from the American Type Culture Collection (ATCC, USA). The human normal biliary epithelium cell

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line (HIBEpic) was purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 (Corning, NY, USA) supplemented with 10% fetal bovine serum (Corning), 100 U/mL penicillin, and 100 µg/mL streptomycin (Corning) in a humidified atmosphere at 37°C and 5% $CO_2$.

## RNA extraction and quantitative reverse transcriptase PCR (qRT-PCR) analyses

Total RNA was extracted from frozen tissues and cell lines using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. qRT-PCR assays were performed to detect iNOS expression using the PrimeScript RT reagent Kit and SYBR Premix Ex Taq (GeneCopoeia, USA) following the manufacturer's instructions. The primers for real-time PCR were designed as Supplement Table 1. The relative levels of target gene mRNA were expressed as the ratio of target to β-actin and calculated from the standard curve as directed. All of the reported results represent the average ratios of three different independent experiments.

## Vector construction and transfection

siRNA-1, siRNA-2, and siRNA-3 of iNOS were constructed by GeneChem Biomedical Co., Ltd (Shanghai, China) and synthesized as follows: CCATCCGC CCAC CTAA CAGTGGAATGAGTCCTTTA, and AC CGTC GAA TCCAA, respectively. A blank vector was used as a negative control. A total of $5\times10^5$ QBC-939 or ICC-9810 cells were seeded into each well of a six-well plate. When the cells reached 80–90% confluence on the day of transfection, iNOS siRNA or non-T small interfering RNA (non-T siRNA) was transfected into cells. Five groups of cells were used in the present study, including untransfected cells ("untreated"), cells transfected with "non-T siRNA", and cells transfected with iNOS-siRNA ('iNOS siRNA-1, siRNA-2, siRNA-3'). Expression of iNOS was performed at the mRNA level by relative quantitative real-time PCR and at the protein level by Western blotting.

**Table 1** Clinicopathological correlation with iNOS protein expression

| Variable | iNOS | | *p* |
|---|---|---|---|
| | Positive, n=34 | Negative, n=11 | |
| Age | 66.1 | 65.7 | 0.324 |
| M/F | 4 1 | 7/4 | 1 |
| Complicated bile duct stone ± | 27/7 | 2/9 | 0.037 |
| Tumor size, cm | 5.312 | 4.835 | 0.435 |
| Well/moderate/poorly | 2/13/19 | 6/3/2 | 0.032 |
| Pathology T (T1+2)/(T3+4) | 15/19 | 9/2 | 0.002 |
| Pathology N 0/1 | 26/8 | 8/3 | 0.16 |
| Pathology M 0/1 | 32/2 | 10/1 | 0.029 |
| Wip1+/– | 20/14 | 2/9 | 0.019 |
| MMP-2+/– | 30/4 | 6/5 | 0.028 |
| MMP-9+/– | 21/13 | 3/8 | 0.046 |

**Abbreviation:** iNOS, inducible nitric oxide synthase.

## Cell proliferation assay

Cell proliferation was evaluated using CCK8 (Sigma). Five groups of cells including untreated, Non-T siRNA, iNOS siRNA-1, iNOS siRNA-2, and iNOS siRNA-3, were allowed to grow in 96-well plates. Cell proliferation was documented at 0, 12, 24, 36, and 60 hrs following the manufacturer's protocol. CCK-8 reagent was added to each well 2 hrs before the endpoint of incubation. The optical density (OD) 450 nm value was determined by a microplate reader. All experiments were repeated at least three times.

## Cell invasion assay

Transwell invasion assays were performed in 24-well 8-mm pore size transwell plates following the manufacturer's instructions (Corning, New York, NY, USA). The bottom of the transwell chamber was coated with BD Matrigel Basement Membrane Matrix (BD Biosciences, San Diego, CA, USA). The upper chamber was filled with $1\times10^5$ cells in RPMI 1640 containing 5% fetal bovine serum. The lower chamber was filled with RPMI 1640 containing 25% fetal bovine serum as a chemoattractant. After the chambers were incubated for 24 hrs at 37°C, non-invading cells on the upper side of the chamber were removed from the surface of the membrane by scrubbing, and invading cells on the lower surface of the membrane were fixed with methanol, mounted and dried. The number of cells invading through the Matrigel was counted by a technician who was blinded to the experimental settings in four randomly selected microscopic fields of each filter. The experiments were repeated three times.

## Scratch wound-healing assay

Cells ($1\times10^6$ cells) were seeded onto six-well plates, grown overnight and transfected with iNOS siRNA-2 or non-T siRNA. Monolayers of cells were wounded by dragging a

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10 μL pipette tip. Then, the cells were washed to remove cellular debris and cultured again for up to 24 hrs. Images were taken at different time points (0 and 24 hrs) after wounding under an inverted microscope. Every experiment was repeated three times.

## Apoptosis assay by flow cytometry

Cells were seeded onto six-well plates for 48 hrs and then harvested for Annexin V-FITC and PI staining according to the manufacturer's recommended protocol. The cells were subsequently analyzed by flow cytometry using a fluorescence-activated cell-sorting flow cytometer (BD Biosciences). All assays were performed independently in triplicate.

## Cell cycle analysis

Cells were harvested 48 hrs after seeding, and single-cell suspensions containing $1\times10^6$ cells were fixed with 75% alcohol. The cell cycle was monitored using propidium iodide staining of the nuclei. The fluorescence of DNA-bound propidium iodide in cells was measured with a FACScan flow cytometer (BD Biosciences, San Diego, CA, USA).

## NO analysis

Nitrate reductase method (NO assay kit, A012, Nanjing Jiancheng Bioengineering institute) was used to detect the NO content of supernatant in each group. Check test agent from the icebox and closed sets of each sample set in 37°C water bath after 5 mins, according to the NO kit instructions. After all the reactions, the distilled water was adjusted to zero, and the absorbance (OD) was measured under the wavelength of spectrophotometer 550 nm. NO content is calculated according to the following formula: NO (with the presence of mol/L) =[(with the presence of tube OD value)/(with the presence of tube OD value – with the absence of tube OD value)] x the concentration of the standard product (100 mol/L) x the dilution factor before the test. The average value of the results of the same five experiments was the content of NO.

## Viability assays

Cells were treated with different concentrations (1, 10, 25, 50, 100, and 200μmol/L) of 1400 W. And then, the effect of 1400 W on the viability of cells was determined by CCK-8 assay as described previously.[19]

## Western blotting

Cells were harvested and washed with pre-chilled PBS before being lysed using RIPA buffer containing protease inhibitors. Cells were incubated with the lysis buffer for 30 mins on ice and then centrifuged at 12,000 rpm at 4°C for 15 mins. The protein concentration was measured by the BCA assay following the manufacturer's protocol. Equal amounts of protein were separated by SDS-PAGE gel and subsequently electrotransferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skimmed milk for 1 hr at room temperature and then incubated with a primary anti-iNOS antibody (Abcam, USA) at a 1:200 dilution against iNOS overnight at 4°C. β-actin was used as protein-loading control. After washing three times with TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. After washing again with TBST, the protein bands were detected by chemiluminescence. The blots shown in the present study are representative of a minimum of three separate experiments.

## Statistical analysis

Statistical analyses were performed using SPSS software version 16.0 for Microsoft Windows (SPSS, Inc., Chicago, IL, USA). Differences between groups were analyzed using Student's *t* test when there were only two groups or assessed by one-way ANOVA when there were more than two groups. Survival of patients was stratified using the Kaplan–Meier method and statistically analyzed using the log-rank statistic. Univariate and multivariate analyses using Cox proportional hazard models were conducted to measure correlations between clinicopathological factors and survival. $p<0.05$ was considered statistically significant.

# Results

## Expression of iNOS is associated with clinicopathological characteristics in ICC patients

The correlations between the iNOS levels and clinicopathological parameters are summarized in Table 1. iNOS expression was observed in 34 (75.6%) of 45 ICC patients. These subjects were verified by a histopathological study and comprised poorly (21 patients), moderately (16 patients), and well (8 patients) differentiated adenocarcinoma. The tumor size was 2–5 cm (32 patients), 6–8 cm (8 patients), and >8 cm

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(5 patients). The increased expression of the iNOS protein showed a significant correlation with complicated bile duct stone ($p$=0.037) and differentiation ($p$=0.032). Furthermore, these data also demonstrated that high iNOS expression was dramatically associated with pathology T ($p$=0.002) and pathology M ($p$=0.029), which serve as important prognostic markers for patients with ICC. iNOS-positive expression in ICC tended to be correlated with Wip1-positive/MMP-2-positive or MMP-9-positive ($p$=0.019, $p$=0.028, $p$=0.046, respectively), which is in agreement with our previous and other studies.[16,20–23]

## Expression of iNOS is up-regulated in ICC tissues and cell lines

In a previous study, researchers observed that iNOS was highly expressed in ICC.[14,15] To confirm this finding, we evaluated the expression of iNOS in 45 pairs of frozen ICC tissues and corresponding normal tissues located 5 cm from the tumor by immunostaining and qRT-PCR. Immunohistochemistry data showed that iNOS expression was significantly up-regulated in ICC tissues (Figure 1A) compared with normal samples (Figure 1B). Consistently, expression of iNOS mRNA was significantly higher in ICC specimens than in normal tissues ($p$<0.05; Figure 1C). iNOS expression was significantly inversely associated with metastasis and the pathological type of the patients (Figure 1D and E; Table 1, $p$<0.05). Furthermore, we also evaluated iNOS expression in three ICC cell lines (QBC939, ICC-9810, and SSP-25) and a normal human normal biliary epithelium cell line (HIBEpic). The relative expression levels for iNOS in these three ICC cell lines were 2.484, 3.372, and 1.4[illegible], respectively, compared with that of HIBEpic cells (Figure 1F).

## iNOS expression is essential for ICC cell proliferation and invasion

To determine the functional significance of iNOS expression in ICC, we perturbed the iNOS levels in ICC cells and investigated the effect of this modulation on cell proliferation, migration, and invasion. We used transient RNA interference strategies targeting iNOS in aggressive ICC-9810 and QBC939 cells. The efficiency of iNOS knockdown was confirmed by qPCR (Figure 2.1A) and immunoblot (Figure 2.1B and C) analyses. We observed a significant decrease in cell proliferation upon transient knockdown of iNOS compared with control cells transfected with non-T siRNA (Figure 2.2D). Since the effects of iNOS siRNA-2 targeting iNOS on growth inhibition were most significant in ICC cells, we selected iNOS siRNA-2 for use in the following experiments. Additionally, iNOS knockdown in ICC-9810 and QBC939 reduced the invasive potential of these cells, as assessed by the Boyden chamber Matrigel invasion assay (Figure 2.2E). NO production was also decreased in QBC939 cells when iNOS was knocked down with siRNA; however, the cell viability showed no significant change in these cells (Figure 2.2F). Similar results were observed in ICC-9810 cells (Figure S1). Taken together, these observations demonstrate the involvement of iNOS in the proliferation and migration of ICC cells in vitro.

## iNOS knockdown induces G0/G1 cell cycle arrest and apoptosis in vitro

We then determined the effects of iNOS on the cell cycle in ICC cell lines (QBC939 and ICC9810). As shown in Figure 3A and B, treatment with iNOS siRNA-2-induced G0/G1 phase cell cycle arrest in QBC939 cells. Furthermore, flow cytometry was employed to determine whether ICC growth inhibition mediated by iNOS siRNA-2 resulted from apoptosis. The results indicated that QBC939 cells showed high apoptosis in an iNOS siRNA-2 treatment, including early and late apoptosis (Figure 3C and D). Similar results were observed in ICC-9810 cells (Figure S2). Therefore, knockdown of iNOS dramatically induced G0/G1 cell cycle arrest and apoptosis of QBC939 and ICC-9810 cells in vitro.

## 1400 W inhibits tumor growth and metastasis of ICC cells in vitro

To functionally characterize iNOS in ICC, we next analyzed the effects of a potent and specific iNOS inhibitor (1400 W). QBC939 and ICC9810 were treated with 1400 W (from 1 to 200 μmol/L), while control cells were treated with PBS. Treatment with 1400 W was well tolerated and resulted in no significant on cell viability but low NO production at a concentration of 25 μmol/L (Figure 4A and B; Figure S3). Therefore, we used the 25 μmol/L concentration for subsequent experiments. To demonstrate the effect of the iNOS inhibitor 1400 W on the migration and mobility of ICC cells, the in vitro scratch wound-healing assay was performed. As shown in Figure 4C and E, the 1400 W treated group of QBC939 cells significantly migrated slower compared with the control group ($p$<0.05). To provide further support for the effect of 1400 W on cell migration, an in

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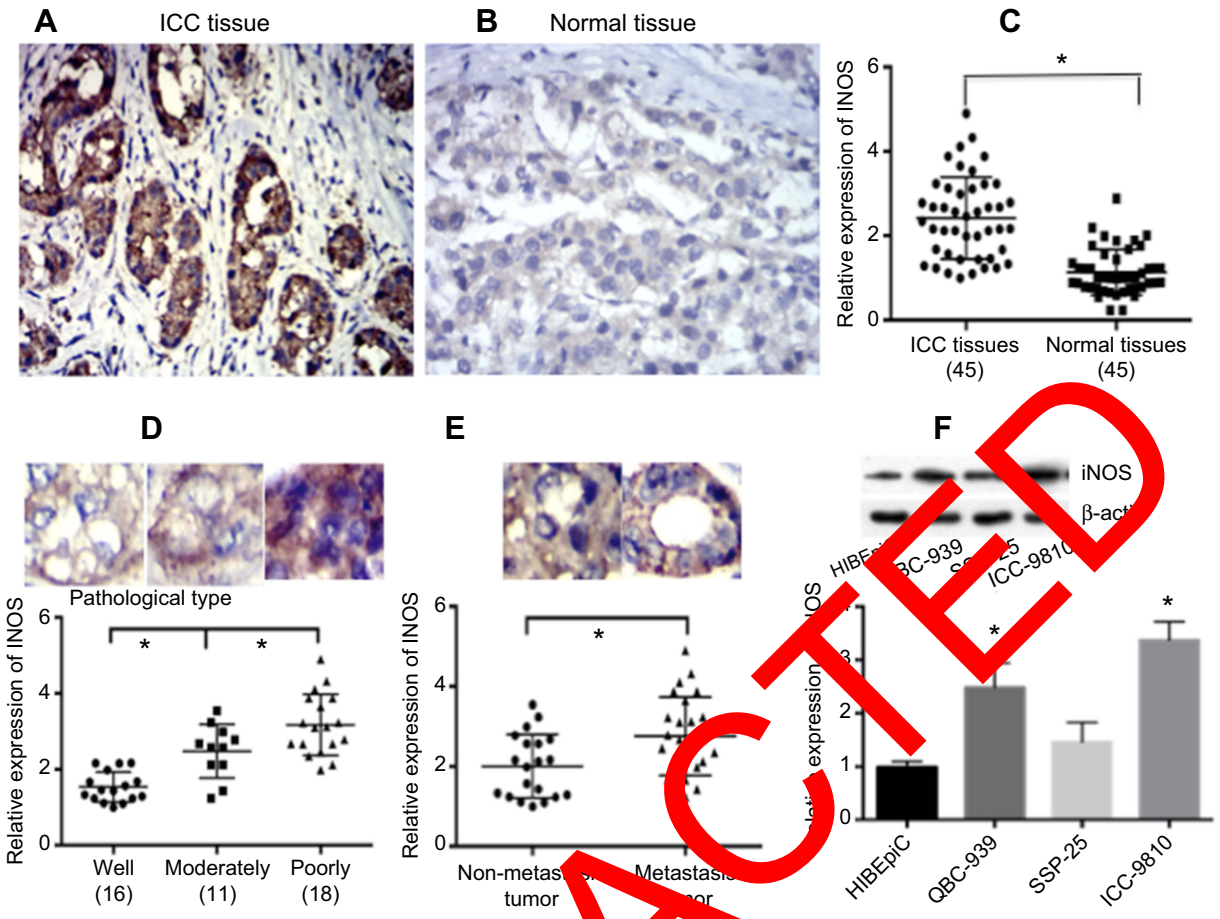


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**Figure 1** Expression of iNOS in ICC tissues and cell lines. Represen[illegible]aining of [illegible]S in ICC tissues (**A**) and adjacent normal tissues (**B**) by immunohistochemistry (the same donor). (**C**) Expression of iNOS mRNA was frequently up[illegible]egulated[illegible] ICC tiss[illegible]s compared with adjacent normal samples according to quantitative real-time PCR analysis. (**D**) iNOS mRNA expression was significantly inv[illegible]ly associa[illegible]d with tum[illegible] differentiation according to quantitative real-time PCR analysis (upper panel): Representative staining of iNOS in tumor differentiation. (**E**) [illegible]reased i[illegible]A exp[illegible]ession in metastasis tumor compared with non-metastasis tumor was detected by real-time quantitative PCR (upper panel): Representati[illegible]staini[illegible]f iNOS in metastasis tumor or not. (**F**) iNOS mRNA expression in the human normal biliary epithelium cell line (HIBEpic) and three ICC cell lines (QBC-93[illegible]CC-9810, a[illegible]SSP-25) using qRT-PCR. Data are represented as the means ± SEM of three independent experiments (upper panel): iNOS protein expression in (HIBE[illegible]QBC-939, ICC-[illegible]0, and SSP-25) cells. *$p$<0.05.
**Abbreviations:** iNOS, inducible nitric oxide[illegible]nthas[illegible]CC, intrahepatic[illegible]holangiocarcinoma.

vitro cell invasion assa[illegible] was u[illegible]ed. Cells [illegible]hat migrated through the Matrigel [illegible]atrix[illegible]re shown in Figure 4D. When iNOS wa[illegible]inhibite[illegible] the nu[illegible]ber of tumor cells migrating th[illegible]ugh M[illegible]rigel s[illegible]ficantly decreased compared with[illegible]e cont[illegible]up (Figure 4D and F) ($p$<0.05). Similar resul[illegible] were observed in ICC-9810 cells (Figure S4). These[illegible]sults imply that iNOS facilitates ICC cell migration and invasion partly through NO.

## Effect of iNOS on gene expression in vitro

ICC is one of the most highly metastatic cancers. iNOS expression has lots of pro-tumorigenic effect such as increasing vascularization, support growth and metastasis, and inhibiting apoptosis. The expression level of Wip1, MMP-2, MMP-9, STAT-3, VEGF-C, Bcl-2, Caspase-3, and JNK in ICC-9810 tumor cells in the ICC cells was determined by quantitative real-time PCR analysis. As shown in Figure 5, Wip1, MMP-2, and MMP-9 were decreased when down-regulation of iNOS ($p$<0.05); however, Caspase 3, STAT-3, VEGF-C, Bcl-2, Caspase-3, and JNK have no differences.

## Association of iNOS expression with mortality of ICC patients

Univariate and multivariate analyses were performed to compare the impact of iNOS expression and other clinical and pathological parameters on survival in ICC patients. Univariate COX analysis showed that a complicated bile duct stone, maximum tumor diameter, tumor differentiation,

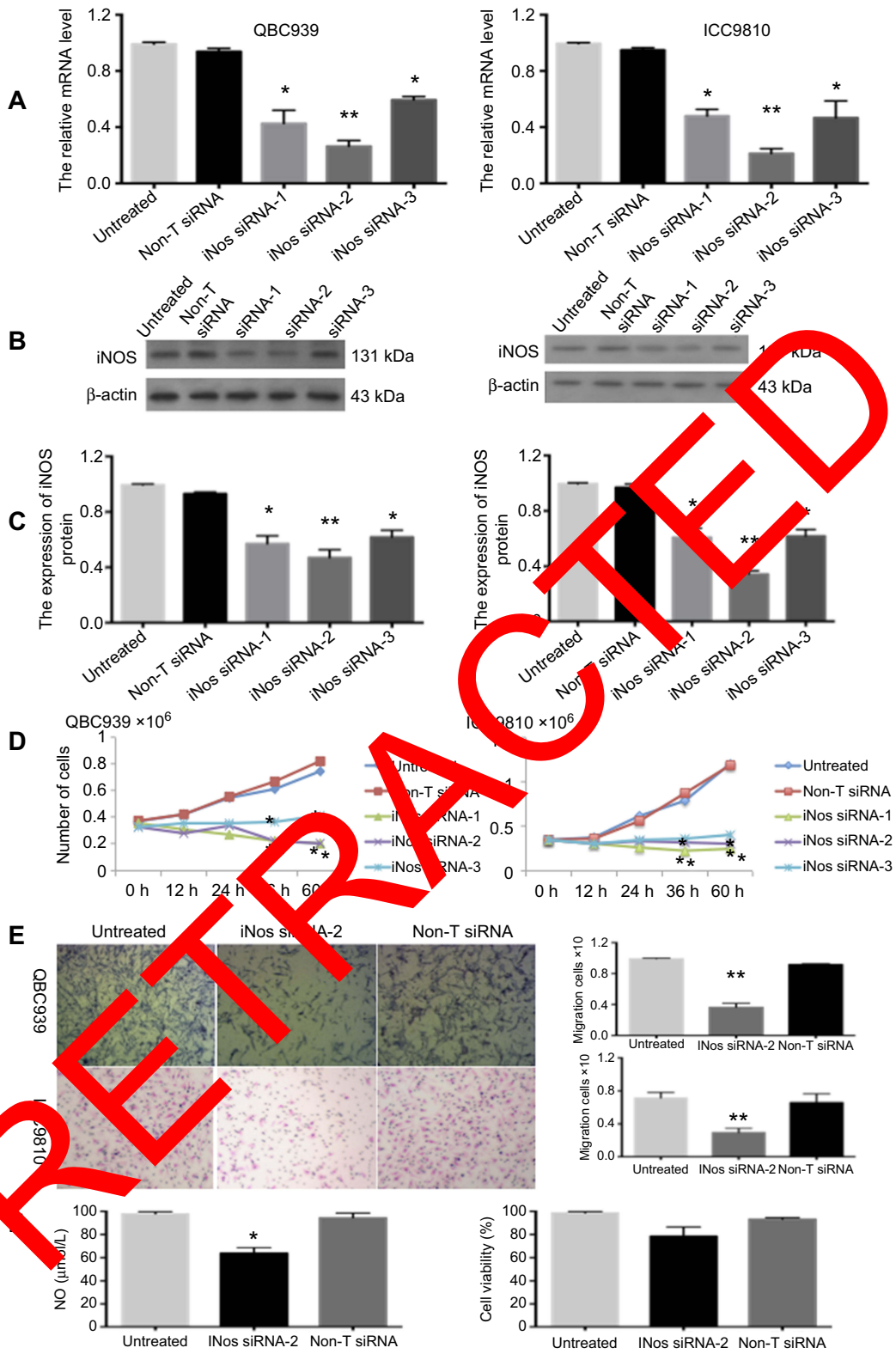


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**Figure 2** iNOS is essential for ICC cell proliferation and invasion. (**A**) iNOS siRNA or non-T small interfering RNA was transfected into QBC-939 and ICC-9810 cells. Five groups of cells were used in the present study, including untransfected cells ("untreated"), cells transfected with non-T small interfering RNA ("Non-T siRNA"), and cells transfected with iNOS-siRNA ('iNOS siRNA-1, siRNA-2, and siRNA-3'). The mRNA levels of iNOS in the five groups of QBC-939 and ICC-9810 cells were assayed by quantitative real-time PCR. (**B,C**) The protein levels of iNOS in the five groups of cells were assayed by Western blotting, and β-Actin was used as an internal control. Bar graphs are derived from densitometric scanning of the blots. (**D**) Knockdown of iNOS-inhibited cell proliferation in both QBC-939 and ICC-9810 cells according to the CCK-8 assay. (**E**) Down-regulated iNOS-suppressed migration and invasion in both QBC-939 and ICC-9810 cells. (**F**) NO production and cell viability under knockdown of iNOS in QBC-939 cells. Data are represented as the means ± SEM of three independent experiments. *$p<0.05$, **$p<0.01$ (All were compared with the untreated control group).

**Abbreviations:** iNOS, inducible nitric oxide synthase; ICC, intrahepatic cholangiocarcinoma.

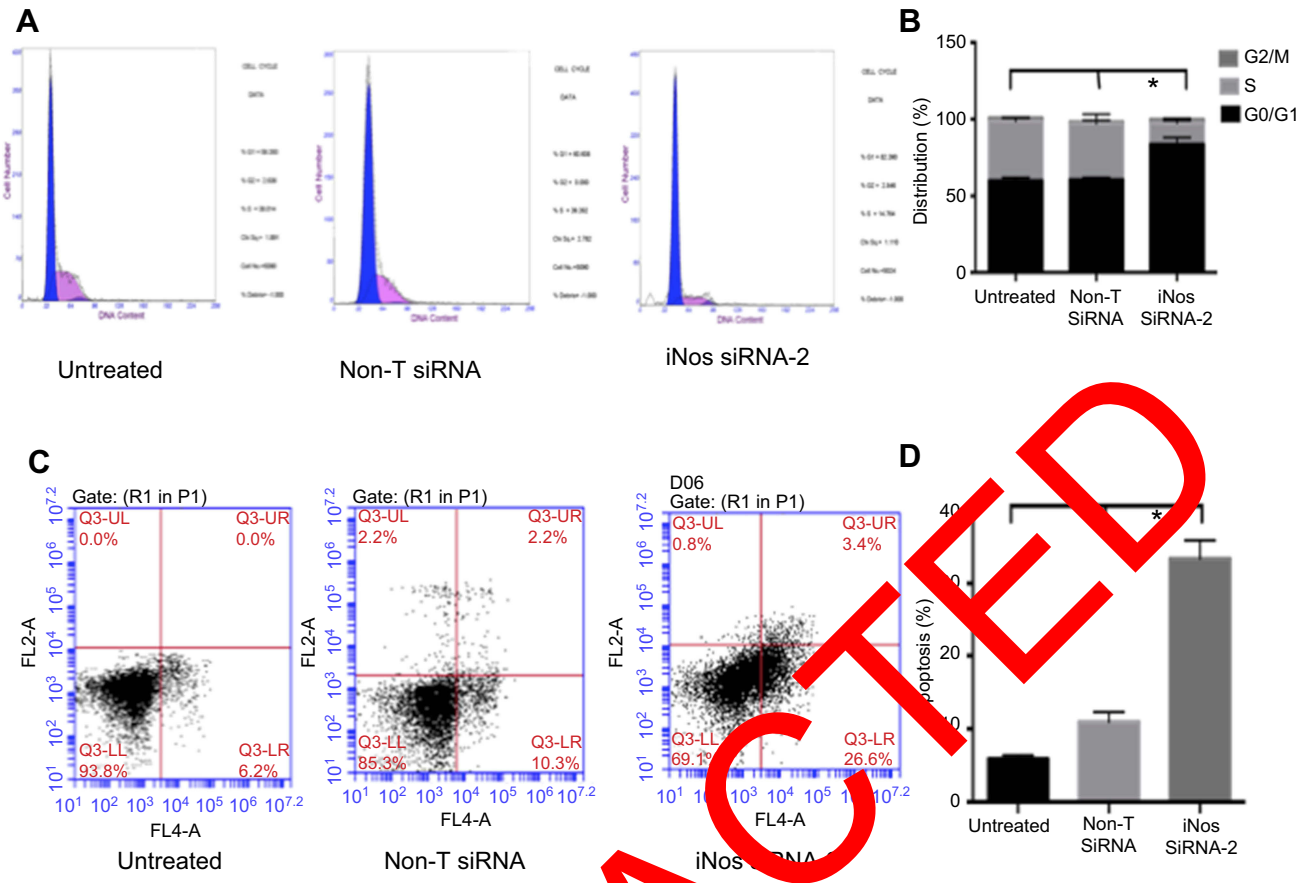


**Figure 3** iNOS knockdown induces G0/G1 cell cycle arrest and ICC cell apoptosis. (**A, B**) Flow cytometry analysis of iNOS-induced G0/G1 phase cell cycle arrest in QBC939 cells according to flow cytometry analysis. (**C, D**) Knockdown of iNOS expression increased cell apoptosis in QBC-939 cells by flow cytometry analysis. Data are presented as the means ± SEM of three independent experiments. *$p$<0.05.

**Abbreviations:** iNOS, inducible nitric oxide synthase; ICC, intrahepatic cholangiocarcinoma.

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pathology T/N, Wip1, MMP-2, MMP-9, and iNOS were prognostic factors that affected overall survival (OS) in patients who underwent radical resection for ICC ($p$<0.05). The significant prognostic factors determined by the univariate analysis were included in a multivariate Cox analysis, which showed that the maximum tumor diameter, tumor differentiation, complicated bile duct stone, pathology T/N, Wip1, MMP-2, and iNOS expression were independent adverse prognostic factors that affected OS of patients who underwent radical resection for ICC (Table 2).

The univariate Cox analysis showed that tumor differentiation, complicated bile duct stone, pathology T/N, Wip1, MMP-2, MMP-9, and iNOS were the prognostic factors for tumor-free survival (TFS) in patients who underwent radical resection for ICC ($p$<0.05). The significant prognostic factors determined by the univariate analysis were included in a multivariate Cox analysis, which showed that tumor differentiation, complicated bile duct stone, pathology T/N, Wip1, MMP-2, and iNOS expression were independent adverse prognostic factors that affected TFS of patients who underwent radical resection for ICC (Table 3).

We conducted Kaplan–Meier analysis and a log-rank test to study the relationship between iNOS protein expression and survival. Patients were divided into two groups according to iNOS expression (IHC). MRI, CT, or B-mode ultrasound were performed every 6 months following surgery. There were 32 patients who experienced relapse in this cohort of patients. Both OS and TFS were significantly shorter in patients expressing iNOS (log-rank test $p$=0.0232, $p$=0.0416, respectively) (Figure 6). Thus, high expression of iNOS is correlated with poor prognosis in ICC patients.

## Discussion

ICC represents the second most common primary liver cancer after hepatocellular carcinoma (HCC), with increasing incidence and poor prognosis.[4] Complete resection is the only potentially curative treatment for patients with resectable

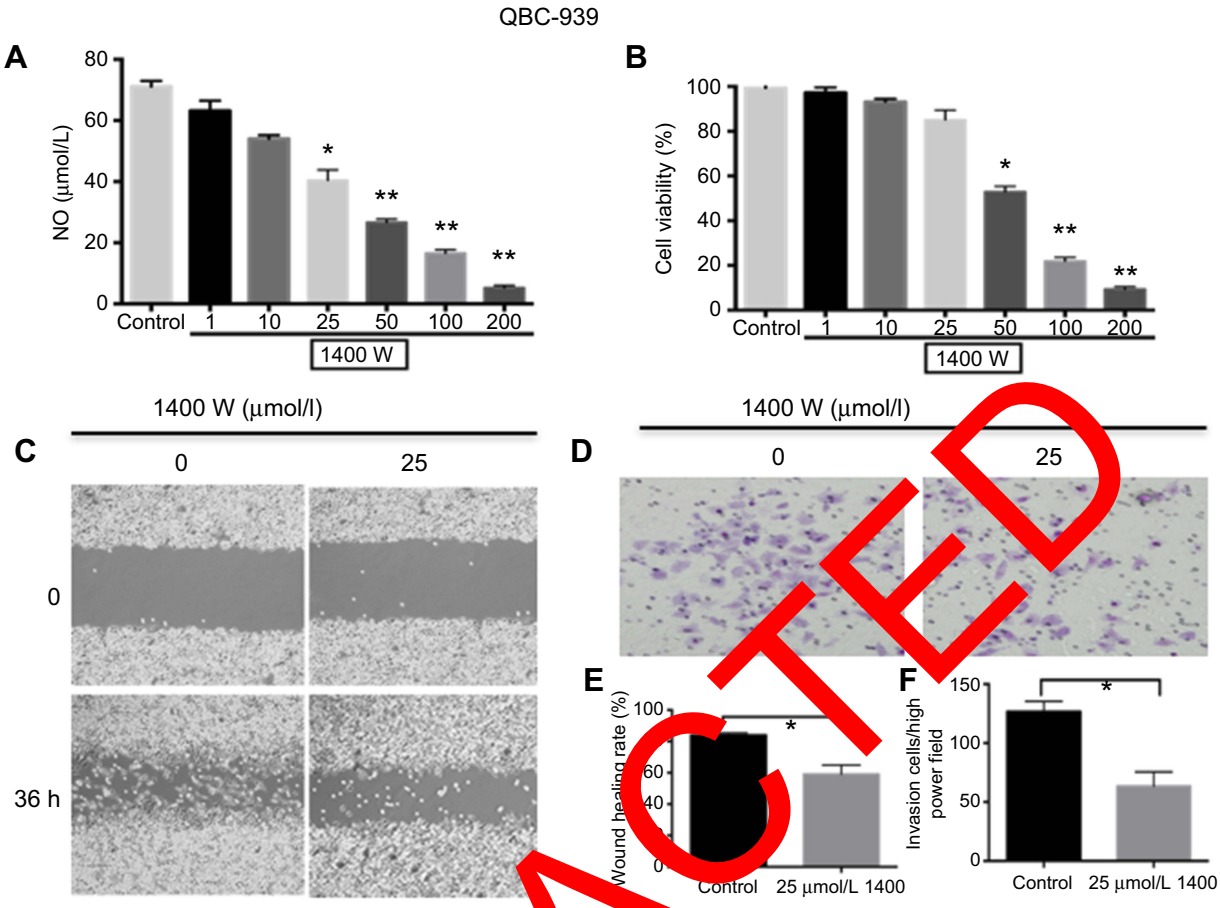


**Figure 4** iNOS inhibitor (1400 W) inhibits tumor growth and metastasis of ICC cells in vitro. (**A**) The effects of different concentrations of 1400 W on the NO content. (**B**) The influence of different concentrations of 1400 W on cell viability. (**C**) Scratch wound healing assay. 1400 W-treated cells and control group cells at 2 time points (0 and 36 hrs) are shown. (**E**) Graphical presentation of the wound-healing rate was measured with the following formula: (0 hrs width-36 hrs width of wound)/(0 hrs width of wound). (**D**) The invasive properties of the cells were analyzed by an invasion assay using a Matrigel-coated plate. 1400 W suppressed migration and invasion in vitro in QBC939 cells. (**F**) Bar graphs are derived from the number of invasive cells in the 1400 W group and control group. Data are presented as the means ± SEM of three independent experiments. *$p$<0.05 (All were compared with the untreated control group), **
**Abbreviations:** iNOS, inducible nitric oxide synthase; ICC, intrahepatic cholangiocarcinoma; NO, nitric oxide.

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disease. Unfortunately, most patients are diagnosed at advanced stages and are unresectable.[5,24] Furthermore, despite the use of systemic therapy, the median survival of patients with advanced disease still remains short (only 7–12 months), with 5-year survival being less than 5%.[1,25,26] Considering the overall poor prognosis of patients with ICC, it is of paramount importance to explore the molecular mechanisms involved in carcinogenesis and tumor progression in this disease to identify novel therapeutic targets and develop effective treatment strategies.

Nitric oxide synthase (NOS) is a member of a family of key enzymes that are responsible for generating NO from L-arginine. There are three distinct isoforms of NOS. Neuronal (nNOS and NOS1) and endothelial (eNOS and NOS3) NOS proteins are constitutive calcium-dependent forms and produce a small amount of NO. The third isoform (iNOS, NOS2) is calcium-independent and is inducible to produce a higher level of NO in response to inflammatory stimuli.[27] iNOS has been associated with many tumors and their progression into metastasis. iNOS positivity has consistently been reported in human cancers at a variety of sites, including the prostate, lung, bladder, breast, pancreas, gastric, oral cavity, esophagus, and colon.[28–33] However, there are many conflicting reports. The role of iNOS in tumor biology is complex and quite perplexing, with both promoting and inhibiting actions having been described.[12,13,27] The main reasons for the conflicting roles of iNOS in tumor biology are the genetic make-up of tumor cells and the concentration of NO in the tumor-cell microenvironment.[34] In general, it has been suggested that at high concentrations NO may have an anti-neoplastic function whereas at low levels it can be pro-angiogenic and pro-tumor formation.[35,36] Our results show iNOS protein expression and associated high-output

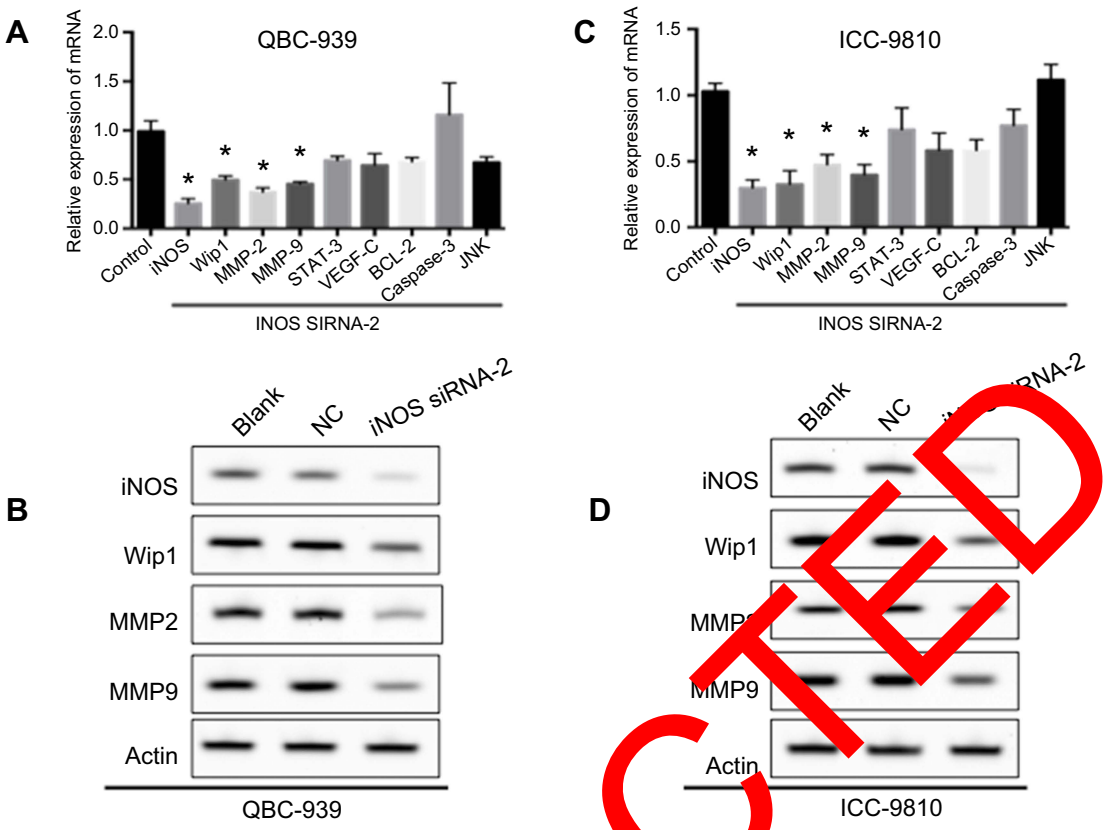


**Figure 5** Gene expression results for iNOS down-regulation. (**A, B**), Wip1, MMP-2, MMP-9 were down-regulation in QBC-939 tumor cells following SiRNA-iNOS treatment. (**C, D**) Similar results were shown in ICC-9810 cells. The values are mean ± SD relative to control values, and normalized based on GAPDH (n=3). The * indicates statistical significance at $p<0.05$ in comparison to control.

**Abbreviations:** iNOS, inducible nitric oxide synthase; ICC, intrahepatic cholangiocarcinoma.

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NO production, which related to ICC cell proliferation and invasion.

Primary sclerosing cholangitis, liver fluke infection, and hepatolithiasis are well-established risk factors for ICC. These risk factors, as well as viral or parasitic infections, are associated with the presence of chronic inflammation. Chronic inflammation is estimated to contribute to approximately 25% of the human cancers.[37] Sustained induction of iNOS in chronic inflammation can produce ROS and RNS, causing DNA damage which leads to the development and progression of cholangiocarcinoma.[38,39] Increased iNOS activity has been positively correlated with the degree of malignancy in gynecological tumors, gastric cancer, squamous cell carcinoma, hepatocellular carcinoma, melanoma, and leukemia.[27] However, the precise role of iNOS in ICC or its precursors or metastatic lesions has largely remained elusive. In the present study, we observed that iNOS was frequently up-regulated in ICC tissues compared with the adjacent normal samples and observed that iNOS expression was significantly inversely associated with tumor differentiation. Thus, the expression level of iNOS was significantly higher in poorly differentiated ICC tissues compared with their well-differentiated counterpart. These results imply that iNOS facilitates ICC carcinogenesis.

The effect of iNOS on tumor metastasis cannot be easily classified as "pro-metastasis" or "anti-metastasis", as it may rely on other factors, such as the cell type, NO concentration, organs involved, or even which step of metastasis iNOS influences.[10] Previous studies have shown that circulating metastatic cells have increased iNOS expression in lung cancer patients with bone metastasis, suggesting that iNOS serves as a biomarker for the metastasis of lung cancer.[28] In the present study, we certified that iNOS was frequently up-regulated in ICC and found that increased iNOS expression was closely related to the vessel invasion and lymph node metastasis of ICC. Furthermore, we demonstrated that knockdown of iNOS and the iNOS inhibitor 1400 W could suppress ICC cell migration and invasion through NO in vitro. Taken together, these findings imply that iNOS promotes cell migration and invasion in ICC. In the process of invasion and metastasis of tumor cells, destruction of the extracellular matrix (ECM) is an

**Table 2** Analysis of the impact on OS after radical resection

| Clinicopathological characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | *p* | HR (95% CI) | *p* | HR (95% CI) |
| Gender | | | | |
| Male | 1 | | | |
| Female | 0.621 | 0.932 (0.716–1.231) | | |
| Age | | | | |
| >55 years | 1 | | | |
| ≤55 years | 0.276 | 1.134 (0.873–1.534) | | |
| Complicated bile duct stone | | | | |
| Yes | 1 | | | |
| No | 0.003 | 1.423 (1.102–1.765) | 0.00 | 1. (1.245–3.061) |
| Maximum tumor diameter | | | | |
| ≤5 | 1 | | | |
| 5<d≤10 | 0.002 | 1.712 (1.089–2.534) | 0.0 | 1.826 (1.201–2.743) |
| Differentiated | | | | |
| Well | 1 | | | |
| Moderate | 0.022 | 1.622 (1.43 .455) | 0.0 | 1.922 (1.231–3.256) |
| Poorly | 0.002 | 2.925 (1.765–4.276) | 0.011 | 2.327 (1.652–3.986) |
| Pathology T (T1+2)/(T3+4) | | | | |
| Yes | 1 | | | |
| No | 0.001 | 1. (1.438–1.93 | 0.015 | 1.325 (0.897–2.674) |
| Pathology N 0/1 | | | | |
| Yes | 1 | | | |
| No | 0.009 | .746 (1.098–2.543) | 0.022 | 1.856 (1.743–2.854) |
| Pathology M 0/1 | | | | |
| Yes | 1 | | | |
| No | 0. | 1.534 (1.098–2.512) | | |
| Wip1 | | | | |
| Yes | 1 | | | |
| No | 0.008 | 1.274 (1.358–2.743) | 0.022 | 1.724 (1.356–3.098) |
| MMP-2 | | | | |
| Yes | 1 | | | |
| No | 0.022 | 1.586 (1.128–2.582) | 0.016 | 1.923 (1.452–3.277) |
| MMP-9 | | | | |
| Yes | 1 | | | |
| No | 0.005 | 2.106 (1.376–3.751) | 0.102 | 1.312 (0.935–2.628) |
| iNOS | | | | |
| Yes | 1 | | | |
| No | 0.008 | 1.738 (1.192–2.461) | 0.008 | 1.862 (1.736–3.628) |

**Abbreviations:** OS, overall survival; iNOS, inducible nitric oxide synthase.

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essential initial step. A large body of experimental data has shown that matrix metalloproteinases (MMPs) play a critical role in ECM degradation, which is closely related to the invasiveness and metastasis of tumor cells.[10,40] iNOS has been implicated in modulating MMP-1, −2, −3, −8, and −9 expression and therefore affects tumor-cell migration and invasion.[41,42] Previous studies have reported that the expression level of iNOS was positively correlated with

**Table 3** Analysis of the impact on TFS after radical resection

| Clinicopathological characteristics | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | *p* | HR (95% CI) | *p* | HR (95% CI) |
| Gender | | | | |
| Male | 1 | | | |
| Female | 0.526 | 0.847 (0.617–1.342) | | |
| Age | | | | |
| >55 years | 1 | | | |
| ≤55 years | 0.361 | 1.229 (0.910–1.729) | | |
| Complicated bile duct stone | | | | |
| Yes | 1 | | | |
| No | 0.004 | 1.226 (0.957–1.425) 0.034 | | 1.345 ( 57–2.692) |
| Maximum tumor diameter | | | | |
| ≤5 | 1 | | | |
| 5<d≤10 | 0.362 | 1.712 (1.058–3.281) | | |
| Differentiated | | | | |
| Well | 1 | | | |
| Moderate | 0 | 1.317 (1.065–1.453) | 0.011 | 1.526 (1.048–2.848) |
| Poorly | 0.007 | 1.926 (1.177–3.023) | 0.001 | 2.174 (1.481–4.025) |
| Pathology T (T1+2)/(T3+4) | | | | |
| Yes | 1 | | | |
| No | 0.007 | 1.821 (1.1 2.659) | 0.612 | 2.251 (1.401–3.519) |
| Pathology N 0/1 | | | | |
| Yes | 1 | | | |
| No | 0.001 | 1 56 ( 57–2. 3) | 0.003 | 1.935 (1.386–3.572) |
| Pathology M 0/1 | | | | |
| Yes | | | | |
| No | 0 24 | (1.087–2.213) | | |
| Wip1 | | | | |
| Yes | 1 | | | |
| No | 0.015 | 1.045 (0.845–1.962) | 0.012 | 1.381 (1.162–3.491) |
| MMP-2 | | | | |
| Yes | 1 | | | |
| No | 0.002 | 1.262 (1.085–2.352) | 0.022 | 1.617 (1.628–4.136) |
| MMP-9 | | | | |
| Yes | 1 | | | |
| No | 0.022 | 1.162 (1.066–2.913) | 0.055 | 1.732 (1.453–2.735) |
| iNOS | | | | |
| Yes | 1 | | | |
| No | 0.001 | 1.380 (1.021–2.159) | 0.003 | 1.562 (1.683–2.835) |

**Abbreviations:** TFS, tumor-free survival; iNOS, inducible nitric oxide synthase.

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that of MMP-9 in HCC tissues.[33] In human prostate cancer cells, enhanced migration and invasion of the photostressed surviving cells was due to NO-mediated activation of MMP-9.[31,36] Ishii et al, also reported that NO generation may promote tumor progression through the activation of various MMP.[43] The present study demonstrated that iNOS-positive expression was correlated with that of MMP-2 or MMP-9 in ICC tissues, suggesting that iNOS promotes cell migration and invasion in ICC by up-regulating MMP expression. Wip1 functions as an oncogene and inhibits

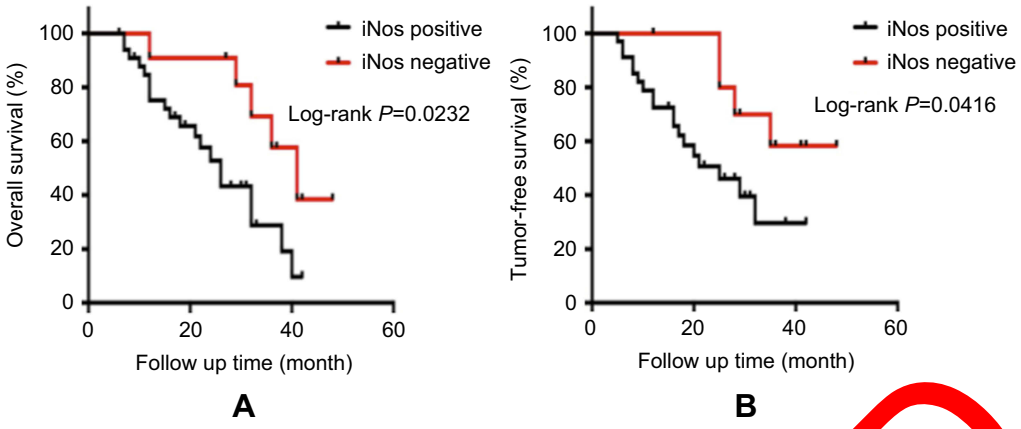


**Figure 6** Kaplan–Meier survival curves for iNOS expression. (**A**) Overall survival (**B**) tumor-free survival. Patients with positive iNOS protein expression had a significantly worse outcome compared to patients with negative iNOS expression. ($p$=0.0232, log-rank test) and ($p$=0.0416, log-rank test).
**Abbreviation:** iNOS, inducible nitric oxide synthase.

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p53 functions and activity in cancer cells.[16,17] In the present study, these data demonstrated that iNOS-positive expression was positively correlated with that of Wip1, MMP-2, and MMP-9 in ICC tissues. These findings indicate that Wip1 is involved in the tumorigenicity and invasion of human ICC at least in part through the MMP-2 signaling pathway.[16] Both iNOS and Wip1 promote ICC cell migration and invasion by upregulating MMP expressi To evaluate the underlying mechanism, further investiga ions are needed.

In the present study, the data show tha high pressi n of iNOS was correlated with poo prognos in ICC patients after radical resection. M tivari te Cox analysis demonstrated that iNOS expr ion was a independent adverse prognostic factor fo both S and TFS, uggesting that iNOS expression y be a po tial biomarker for poor prognosis in IC . Tumor invasion and lymph node metastasis are sig ficant egative prognostic factors for ICC patients.[1,6,44] Cli al data sh w that iNOS expression is an ind ender predi r poor survival in women with estrog receptor-negative breast tumor.[11] In other clin studies of gastric carcinoma, patients with intermediate high iNOS expression had much poorer prognosis accor ng to the 5-year survival analysis.[45]

In conclusion, we have demonstrated that iNOS may be a useful biomarker to predict disease progression in ICC. Overexpression of iNOS is correlated with tumor recurrence and promotes tumor progression. In order for this protein to be established as a powerful prognostic factor and potential therapeutic target, subsequent studies are required for clarifying the mechanisms underlying iNOS-induced migration and invasion by ICC. Our study provides evide e sh g that i OS may be a potential target for t management of C.

## Abbreviations

C, intrahepatic cholangiocarcinoma; iNOS, inducible itric oxide nthase; RT-PCR, real-time quantitative PCR; NS, reactiv nitrogen species; ROS, reactive oxygen species; TME, tumor microenvironment; OD, optical density; NO, nitric oxide; ER, estrogen receptor; Wip1, wild-type p53-induced phosphatase; MMP, matrix metalloproteinase; STAT-3, signal transducer and activator of transcription-3; VEGF-C, vascular endothelial growth factor C; Bcl-2, B-cell lymphoma 2; JNK, Jun N-terminal kinases.

## Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the ethical committee of Hunan Normal University. Informed consent was obtained from all individual participants included in the study.

## Availability of data and materials

Data and materials are included in the manuscript.

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## Disclosure

The authors report no financial or commercial conflicts of interest in this work.

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# Supplementary materials

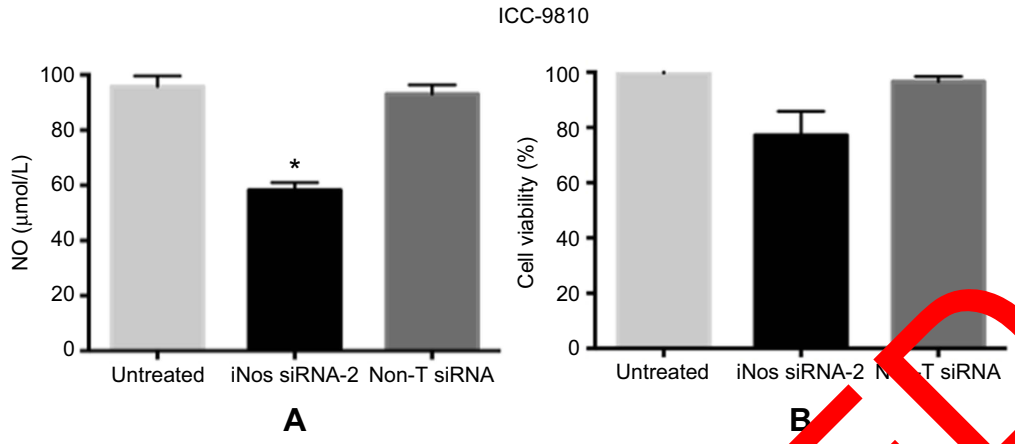


**Figure S1** NO (**A**) production and cell viability (**B**) under knockdown of iNOS in ICC-9810 cells. Data are represented as the means ± SEM of three independent experiments. *$p$<0.05 (All were compared with the untreated control group).
**Abbreviations:** NO, nitric oxide; iNOS, inducible nitric oxide synthase; ICC, intrahepatic cholangiocarcinoma.

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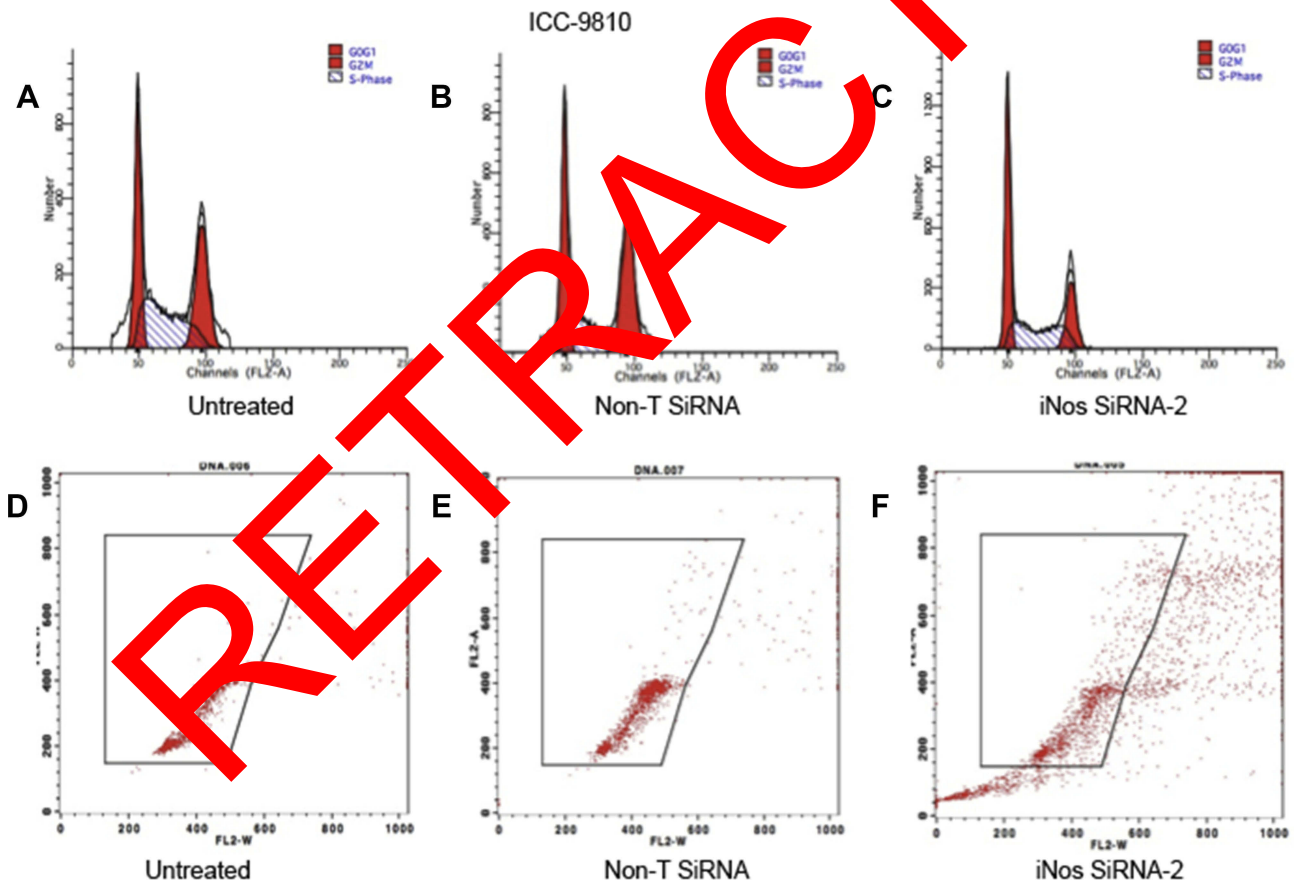


**Figure S2** iNOS knockdown induces G0/G1 cell cycle arrest and ICC cell apoptosis. (**A–C**) Down-regulation of iNOS-induced G0/G1 phase cell cycle arrest in ICC-9810 cells according to flow cytometry analysis. (**D–F**) Knockdown of iNOS expression increased apoptosis in ICC-9810 cells by flow cytometry analysis. Data are presented as the means ± SEM of three independent experiments.
**Abbreviations:** iNOS, inducible nitric oxide synthase; ICC, intrahepatic cholangiocarcinoma.

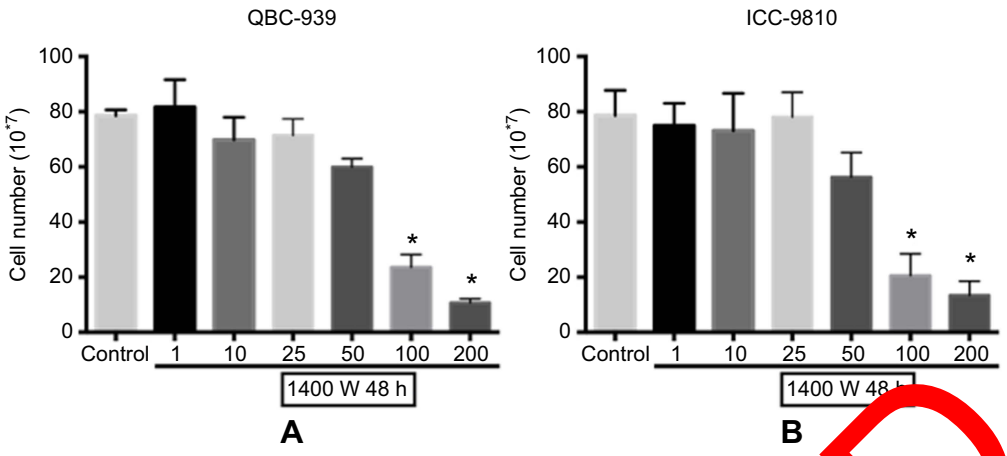


**Figure S3** QBC-939 (**A**) and ICC-9810 (**B**) cells were treated with 0–200 μmol/L 1400 W for 48 hrs, and CCK-8 was performed to measure cell proliferation. *$p$<0.05.
**Abbreviation:** ICC, intrahepatic cholangiocarcinoma.

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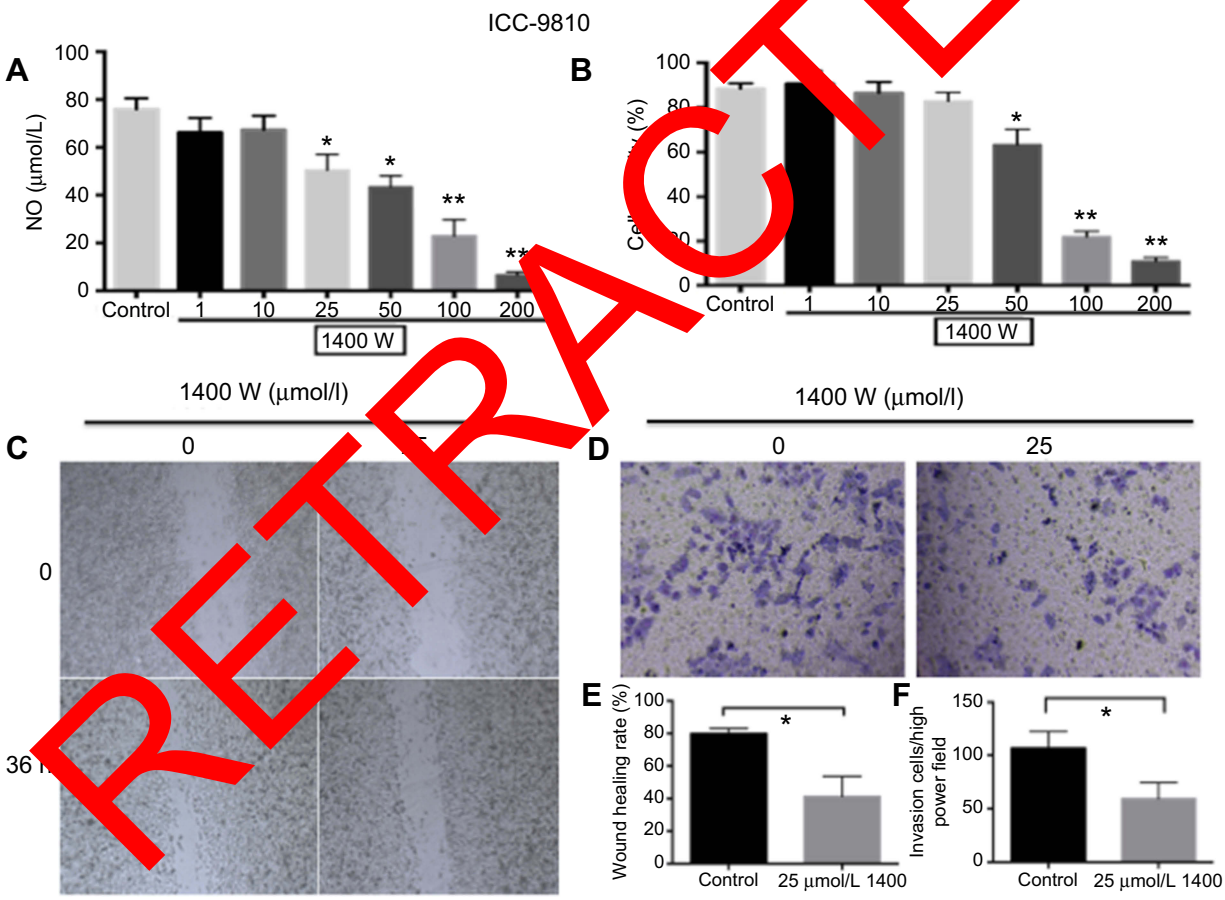


**Figure S4** iNOS inhibitor (1400 W) inhibits tumor growth and metastasis of ICC cells in vitro. (**A**) The effects of different concentrations of 1400 W on the NO content. (**B**) The influence of different concentrations of 1400 W on cell viability. (**C**) Scratch wound-healing assay. 1400 W-treated cells and control group cells at two time points (0 and 36 hrs) are shown. (**E**) Graphical presentation of the wound-healing rate was measured with the following formula: (0 hrs width-36 hrs width of wound)/(0 hrs width of wound). (**D**) The invasive properties of the cells were analyzed by an invasion assay using a Matrigel-coated plate. 1400 W suppressed migration and invasion in vitro in ICC-9810 cells. (**F**) Bar graphs are derived from the number of invasive cells in the 1400 W group and control group. Data are presented as the means ± SEM of three independent experiments. *$p$<0.05, **$p$<0.01 (All were compared with the untreated control group).
**Abbreviations:** iNOS, inducible nitric oxide synthase; ICC, intrahepatic cholangiocarcinoma; NO, nitric oxide.

**Table S1** Primer sequences

| Gene name | Forward primer sequence (5′–3′) | Reverse primer sequence (5′–3′) |
|---|---|---|
| *iNOS* | CACTTCCAACGCAACATGGG | CTTTGACCCAGTAGCTGCCA |
| *STAT-3* | CCCCCTACCAAGAAGCACTG | ACCGACAGCCAGTCAAAGAG |
| *MMP-9* | TCGTGGTTCCAACTCGGTTT | CGGCCCTCGAAGATGAAGG |
| *VEGF-C* | AGGCCAACCTCAACTCAAGG | TCGCGACTCCAAACTCCTTC |
| *Bcl-2* | TGGGAGAACGGGGTACGATA | CATGACCCCACCGAACTCAA |
| *caspase 3* | GTTGGCGTCGCCTTGAAATC | TGAGGTTTGCTGCATCGACA |
| *Wip1* | GAAGGATGACTTTGTCAG | CCCAGACTTGTTCATTAC |
| *MMP-2* | GCAGCCCATGAGTTCGGCCAT | AGCATCAGGGGAGGGCCCATA |
| *JNK* | CTACAAGTCCTCGCTGGTC | CCTTGTTGCTACCGTCGAAG |
| *GAPDH* | GACAGTCAGCCGCATCTTCT | GCGCCCA[illegible]CCAAATC |

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