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SOX9 expression decreases survival of patients with intrahepatic cholangiocarcinoma by conferring chemoresistance

Running title: SOX9 in intrahepatic cholangiocarcinoma

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Abbreviation

CHEK1, Checkpoint kinase 1
CK19, cytokeratin 19
CSC, cancer stem cell
DFS, disease free survival
DR, ductular reaction
EpCAM, epithelial cell adhesion molecule
eCCA, extrahepatic cholangiocarcinoma
iCCA, intrahepatic cholangiocarcinoma
MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
OS, overall survival
PBS, phosphate-buffered saline
RIPA, radio immunoprecipitation assay
RNAi, RNA interference
SD, standard deviation
SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis
siRNA, short interfering RNA
SOX9, Sex-determining region Y (SRY)-box 9
TBST, Tris-buffered saline with Tween 20

Abstract

Background: Sex-determining region Y (SRY)-box 9 (SOX9) expression confers cancer stem cell features. However, SOX9 function in intrahepatic cholangiocarcinoma (iCCA) is unknown. This study investigated the effects and underlying mechanisms of SOX9 in iCCA.

Methods: SOX9 expression in 59 iCCA patients was examined by immunohistochemistry. The association between SOX9 expression and clinical outcome was evaluated. Gene signature and biological functions of SOX9 in iCCA were examined *in vitro*.

Results: iCCA patients with high SOX9 expression had shorter survival time than those with low SOX9. In patients receiving chemotherapy, median survival time in patients with low and high levels of SOX9 were 62 and 22 months, respectively. *In vitro*, gemcitabine increased SOX9 expression in iCCA cells. When SOX9 was knocked down, gemcitabine-induced apoptosis was markedly increased. Silencing SOX9 significantly inhibited gemcitabine-induced phosphorylation of checkpoint kinase 1, a key cell cycle check point protein that coordinates the DNA damage response, and inhibited the expression of multidrug resistance genes. Microarray analyses showed that SOX9 knockdown in CCA cells altered gene signatures associated with multidrug resistance and p53 signaling.

Conclusions: SOX9 governs the response of CCA cells to chemotherapy. SOX9 is a biomarker to select iCCA patients eligible for efficient chemotherapy.

(Words: 197)

Introduction

Cholangiocarcinoma (CCA) is the second most common primary liver cancer following hepatocellular carcinoma (HCC), and accounts for approximately 10%–15% of all primary liver malignancies (1). The global incidence and mortality rate for CCA have been increasing over the last decades (2, 3). Anatomically, CCA is classified into intrahepatic (iCCA) and extrahepatic cholangiocarcinoma (eCCA) depending on the location of the tumor along the biliary tract (4). To date, curative surgical resection is the most efficient treatment for long-term survival of selected iCCA patients (5, 6). However, in most cases, the tumors are quite advanced at the time of diagnosis and surgical resection is not possible (5). Systemic chemotherapy and radiotherapy regimens remain the only approach to render patient eligible for surgery and palliative treatment (7). However, the response of iCCA to these treatments is very weak (7). Therefore, elucidating the underlying mechanisms of iCCA chemoresistance is one key issue to improve survival of patients.

Sex-determining region Y-box (SRY-box) containing gene 9 (SOX9) belongs to the SOX family of transcription factors (8). It is widely expressed in multiple organs during embryonic development, including the liver (9, 10). In liver embryogenesis, SOX9 expression is the most specific and earliest marker of hepatoblasts and determines the timing of intrahepatic bile duct morphogenesis (11, 12). In normal adult liver, SOX9 is expressed in the periportal small intrahepatic ducts, and peribiliary glands lining the large bile ducts (13). SOX9 plays important roles in maintaining liver homeostasis, regulating liver regeneration, and eventually in liver cancer development (14). In acute or chronic liver disease, SOX9 expression robustly manifests in ductular reactions (DRs), which contain putative progenitor cells capable of differentiating into both cholangiocytes and hepatocytes (15). Moreover, SOX9 positive cells express stem cell markers, such as epithelial cell adhesion molecule (EpCAM), neural cell adhesion molecule, CD133, CXC motif chemokine receptor 4 (13, 16). In contrast to normal hepatocytes, where SOX9 is not expressed, a subset of HCC cells displayed SOX9 expression. These patients usually demonstrate severe venous cancer invasion, advanced tumor stage and shorter survival (17). Recent studies reported that SOX9 positive HCC cells exhibit liver cancer stem cell-like features, and that SOX9 in cancer cells confers self-renewal and tumorigenicity by promoting symmetrical cell division (17, 18). To date, only few studies had addressed the role of SOX9 in CCA (19). Here, we report clinical and functional data supporting an oncogenic role and therapeutic significance of SOX9 expression in iCCA.

Materials and Methods

Patients and liver tissues

This study enrolled 59 iCCA patients from Tübingen, Germany (18 iCCA patients) and Rennes, France (41 iCCA patients) between 2002 and 2010. In addition, 21 liver tissues from patients with chronic hepatitis B infection were enrolled in Mannheim, Germany. Basic characteristics of the enrolled chronic hepatitis B and iCCA patients are shown in **Supplementary Table 1 and 2**. The study protocol fulfilled national laws and regulations and was approved by the local Ethics Committees.

Cell culture and treatment

The following cell lines were investigated in the study: CC-SW-1 and HuCCT-1 (iCCA lines), EGI-1 and TFK-1 (eCCA lines), HCCC-9810 (mix CCA line) and MMNK-1 (normal cholangiocyte line). EGI-1, CC-SW-1 and MMNK-1 were cultured in DMEM (BE12-709F, Lonza) supplemented with 10% FBS (10270-098, Invitrogen), 4mM L-glutamine (17-605C, Lonza) and 100 U/mL penicillin/streptomycin (A2210 Biochrom KG). TFK-1 and HuCCT-1 were cultured in RPMI1640 supplemented with 10% FBS (10270-098, Invitrogen), 4mM L-glutamine (17-605C, Lonza) and 100 U/mL penicillin/streptomycin (A2210 Biochrom KG). All the cell lines were cultured in a humidified incubator at 37° and with 5% CO_2 atmosphere.

Cells underwent starvation without FBS medium for 10 to 16 hours before treatment with gemcitabine and cisplatin (Kindly provided by Prof. Lu LG, Shanghai Jiao Tong University School of Medicine), which was dissolved in phosphate-buffered saline (PBS) to make a 100mM stock solution and diluted with cell culture medium to indicated concentrations during treatment.

Immunohistochemistry and staining evaluation

Tissue microarray assay was performed as previously described (20). In brief, formalin-fixed, paraffin-embedded specimens were deparaffinized in serial ethanol dilutions and rehydrated. After a single PBS wash, heat-induced antigen retrieval was performed with 1mM EDTA solution, pH 8.4 (03677; Sigma-Aldrich, Steinheim, Germany) at 98°C for 10 minutes. Endogenous peroxidase activity was blocked with Dako dual endogenous enzyme blocking reagent (S2003; Dako,Via Real Carpinteria, California, USA), followed by blocking with 3 % hydrogen peroxidase for 5 minutes at room temperature to prevent unspecific binding of antibodies. The tissue sections were incubated with polyclonal rabbit anti-SOX9 antibody

(HPA001758; Sigma-Aldrich) at a dilution of 1:100, or monoclonal mouse anti-CK19 antibody (SC-6287; Santa Cruz) at a dilution of 1:100 overnight at 4°C. The specimens were subsequently washed in PBS for 3 × 5 minutes, and incubated with anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase for 1 hour at room temperature, and then detected with 3.3'-diaminobenzidine for 7 minutes. The slides were counterstained with hematoxylin. All sections were dehydrated and mounted with malinol mounting medium.

Immunostaining results for SOX9 were scored semi-quantitatively based on the intensity and proportion of positive tumor cell nuclei. In detail, the intensity score of SOX9 nuclear staining was defined as four grades: 0, negative; 1, weak with color yellow; 2, medium with color brown; 3, strong with color black. The number of cells with SOX9 positive nuclei was defined as six grades: 0, no detectable positive cells; 1, positive cells ≤ 1%; 2, positive cells > 1%, and ≤ 10%; 3, positive cells > 10%, and ≤ 33%; 4, positive cells > 33%, and ≤ 66%; 5, positive cells > 66%. The final immune staining scores were calculated as the intensity scores × the proportion scores. The samples with final scores over 10 were defined as "high SOX9 expression", and the remainder as "low SOX9 expression". The representative pictures of SOX9 staining and for semi-quantitative scoring system are presented in **Supplementary Figure 1**. CK19 expression was categorized into high expression and low expression according to the immunoreactivity in tumor cells. The immunoreactivity of CK19 was defined as four grades: 0, positive cells ⩽1%; 1, positive cells > 1% and ⩽ 33%; 2, positive cells > 33% and ⩽66%; 3, positive cells > 66%. The samples with grade 3 were defined as high CK19 expression, and the others were low CK19 expression.

RNA interference (RNAi)

For transient transfection of short interfering RNA (siRNA), cells were treated with indicated culture medium without penicillin/streptomycin. SiRNA targeting human SOX9 (M-021507-00) and control siRNA (D-001206-14) were purchased from Dharmacon. SOX9 siRNA were transfected with RNAiMAX (13778, Invitrogen). The transfection was performed in six-well cell culture vessels. Tumor cells were plated at a density of 1.5×10^5 cells per well with 2ml corresponding growth medium. Briefly, for siRNA transfection, 2μl RNAiMAX was mixed with 20pmol SOX9 siRNA in 200μl Opti-MEM medium. The mixtures were preincubated for 20 min at room temperature before adding to cells. RNA and whole cell protein were extracted 48 and 60 hours after transfection for further examination, respectively.

3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (*MTT) assay*

Cells were incubated with 5mg/ml MTT reagent (M5655, Sigma Aldrich) for 5h. Then, the supernatant was removed carefully and the 100µl solvent solution containing 40µl of 10% SDS, 40µl DMSO and 20µl of 1.2% Acetate acid solution (600µl Acetate acid in 50mL PBS) was added and incubated overnight for measurement. Absorbance was measured at 570 nm with a reference to 630 nm. For cell viability assay and gemcitabine IC50 measurement, cells were incubated in 96-well plate for 48 hours before incubation with MTT.

Cell cycle analysis

Cells were harvested at 48 hours after siRNA treatment and washed with cold PBS, then fixed with 70% cold ethanol. To remove RNA, the cells were re-suspended in solution containing TritonX-100 (0.1%) and 100µg/ml RNase. The samples were stained with propidium iodide (20µg/mL) for 30 minutes in the dark, and then subjected to analysis for DNA content using FACS Calibur (BD Biosciences, Heidelberg, Germany) and data analysis was performed using Flowjo version10 software.

Transwell migration assay

Cell culture inserts with 8µM pore size (Falcon) were used. For tumor cell migration, 2.0×10^5 iCCA tumor cells were suspended in RPMI or DMEM medium with 0.5% FBS and plated in the upper chambers. The lower chambers were filled RPMI or DMEM with 10% FBS. After 16 h, the medium in the inserts were removed and washed with PBS. The inserts were filled with 3.7% formaldehyde for 5 minutes. Thereafter, the inserts were incubated in methanol for 30 minutes. The filters were stained with 10% Gimsa (Sigma, St. Louis, MO) for 15 minutes. The inner side was wiped with cotton swabs. Migrated cells were counted under a light microscope.

Caspase 3 assay

Caspase 3 assay was performed as previously described (21). In brief, cells were lysed in 80µl of lysis buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 1mM DTT, 0.1mM EDTA, pH 7.4). Then, 20µl of cell lysate were incubated in 70µl reaction buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 10mM DTT, 0. mM EDTA, 10% (w/v) glycerol, pH 7.4) and 10µl AC-DEVD-AFC caspase 3 fluorimetric substrate (Biomol, Hamburg, Germany) for 90 min at 37 °C. Subsequently, Caspase 3 activity was detected by fluorometric measurement using

Tecan infinite M200 (excitation 400 nm; emission 505 nm). The caspase 3 activity was normalized to protein levels and reported as relative fluorescent units per minute per mg protein.

Immunoblotting

Immunoblotting assay was performed as previously described (20). Briefly, total cell protein was extracted on ice using radio immunoprecipitation assay (RIPA) buffer with freshly added protease and phosphatase inhibitors. Protein concentrations were assessed with a Bio-Rad protein assay. 20µg of total cell protein extracts were subjected to 10% or 12% sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose membranes. 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) was used to block nonspecific binding. Membranes were probed with primary and secondary antibodies in TBST according to manufacturer's instructions. HRP-linked anti-mouse and anti-rabbit Abs were used as secondary antibodies. Alpha-tubulin and GAPDH were used as loading control. Signal was visualized by incubating the blots in Supersignal Ultra (Pierce, Hamburg, Germany).

RNA isolation and quantitative real-time reverse transcription polymerase chain reaction

Total cell RNA was extracted using the InviTrap spin universal RNA mini kit (Stratec, Berlin, Germany), according to the manufacturer's instructions. For first strand cDNA synthesis, reverse transcription of 500ng RNA was performed with random primers (Thermo Scientific) and RevertAid H Minus M-MuLV reverse transcriptase (Thermo Scientific) according to the manufacturer's instructions and subsequently diluted with nuclease-free water (Invitrogen) to 10ng/µl cDNA. For PCR amplification, 10.4µl mixtures contained 5µl (50ng) template cDNA, 5µl SYBR Green (4367659, Life Technologies), and 4µM forward and reverse primer PCRs were run in triplicate and performed on a StepOnePlus Real-time PCR (Applied Biosystems). PCR amplification cycling conditions comprised 10 min polymerase activation at 95 °C and 40 cycles at 95°C for 15s and 60°C for 1 min. A melting curve analysis was performed for each PCR analysis. Relative quantification of target genes was normalized against the house keeping gene PPIA.

Microarray

Gene expression profiling was performed using arrays of human HuGene-2_0-st-type from Affymetrix. Biotinylated antisense cDNA was prepared according to the Affymetrix standard labelling protocol with the GeneChip® WT Plus Reagent Kit and the GeneChip®

Hybridization, Wash and Stain Kit (both from Affymetrix, Santa Clara, USA). Subsequently, the hybridization on the chip was performed on a GeneChip Hybridization oven 640, then dyed in the GeneChip Fluidics Station 450 and thereafter scanned with a GeneChip Scanner 3000. All of the equipment used was from the Affymetrix-Company (Affymetrix, High Wycombe, UK).

Bioinformatic analyses

A Custom CDF Version 21with ENTREZ based gene definitions was used to annotate the arrays (22). The Raw fluorescence intensity values were normalized applying quantile normalization and RMA background correction. Differential expressed genes were identified by using a commercial software package SAS JMP10 Genomics, version 6, from SAS (SAS Institute, Cary, NC, USA). A false positive rate of a=0.05 with FDR correction was taken as the level of significance.

Statistical analyses

Variables were summarized as means ± standard deviation (S.D) and depicted graphically as means ± S.D. *P* values were calculated using the chi-square test or calculated using a two-sided (unpaired) Student's t test. Kaplan-Meier survival curve and uni-variate Cox analysis was used to evaluate overall survival rates and disease free survival rate of iCCA patients. *P* values were calculated using the log-rank test. $P < 0.05$ was considered significant.

Results

SOX9 has distinct expression patterns in chronic liver disease and CCA

First, we compared expression of SOX9 and CK19, two classic markers of biliary tree, in 80 patients with chronic liver disease or iCCA. Among 21 patients with chronic liver disease, 17 showed SOX9 positive immunoreactivity whereas 4 were negative (**Figure 1A** shows representative patients.). In contrast to SOX9, CK19 immunostaining was positive in all patients (data not shown). The results suggest that SOX9 expression in cholangiocytes is unstable in chronic liver disease compared to CK19. Distinct from CK19, which localized in cytoplasm of cholangiocytes, SOX9 was expressed in the nuclei of cells in the canals of Hering, reactive ductules and bile ducts (Patient 1 and 2, **Figure 1 A**). As in chronic liver disease, SOX9 expression was observed in the nuclei of iCCA tumor cells, while CK19 localized in the cytoplasm of cancer cells (**Figure 1B**). Expressions of SOX9 and CK19 in

cancer cells were heterogeneous. **Figure 1B** display four patterns of SOX9 and CK19 expression in iCCA: $SOX9^{high}CK19^{high}$, $SOX9^{high}CK19^{low}$, $SOX9^{low}CK19^{high}$ and $SOX9^{low}CK19^{low}$. There was no significant correlation between expression of SOX9 and CK19 in iCCA tumor cells ($P > 0.05$). In all examined tissue specimens, neither SOX9 nor CK19 were detected in hepatocytes.

Expression of SOX9 predicts poor clinical outcome of iCCA

Next, we analyzed the correlation of SOX9 and CK19 expression with clinical parameters of the iCCA patients, including age, gender, vascular invasion, existence of cirrhosis and AJCC classification. Among these clinical parameters, CK19 expression was associated with AJCC classification of CCA, while SOX9 did not show any association with these clinical parameters **(Supplementary Table 3)**. However, multivariate analysis showed that among the analyzed variables, only SOX9 expression significantly influenced the overall survival of iCCA patients (Hazard ratio=3.614, 95% Confidence Interval=1.493-9.076， P=0.006, **Supplementary Table 4**). Furthermore, Kaplan-Meier analysis and the log-rank test showed that patients with high SOX9 expression had shorter overall survival (OS) and disease free survival (DFS) rates than those with low SOX9 expression ($P < 0.01$ and $P < 0.05$, respectively, **Figure 2A** and **2C)**. The median OS time in patients with SOX9 low expression was 62 months, whereas the value in those patients with high SOX9 expression was only 22 months (**Figure 2A**). In contrast to SOX9, there was no association between CK19 expression and survival time in these patients ($P > 0.05$, **Figure 2B** and **2D**).

This cohort of iCCA included 9 patients who received chemotherapy (e.g. gemcitabine and Cisplatin) **(Supplementary Table 5).** Among them, 6 patients had low SOX9 expression and 3 patients showed high levels of SOX9. Survival analyses revealed that patients with high SOX9 expression had shorter OS time ($P < 0.05$, **Figure 3A**). In patients who received chemotherapy, the mean survival time in patients with SOX9 low expression was 62 months, whereas the value in those patients with high SOX9 expression was only 22 months (**Figure 3A**). Except 1 patient who received chemotherapy following surgery, additional 8 patients, 5 with low and 3 with high SOX9 expression, received chemotherapy due to the recurrence of iCCA. The survival times until the end of the follow-up in 5 patients with low SOX9 levels were 16, 19, 20, 29 and 34 months, whereas the values in 3 with high SOX9 expression were 13, 14 and 16 months, respectively (**Supplementary Table 5**). Survival difference between the two groups was significant ($P < 0.01$, **Figure 3A**). Notably, 4 out of 5 patients with low SOX9 were still survival when the follow-up ended (**Supplementary Table 5**). However, all

3 patients with high SOX9 expression were dead during follow-up (**Supplementary Table 5**). CK19 expression did not show any correlation with chemotherapy response ($P > 0.05$, data not shown).

SOX9 inhibition sensitizes CCA cells to gemcitabine

To investigate how SOX9 expression in CCA cell might modify their response to chemotherapy, we performed microarray analysis in iCCA CC-SW-1 cells after SOX9 silencing. We found that gene expression associated with drug metabolism and ABC transporters such as *ABCB1* (MDR1) and *ABCC4* (MRP4) was decreased, while genes related with the p53 signaling pathway were increased when SOX9 was knocked down with siRNA (**Supplementary Figure 2A**). Western blot and qPCR further confirmed that the expression of multidrug resistance genes *ABCC4* and *ABCB1* was markedly reduced when SOX9 was inhibited in CC-SW-1 cells (**Supplementary Figure 2B-C**).

Next, we treated different types of CCA cells with gemcitabine, an analog of deoxycytidine, which is widely used in the treatment of CCA. Notably, basal expression of SOX9 in CCA cells was significantly higher than in normal cholangiocytes (**Figure 4A**). More impressively, expression of SOX9 protein was further increased upon gemcitabine treatment in both iCCA CC-SW-1 and eCCA EGI-1 cells **(Figure 4B)**. To examine the function of SOX9 in gemcitabine-treated CCA cells, we knocked down SOX9 expression using siRNA in CC-SW-1 and EGI-1 cells, followed by treatment with gemcitabine for 24 hours **(Figure 4C)**. MTT assay showed that when SOX9 expression was inhibited, the IC50 of gemcitabine-treated cells significantly decreased from 7.1 ± 0.15nM to 2.0 ± 0.23nM in CC-SW-1 cells and from 380.3 ± 249.1nM to 46.3 ± 21.9nM in EGI-1 cells, respectively **(Figure 4D)**.

Given the key role of phosphorylation of checkpoint kinase 1 (CHEK1) in coordinating the DNA damage response and inhibiting the expression of multidrug resistance genes (23), we examined whether disruption of SOX9 impacted CHEK1 activation. Immunoblot analysis showed that SOX9 siRNA remarkably inhibited gemcitabine-dependent pCHEK1 in both CC-SW-1 and EGI-1 cells and MRP4 expression in CC-SW-1 cells **(Figure 4D-F)**. Consistent with reduced expression of MRP4 and pCHK1, analyses based on immunoblot and cleaved Caspase 3 activity assay revealed marked increases in cleaved Caspase 3 and 8 expression and Caspase activity in CCA cells with SOX9 knockdown, indicating that gemcitabine-induced apoptosis was increased when SOX9 expression was inhibited **(Figure 4G-H)**.

In addition to gemcitabine, we examined the role of SOX9 in cisplatin-treated CC-SW-1 and EGI cells. MTT assay showed that knockdown of SOX9 did not have impact on cisplatin-

inhibited cell viability in both cells (**Supplementary Figure 3A-B**). In contrast to gemcitabine, administration of cisplatin and/or knockdown of SOX9 did not influence the expression of pCHEK1 (**Supplementary Figure 3C-D**).

SOX9 is essential for CCA cells proliferation, stemness and migration

Next, we examined the role of SOX9 in CCA cell proliferation, stemness and migration. MTT analyses showed that knockdown of SOX9 expression significantly inhibited cell proliferation in 4 types of CCA cells (**Figure 5A**). In CC-SW-1 and EGI-1 cells, SOX9 inhibition significantly decreased the proportion of cells staying in G1 phase and increased those in G2/M phase **(Figure 5B)**. The results suggest that SOX9 is required for maintaining CCA cell proliferation.

Subsequently, we investigated the effects of SOX9 on stemness of CCA cells. We found that knockdown of SOX9 expression decreased EpCAM expression at both RNA and protein levels in CC-SW-1 cells (**Figure 5C**). In HCC, EpCAM is considered as a crucial factor in the maintenance of cancer stem cell (CSC)-like features in cancer cells (24). To investigate whether SOX9 is implicated to the CSC features of CCA cells, we performed tumor sphere formation assay, a widely recognized method to evaluate cancer stem cell self-renewal and differentiation at the single cell level *in vitro* (25). SOX9 knockdown significantly inhibited the capacity of tumor sphere formation in CC-SW-1 **(Figure 5D)**.

In addition, we also investigated the role of SOX9 in CCA cell migration. Transwell assay showed that knockdown of SOX9 expression significantly inhibited cell migration in CC-SW-1 cells **(Figure 5E)**.

Discussion

The standard treatment for advanced-stage iCCA is systemic chemotherapy with gemcitabine and cisplatin (7). However, the median overall survival time is less than 12 months (7). In patients treated with gemcitabine alone, the survival time is less than 8 months (7), thus improving the sensitivity of cholangiocarcinoma cells to chemotherapy is a key to prolonging the survival of iCCA patients. In the current study, we found that SOX9, the earliest cholangiocyte marker during embryonic liver development (11, 12), plays a crucial role in iCCA cells' resistance to chemotherapy. We examined the expression of SOX9 in 59 iCCA patients who received surgery. High expression of SOX9 in the nuclei of iCCA cancer cells

was significantly associated with shorter survival time (P = 0.0039). Of 9 patients treated with chemotherapy following surgery, the median survival time reached to 62 months in 6 patients who had low levels of SOX9 expression, whereas survival time was only 22 months in the 3 patients who had high SOX9 levels. Although the sample size was small in this study, the difference in survival time between both groups was significant (P = 0.017). Among the 9 patients, 8 received chemotherapy because of the recurrence of cancer. The 5 patients with low SOX9 levels survived between 16 and 34 months, however, the longest survival time in 3 patients with high SOX9 expression was only 16 months. These results suggest that SOX9 expression correlates with cholangiocellular cancer cells' response to chemotherapy. Further *in vitro* studies provided the following mechanistic explanations of the observed differences: (1) Microarray, qPCR and western blot analyses showed that disruption of SOX9 with siRNA significantly decreased expression of genes/proteins associated with drug metabolism and multidrug resistance and increased the abundance of genes associated with p53 signaling pathway; (2) Knockdown of SOX9 markedly inhibited gemcitabine-induced activation of CHK1, a key cell cycle checkpoint protein that coordinates the DNA damage response, and expression of multiple drug resistance protein MRP4, and thus increased cancer cell apoptosis; (3) Gemcitabine dose-dependently induced expression of SOX9, indicating that CCA cells increase SOX9 as a defensive mechanism against treatment with chemotherapeutics such as gemcitabine. **Figure 6** depicts the gemcitabine-induced loop in CCA cells.

The role of SOX9 in the chemo-resistance has been reported in multiple tumors, e.g. chondrosarcoma, breast cancer, glioblastoma, cervical cancer, gastric cancer and lung cancer (26-31). In cervical cancer cells, SOX9 was found to increase cancer cell chemo-resistance through inhibiting miR-130a (27). However, the detailed mechanisms of how SOX9 contributes to chemo-resistance have not been clarified to date. Our observation that SOX9 confers chemo-resistance to cholangiocarcinoma through the activation of CHK1 and the expression of multiple drug resistance proteins might provide a light pointer for further investigation of this aspect.

The current study also investigated the role of SOX9 in CCA cells receiving cisplatin. In contrast to gemcitabine, knockdown of SOX9 did not impact the efficiency of cisplatin. Administration of cisplatin did not alter expression of pChk1 in CCA cells. The discrepancy between the two compounds might be due to their different mechanisms of action.

Gemcitabine results in cell death mainly through the inhibition of DNA synthesis and the inhibition of enzymes relevant to deoxyribonucleotide metabolism (32). Thus, Gemcitabine exerts these actions through impacting multiple pathways, including regulating checkpoint kinases. Different from gemcitabine, cisplatin causes cell death through the formation of [PtCl(guanine-DNA)(NH3)2]+, which inhibits DNA repair and activates apoptosis (33).

The defensive effects of SOX9 for CCA cells are not limited to cancer cells facing chemotherapy. Knockdown of SOX9 in both iCCA and eCCA cells remarkably inhibited the capacity of cancer cell proliferation and migration, decreased CSC stemness and increased apoptosis. These results provide an explanation why SOX9 expression is associated with survival time of patients receiving chemotherapy, but also in those who are treated by surgery alone. On the other hand, it should be kept in mind that these *in vitro* findings are not always consistent with the observation obtained in patients. For example, there was no correlation between the expression of SOX9 and cell proliferation markers, e.g. Ki67 and PCNA, in iCCA patients (data not shown). Given that there are multiple growth factors and proliferative signals governing cancer cell expansion in patients with iCCA, it is not surprising that low levels of SOX9 expression alone does not have a significant impact on the proliferation of cancer cells. In addition, although there is a crucial role of SOX9 in maintaining cell identity, we did not observe a significant correlation between SOX9 expression and histological differentiation in this cohort of iCCA patients (data not shown). The result might have two explanations: (1) SOX9 expression does not impact on differentiation of iCCA, and (2) SOX9 might have a subtle influence on cancer cell differentiation, but the association is to small to be detected in the currently small number of specimens.

In diseased liver, high levels of SOX9 occur not only in CCA, but also in HCC. However, in contrast to cholangiocytes, normal hepatocytes do not express SOX9. Expression of SOX9 in HCC reflects a cancer stem cell/progenitor cell (18). Given that SOX9 is the earliest and dominant phenotype marker of normal cholangiocytes, the induction of a cancer stem cell-like phenotype should not be attributed to the expression of SOX9. However, the current results do suggest that SOX9 plays a role in the maintenance of cancer stem cell phenotypes. Like CCA patients, HCC patients with high levels of SOX9 had poor prognosis. As in CCA, SOX9 in HCC is implicated in maintaining proliferation and self-renewal of cancer cells [18]. In the future, it will be interesting to find out whether SOX9 dependent control of check point protein activation may also play a role in chemo-resistance of HCC.

Besides SOX9, this study analyzed the association between CK19 expression and the clinical outcome of iCCA. CK19 is a classical marker for cholangiocytes. It has been reported that CK19 contributes to the differentiation of iCCA from metastatic adenocarcinoma and is associated with the histological differentiation of iCCA (34). In the current cohort of iCCA patients, CK19 expression was correlated with AJCC classification of iCCA, whereas it did not show any association with the survival of iCCA patients.

Taken together, SOX9 expression is a sensitive marker that predicts the survival time of iCCA patients, particularly in those receiving chemotherapy. Our study demonstrates that SOX9 is a key transcription factor that prevents iCCA cells from apoptosis when the cells are attacked by drugs such as gemcitabine. SOX9 exerts the observed effects on CCA cells, at least in part, through the activation of Chk1 and upregulation of multidrug resistance genes. Limitations of this study are: 1) the low number of samples precludes more general conclusions; 2) it is not clear how SOX9 exerts the observed effects on the expression of multidrug resistance genes; 3) we cannot conclude whether SOX9 expression has a similar effect in eCCA. Our study also enrolled 5 eCCA patients who demonstrate a similar biological behavior as iCCA (data not shown). Results in eCCA cell line EGI-1 indicate that the SOX9 expression has similar effects as observed in iCCA. Further investigation based on a large size of patient cohorts is required in the future.

Conflict of interest: The authors disclose no conflicts.

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Figure legends

Fig. 1. Expression of SOX9 and CK19 in patients with iCCA. (**A**) SOX9 express in cells of bile ducts (Patient 1) and reactive ducts (Patient 2) of liver tissues with chronic HBV infection. Patient 3 shows negative SOX9 immune reaction in the liver. (**B**) Four patterns of SOX9 and CK19 expression in iCCA patients.

Fig. 2. SOX9, but not CK19, is associated with survival time of iCCA patients. Kaplan-Meier survival analysis shows the association between SOX9 (**A** and **C**)/CK19 expression (**B** and **D**) and survival time in 59 iCCA patients.

Fig. 3. SOX9 is associated with survival time of iCCA patients receiving chemotherapy. (A) Kaplan-Meier plot for OS rate of 9 iCCA patients received chemotherapy before or after cancer recurrence. (**B**) Kaplan-Meier plot for OS rate of 8 iCCA patients received chemotherapy after cancer recurrence.

Fig. 4. Disruption of SOX9 increases gemcitabine-induced iCCA cell apoptosis. (**A**) The expression of SOX9 in normal cholangiocytes and different CCA cells. (**B**) Administration of gemcitabine dose-dependently induced expression of SOX9 in both CC-SW1 and EGI-1 cells. (**C**) SOX9 was knocked down by siRNA. (**D**) MTT analyses showed cell viability in gemcitabine-treated CC-SW1 and EGI-1 cells with or without SOX9 knockdown. (**E-G**) Phosphorylation of CHK1, MRP4, cleaved Caspase 3 and 8 were measured in gemcitabine-treated CC-SW1 and EGI-1 cells with or without SOX9 knockdown. (**H**) Caspase 3 assay was used to measure Caspase 3 activity in gemcitabine-treated CC-SW1 and EGI-1 cells with or without SOX9 knockdown. All Western blot analyses in **A-C** and **E-G** were performed at least for three times. Quantification of western blot is shown by the numbers between bands.

Fig. 5. Disruption of SOX9 impact biological behaviors of CCA cells. (**A**) MTT analyses were performed to measure cell viability in different CCA cells with or without SOX9 knockdown. (**B**) FACS analyses were used to examine the impact of SOX9 on cell cycle of CC-SW1 and EGI-1 cells. (**C**) mRNA and protein expression of EpCAM were measured by qPCR and Western blot in CC-SW1 and EGI-1 cells with or without SOX9 knockdown. (**D**)

Tumor sphere formation assay was used to assess self-renewal of CC-SW-1 cells with or without SXO9 knockdown. (**E**) The impact of SOX9 on CC-SW-1 cell migration was examined with transwell migration assay.

Fig. 6. A scheme depicts the mechanisms on how SOX9 prevent cells from apoptosis in gemcitabine-treated CCA cells.

Supplementary material

Supplementary Fig 1. Representative IHC staining showing how to identify SOX9 intensity score.

Supplementary Fig 2. Knockdown of SOX9 alter gene and protein expression-associated with adenosine triphosphate–binding cassette transporters, drug metabolism enzymes and with p53 signaling in CC-SW-1 cells. (**A**) Microarray analysis (see Materials and Methods); (**B**) Western blot for MRP4 expression; (**C**) qPCR for ABCB1 gene expression.

Supplementary Fig 3. Knockdown of SOX9 did not impact cisplatin-inhibited cell viability in CCA cells. (**A-B**) MTT analyses showed cell viability in cisplatin-treated CC-SW1 and EGI-1 cells with or without SOX9 knockdown. (**C-D**) Phosphorylation of CHK1 and total CHK1 were measured in cisplatin-treated CC-SW1 and EGI-1 cells with or without SOX9 knockdown.

Supplementary tables

Supplementary Table 1. Clinicopathological features of patients with chronic liver disease

Supplementary Table 2. Clinicopathological features of validating set iCCA

Supplementary Table 3. Patient characteristics in iCCA patients with different levels of SOX9/CK19

Supplementary Table 4. Multivariate Analysis for Overall Survival of iCCA patients

Supplementary Table 5. SOX9 and CK19 expression and the clinical outcome of iCCA patients that received chemotherapy

Figure 1

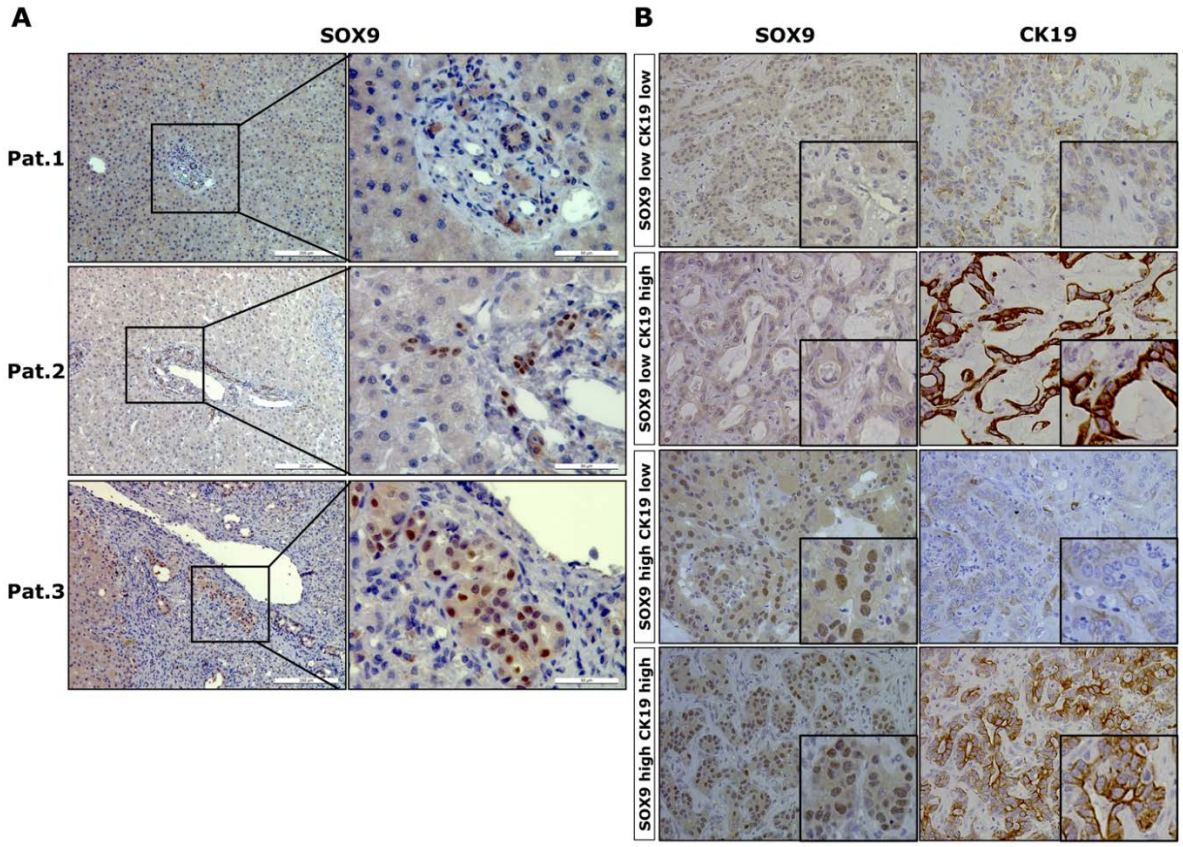

A
SOX9
Pat.1
Pat.2
Pat.3
B
SOX9
CK19
SOX9 low CK19 low
SOX9 low CK19 high
SOX9 high CK19 low
SOX9 high CK19 high

Figure 2

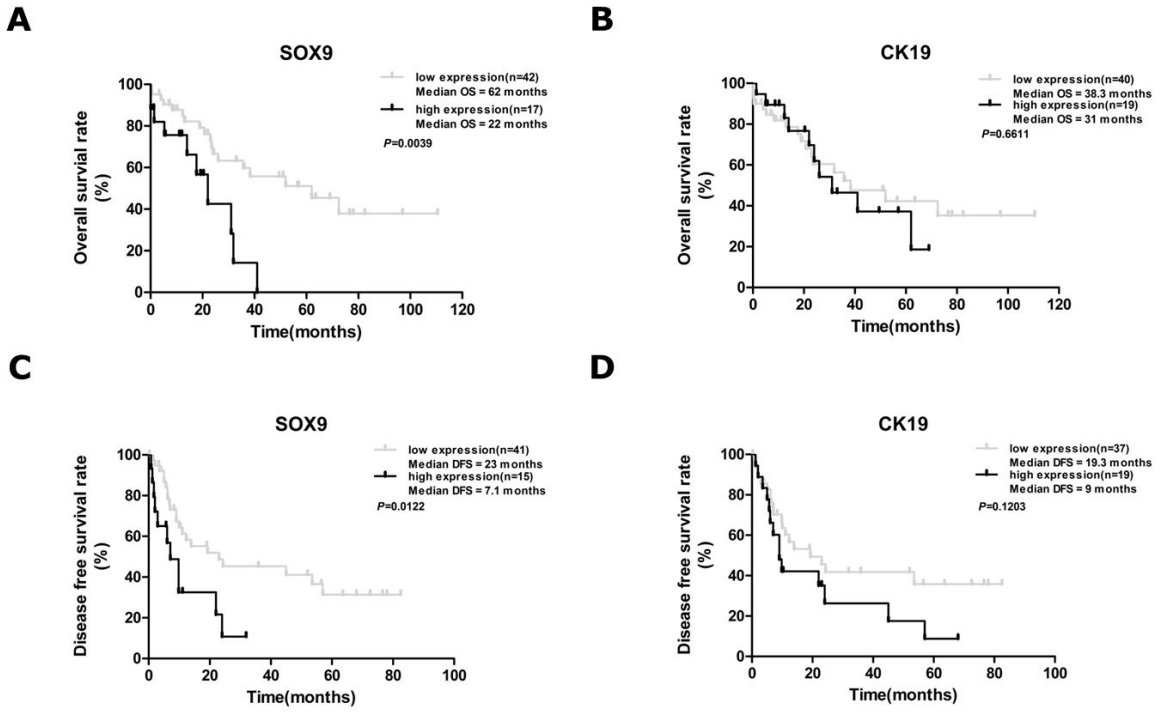


Figure 3

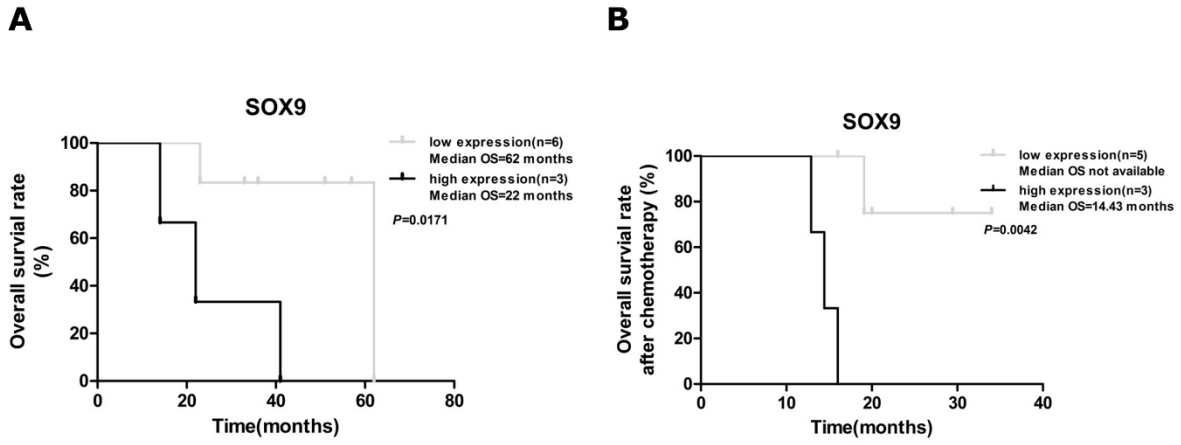


Figure 4

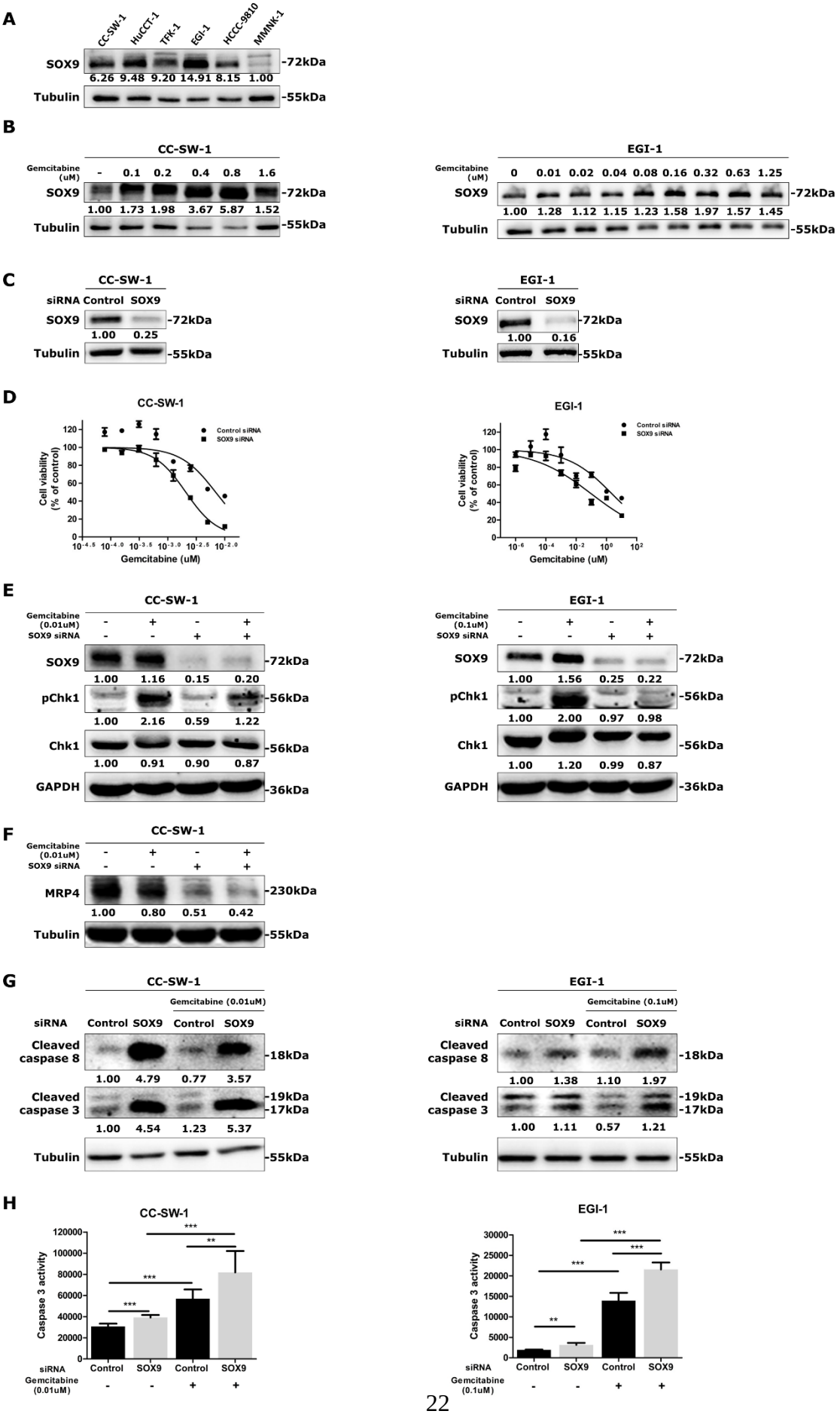

A
CC-SW-1
HuCCT-1
TFK-1
EGI-1
HCCC-9810
MMNK-1
SOX9
6.26 9.48 9.20 14.91 8.15 1.00
Tubulin
-72kDa
-55kDa
B
CC-SW-1
Gemcitabine (uM)
- 0.1 0.2 0.4 0.8 1.6
SOX9
1.00 1.73 1.98 3.67 5.87 1.52
Tubulin
EGI-1
Gemcitabine (uM)
0 0.01 0.02 0.04 0.08 0.16 0.32 0.63 1.25
SOX9
1.00 1.28 1.12 1.15 1.23 1.58 1.97 1.57 1.45
Tubulin
C
CC-SW-1
siRNA Control SOX9
SOX9
1.00 0.25
Tubulin
EGI-1
siRNA Control SOX9
SOX9
1.00 0.16
Tubulin
D
CC-SW-1
Control siRNA
SOX9 siRNA
Cell viability (% of control)
Gemcitabine (uM)
EGI-1
Control siRNA
SOX9 siRNA
Cell viability (% of control)
Gemcitabine (uM)
E
CC-SW-1
Gemcitabine (0.01uM)
SOX9 siRNA
SOX9
1.00 1.16 0.15 0.20
pChk1
1.00 2.16 0.59 1.22
Chk1
1.00 0.91 0.90 0.87
GAPDH
-72kDa
-56kDa
-56kDa
-36kDa
EGI-1
Gemcitabine (0.1uM)
SOX9 siRNA
SOX9
1.00 1.56 0.25 0.22
pChk1
1.00 2.00 0.97 0.98
Chk1
1.00 1.20 0.99 0.87
GAPDH
F
CC-SW-1
Gemcitabine (0.01uM)
SOX9 siRNA
MRP4
1.00 0.80 0.51 0.42
Tubulin
-230kDa
-55kDa
G
CC-SW-1
Gemcitabine (0.01uM)
siRNA Control SOX9 Control SOX9
Cleaved caspase 8
1.00 4.79 0.77 3.57
Cleaved caspase 3
1.00 4.54 1.23 5.37
Tubulin
-18kDa
-19kDa
-17kDa
-55kDa
EGI-1
Gemcitabine (0.1uM)
siRNA Control SOX9 Control SOX9
Cleaved caspase 8
1.00 1.38 1.10 1.97
Cleaved caspase 3
1.00 1.11 0.57 1.21
Tubulin
H
CC-SW-1
Caspase 3 activity
siRNA Control SOX9 Control SOX9
Gemcitabine (0.01uM) - - + +
EGI-1
Caspase 3 activity
siRNA Control SOX9 Control SOX9
Gemcitabine (0.1uM) - - + +

Figure 5

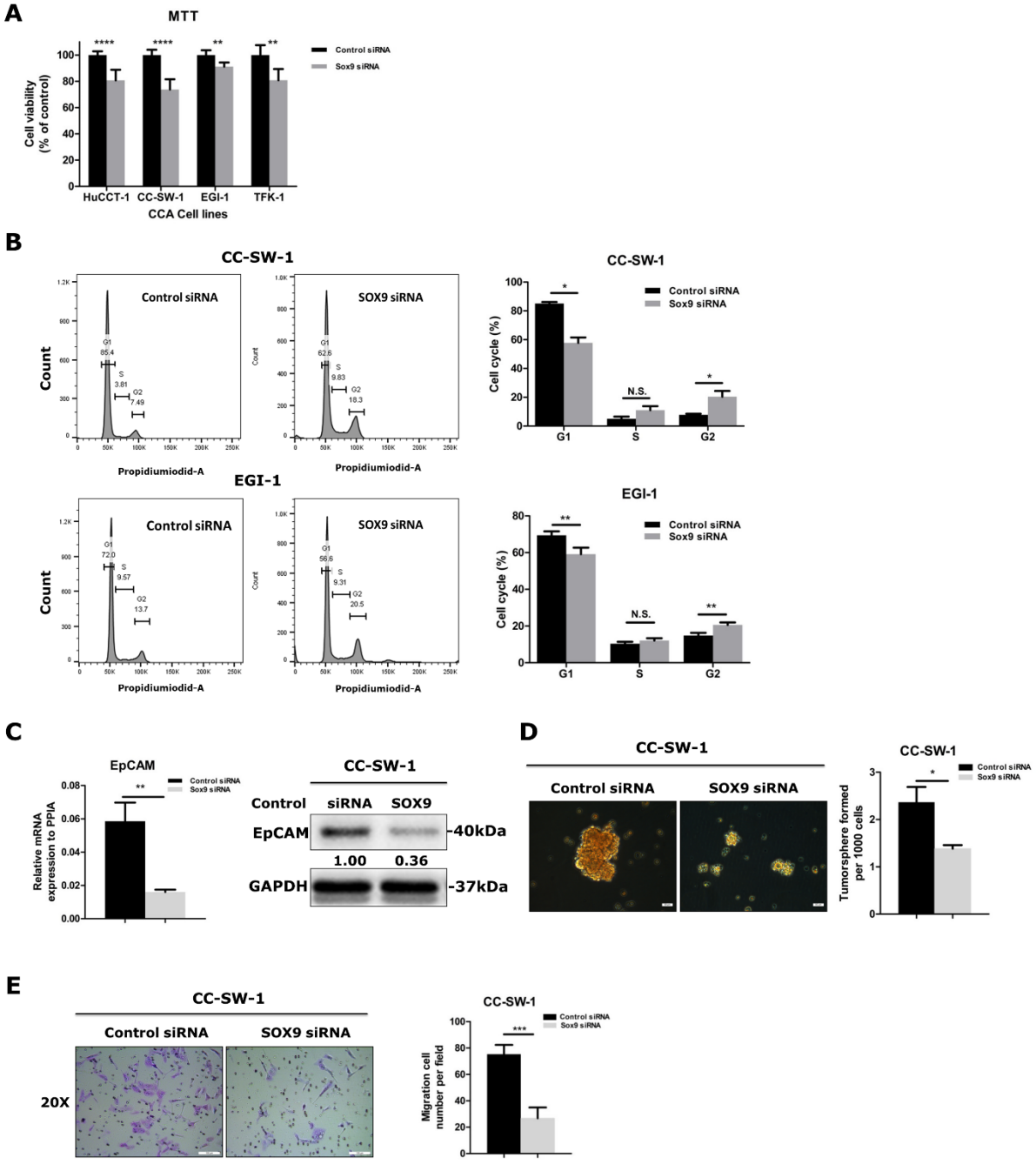
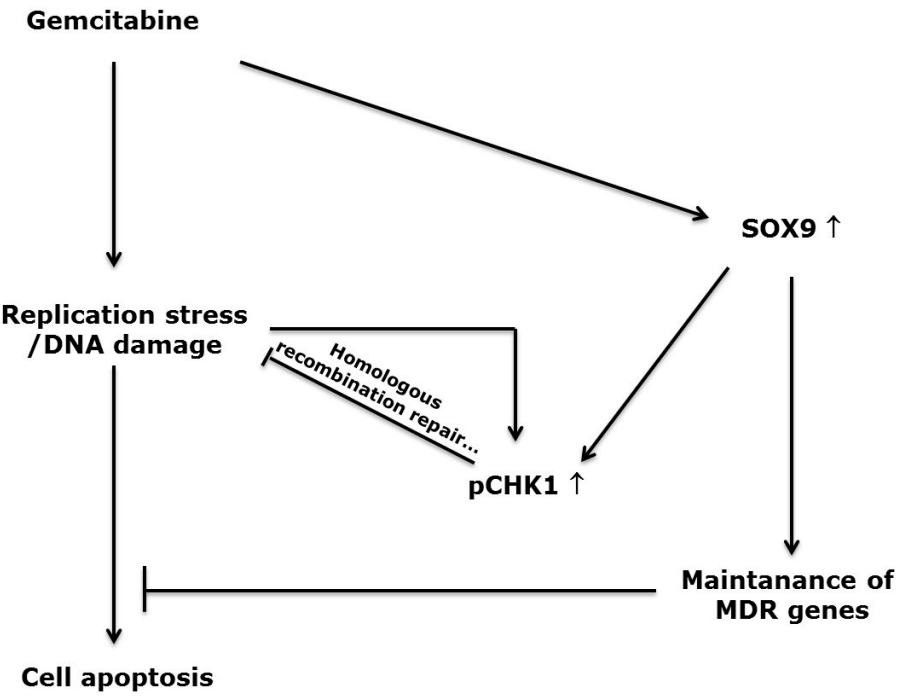
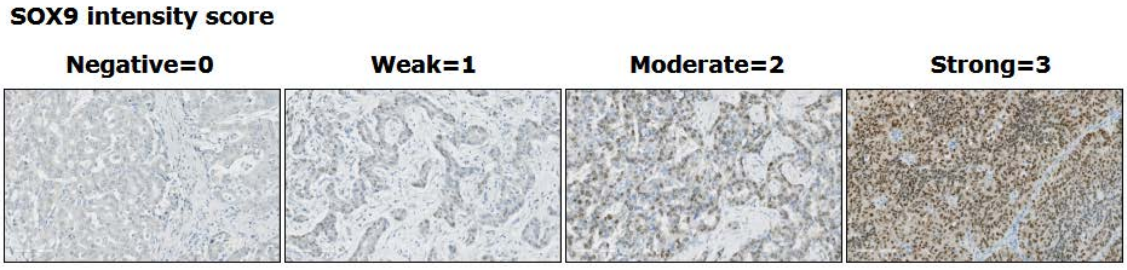

A
MTT
Cell viability (% of control)
HuCCT-1
CC-SW-1
EGI-1
TFK-1
CCA Cell lines
Control siRNA
Sox9 siRNA
B
CC-SW-1
Control siRNA
SOX9 siRNA
Count
Propidiumiodid-A
Cell cycle (%)
G1
S
G2
N.S.
EGI-1
C
EpCAM
Relative mRNA expression to PPIA
Control
siRNA
SOX9
EpCAM
GAPDH
40kDa
37kDa
1.00
0.36
D
Tumorsphere formed per 1000 cells
E
20X
Migration cell number per field

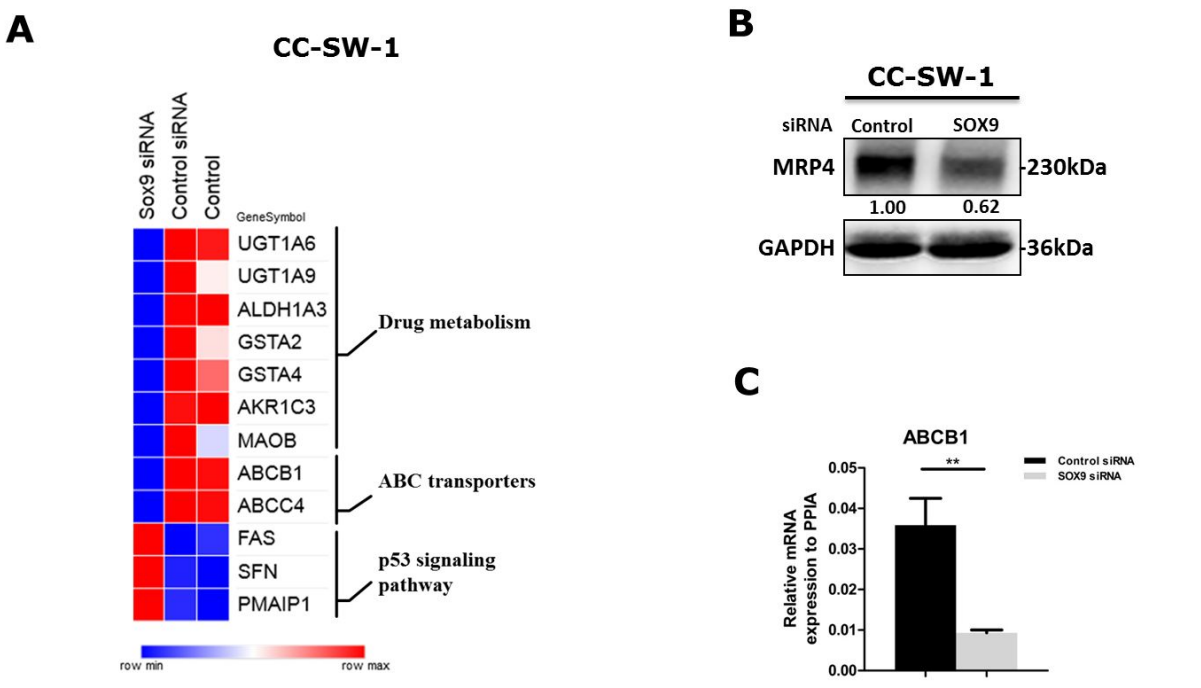
Figure 6



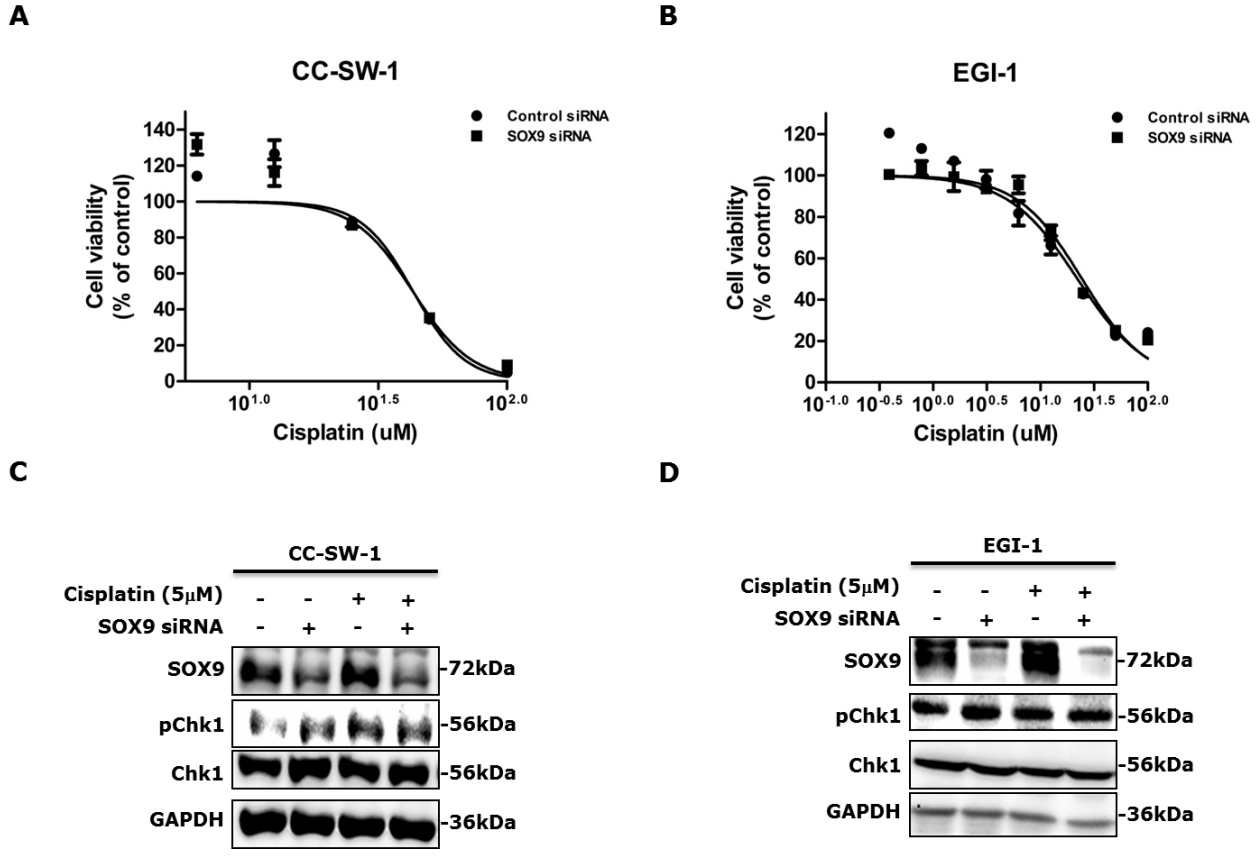
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Table 1. Clinicopathological features of patients with chronic liver disease

Clinicopathological Features		N=21
Age (years)		19 - 65
Gender (male/female)		18/3
Inflammatory grade*		
	0	3
	1	5
	2	5
	3	5
	4	3
Fibrotic Stage*		
	0	3
	1	3
	2	5
	3	4
	4	6

*Inflammation and fibrosis was assessed according to Scheuer criterion.

Supplementary Table 2. Clinicopathological features of validating set iCCA

Clinicopathological Features		N=59
Age (years)		63.47 ± 9.96
Gender (male/female)		44/15
Vascular invasion		8 (13.6%)
Cirrhosis		18 (30.5%)
AJCC classification*		
	I	21 (35.6%)
	II	18 (30.5%)
	III	9 (15.2%)
	IV	11 (18.6%)
Follow up (months)		28.91 ± 26.60
Death		28 (47.5%)

*AJCC 7th Edition of TNM Staging.

Supplementary Table 3. Patient characteristics in iCCA patients with different levels of SOX9/CK19

Clinicopathological factors	SOX9 expression			CK19 expression		
	low expression (n=42)	high expression (n=17)	*P* value	low expression (n=40)	high expression (n=19)	*P* value
Age (year)	64.55 ± 9.60	60.82 ± 10.62	0.20	60.00 ± 10.70	65.13 ± 9.30	0.064
60>	12	7	0.34	10	9	0.086
≥60	30	10		30	10	
Gender						
male	30	14	0.58	31	13	0.454
female	12	3		9	6	
Vascular invasion						
Yes	4	4	0.32	5	3	0.730
No	38	13		35	16	
Cirrhosis						
Yes	11	7	0.26	11	7	0.466
No	31	10		29	12	
AJCC classification						
I+II	26	13	0.44	30	9	0.036
II+III	16	4		10	10	

Supplementary Table 4. Multivariate Analysis for Overall Survival of iCCA patients

Variable	Hazard Ratio	95% Confidence Interval	*P* Value
Age	1.049	1.000-1.100	0.048
Gender (Male *vs.* Female)	0.554	0.182-1.689	0.299
Cirrhosis (Yes *vs.* No)	1.086	0.494-2.391	0.837
AJCC (I+II *vs.* III-IV)	1.955	0.778-4.193	0.154
Vascular Invasion (Yes *vs.* No)	0.633	0.132-3.038	0.568
SOX9 (High *vs.* Low)	**3.614**	**1.493-9.076**	**0.006**
CK19 (High *vs.* Low)	0.949	0.383-2.348	0.910

Supplementary Table 5. SOX9 and CK19 expression and the clinical outcome of iCCA patients that received chemotherapy

SOX9 IHC intensity	CK19 IHC intensity	Death or survival (1=survival, 0=death)	Survival time (months)	Survival time after CTx (months)	Treatment
low	low	0	23	19	Gemcitabine and XELOX
low	strong	0	62	62*	Gemcitabine plus Cisplatin and XELOX
low	strong	1	33	29	Gemcitabine
low	low	1	36	34	Palliative chemotherapy
low	strong	1	57	16	Cisplatin and gemcitabine
low	low	1	51	20	Palliative chemotherapy
strong	strong	0	14	13	Erlotinib
strong	strong	0	22	16	Gemcitabine and cisplatin
strong	strong	0	41	14	Palliative chemotherapy

CTx: Chemotherapy; XELOC: Capecitabine plus Oxaliplatin.

* The patient received chemotherapy following surgery.