Liver, Pancreas and Biliary Tract

Sox9 expression in carcinogenesis and its clinical significance in intrahepatic cholangiocarcinoma

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ABSTRACT

Background: Intrahepatic cholangiocarcinomas develop through a multi-step carcinogenesis. Precancerous lesions are defined as biliary intraepithelial neoplasia. Sex determining region Y-box9 (Sox9) is required for the normal differentiation of the biliary tract.
Aims: To evaluate the Sox9 expression in carcinogenesis and its correlation with clinicopathological features in intrahepatic cholangiocarcinoma.
Methods: Sox9 expression in normal epithelium, biliary intraepithelial neoplasia, and intrahepatic cholangiocarcinoma were investigated immunohistochemically using 43 specimens of intrahepatic cholangiocarcinoma. Sox9 expression in intrahepatic cholangiocarcinoma was compared with the clinicopathological features. The molecular effects of Sox9 were investigated by gene transfection to intrahepatic cholangiocarcinoma cell lines.
Results: Sox9 expression was decreased from the normal epithelium to the biliary intraepithelial neoplasia in a stepwise fashion. In 51.2% (22/43) of the patients with intrahepatic cholangiocarcinoma, Sox9 expression was positive, and Sox9 expression was significantly associated with the biliary infiltration ($P = 0.034$) and poor overall survival ($P = 0.039$). Upregulation of Sox9 promoted the cell migration and invasion, and decreased the E-cadherin expression and increased the vimentin and α-SMA expression in cell lines.
Conclusions: Decreased Sox9 expression may be related to the early stage of the carcinogenesis of intrahepatic cholangiocarcinoma. Sox9 overexpression in intrahepatic cholangiocarcinoma is related to biliary infiltration and poorer prognosis, and it promotes cell migration and invasion, via the epithelial-to-mesenchymal transition.

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1. Introduction

Intrahepatic cholangiocarcinoma (ICC) is an epithelial cancer of the intrahepatic biliary tract. The incidence of ICC is rare, but increasing worldwide [1]. The most effective treatment for an ICC is radical resection, but ICCs are often diagnosed at an advanced stage, at which surgery may not be possible [2–4]. The prognosis for ICCs is still poor despite the introduction of chemotherapy with gemcitabine or S-1 (tegafur, gimeracil, oteracil) and radiotherapy [3,5]. The elucidation of the mechanisms underlying the carcinogenesis of the biliary tract is a key to improving the prognosis of ICCs.

ICCs originate from epithelial cells of the intrahepatic biliary tract, typically in response to chronic inflammation, and they develop via a multi-step carcinogenesis [6]. Two types of premalignant lesions of cholangiocarcinoma have been identified. Flat or low papillary-type biliary intraepithelial neoplasia (BilINs) and papillary-type intraductal papillary mucinous tumours of the bile

duct (IPN-Bs) are thought to progress to carcinoma via multistep processes [7,8]. BilINs are classified into three grades (BilIN-1, BilIN-2, and BilIN-3) according to the degree of atypia: BilIN-1 corresponds to low-grade dysplasia, BilIN-2, to intermediate-grade dysplasia, and BilIN-3, to high-grade dysplasia.

Sox9 (Sry [sex-determining region Y] – box9) is a member of the Sry-related high-mobility group (HMG) box family. Sox9 plays an important role not only in sex determination, but also in chondrogenesis and the embryonic formation of several tissues and organs, including the testis, heart, lung, pancreas, biliary tract and the central nervous system [9–15]. Abnormality in any of several genes that play a role in the normal differentiation of several organs can cause several neoplasms [16,17]. Several studies have found that digestive system cancers such as hepatocellular carcinoma (HCC), oesophageal cancer, gastric cancer (GC) and colorectal cancer are correlated with Sox9 expression in carcinogenesis [18–21]. Moreover, Sox9 has attracted attention as a progressive or prognostic biomarker in patients diagnosed with HCC or GC [18,22].

In recent years, we have reported that Sox9 expression gradually decreased in the carcinogenesis of intraductal papillary mucinous neoplasm (IPMN) in the pancreas, and that Sox9 expression in IPN-B, which is recognised as the counterpart of IPMN, was significantly lower than the expression in the normal epithelium of the bile duct [23,24]. In addition, a stepwise decrease of Sox9 expression in pancreatic intraepithelial neoplasia, which is recognised as the counterpart of BilIN, was reported by Shroff et al. [25]. In the present study, we analysed Sox9 expression in ICCs including the precancerous lesions, and we determined this expression's correlation with clinicopathological features. We also investigated the molecular effects of Sox9 expression on ICC cell lines, especially in cell migration and invasion.

2. Materials and methods

2.1. Tissue samples and immunohistochemistry

A total of 43 patients [male/female: 26/17, median age (range): 71 (23–88) years] diagnosed with ICC underwent hepatic resection of varying extents in our department between April 2000 and October 2014. The patients' characteristics are shown in Table 1. Tumour staging was classified according to the TNM Classification of Malignant Tumours defined by the International Union Against Cancer (seventh edition) [26]. Biliary infiltration was defined as positive in cases in which the cancer cells had invaded the intrahepatic bile duct. None of the patients received any chemotherapy or radiation therapy before their operation. Surgical specimens were investigated immunohistochemically for Sox9 expression. This study was approved by the Ethics Committee of Nagasaki University, and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from each patients included in this study.

A total of 43 cancerous lesions and 43 normal biliary epithelium samples, one of each from each patient, were investigated. In addition, a total of 47 BilIN-1 lesions, 27 BilIN-2 lesions, and 16 BilIN-3 lesions were obtained from 23 ICC surgical specimens under a microscope. The investigated normal epithelium and BilIN samples were selected from tissue outside of the main cancerous area and obtained from the periphery of the liver.

Immunohistochemistry was performed using mouse monoclonal anti-Sox9 antibody (Abcam, Cambridge, UK) following the protocol described by Tanaka et al. [23]. The quantification of Sox9 expression was performed using an immunoreactive score (IRS) for each lesion, taking into account the percentage (0, 0%; 1, 1–10%; 2, 11–50%; 3, 51–100%) and the intensity (negative: 0, weak: 1, moderate: 2, strong: 3) of stained cells. Strong and moderate

staining could be detected at ×40 and ×100 magnification, respectively, whereas weak staining could be detected at ×200 magnification. The IRS (0–9) was obtained by multiplying the percentage score and the intensity score. An IRS ≥ 1 was defined as Sox9-positive, and the IRS = 0 was defined as Sox9-negative. The correlations between Sox9 expression and the clinicopathological features were investigated. Overall survival was calculated from the day of operation. Two patients who did not undergo curative resection were excluded from the overall survival analysis.

2.2. Cell line culture, transfection and RNA interference

Human ICC cell lines HuCCT1 and HuH28 were purchased from JCRB Cell Bank, Japan. HuCCT1 and HuH28 cells were grown at 37 °C under 5%CO_2 in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco®; Invitrogen, Carlsbad, CA). The Sox9 cDNA ligated into the pCMV6 vector was purchased from OriGene (Rockville, MD). As a negative control (Mock), a pCMV6 vector with no insertion was used.

HuCCT1 and HuH28 cells were transfected with Sox9 cDNA or pCMV6 vector using TransIT®-2020 Transfection Reagent (Mirus, Madison, WI) according to the manufacturer's instructions. HuCCT1 cells were transfected with Sox9 short interference RNA (siRNA) (Silencer® Select siRNA, Ambion, Carlsbad, CA) using Lipofectamine® RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. As a negative control (scramble), Silencer® Select Negative Control siRNA (Ambion) was used. Successful regulation of Sox9 expression in the HuCCT1 and HuH28 cells was verified by Western blotting.

2.3. Western blotting

The samples were lysed with Triton-deoxycholate (DOC) lysis buffer. After centrifugation, the supernatant was stored at −80 °C until analysis. After the amounts of protein were measured using a BCA protein assay kit (Pierce 23227, Life Technologies, Carlsbad, CA), loading buffer [2% SDS, 5% β-mercaptoethanol, 5% sucrose, 0.005% bromophenol blue and 62.5 mM Tris–HCl (pH6.8)] was added to the proteins, and the mixtures were incubated at 95 °C for 5 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% acrylamide gels. The proteins were transferred onto Immobilon-P membranes (IPVH10100, Millipore, Bedford, MA) in a transfer buffer containing 20% methanol.

After blocking in 5% skim milk for 60 min at room temperature, the membranes were reacted with primary antibody overnight at 4 °C, and followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. The following primary antibodies were used: Sox9 (ab3697, Abcam), E-cadherin (ab15148, Abcam), vimentin and α-SMA: (ab157392, Abcam). The proteins were visualised using a chemiluminescence system (RPN2132, GE Healthcare, Buckinghamshire, UK). The quantification of the Western blotting data was performed with GAPDH as a reference, using Image J software (NIH, Bethesda, Maryland).

2.4. Cell proliferation assay

Twenty-four, 48, and 72 hours after the transfection of HuCCT1 and HuH28 cells, Cell Proliferation Reagent WST-1 (Roche Applied Science, Indianapolis, IN) was used according to the manufacturer's instructions. A microplate reader (Multiskan® FC, Thermo Fisher Scientific, Vantaa, Finland) was used to calculate the cleavage of WST-1 to formazan by metabolically active cells.

Table 1
Clinicopathological features and Sox9 expression in 43 patients with intrahepatic cholangiocarcinoma.

Variables	Case	Sox9-positive (n, %)	Sox9-negative (n, %)	P-values
Age (years)				1.00
<60	6	3 (50.0)	3 (50.0)	
≥60	37	19 (51.4)	18 (48.6)	
Gender				0.62
Male	26	12 (46.2)	14 (53.8)	
Female	17	10 (58.8)	7 (41.2)	
HBsAg				0.75
Negative	35	18 (51.4)	17 (48.6)	
Positive	8	4 (50.0)	4 (50.0)	
HCVAb				0.35
Negative	38	18 (47.4)	20 (52.6)	
Positive	5	4 (80.0)	1 (20.0)	
Child–Pugh score				1.00
A	36	18 (50.0)	18 (50.0)	
B	7	4 (57.1)	3 (42.9)	
C	0	0 (0)	0 (0)	
Tumour size				0.22
≤5 cm	30	13 (43.3)	17 (56.7)	
>5 cm	13	9 (69.2)	4 (30.8)	
Gross appearance				0.29
MF + PI	10	7 (70.0)	3 (30.0)	
MF	20	11 (55.0)	9 (45.0)	
PI	6	2 (33.3)	4 (66.7)	
IG	7	2 (28.6)	5 (71.4)	
Histological grade[a]				0.43
G1	9	5 (55.6)	4 (44.4)	
G2	24	14 (58.3)	10 (41.7)	
G3	9	3 (33.3)	6 (66.7)	
T stage				0.38
T1	9	3 (33.3)	6 (66.7)	
T2 (a, b)	12	6 (46.2)	7 (53.8)	
T3	5	4 (80.0)	1 (20.0)	
T4	16	9 (56.3)	7 (43.7)	
Nodal status				0.51
N0	30	14 (46.7)	16 (53.3)	
N1	13	8 (61.5)	5 (38.5)	
Distant metastasis				0.49
No	42	22 (52.4)	20 (47.6)	
Yes	1	0 (0)	1 (100)	
Biliary infiltration				0.034
No	10	2 (20.0)	8 (80.0)	
Yes	33	20 (62.5)	13 (37.5)	
Venous invasion				0.93
No	32	17 (53.1)	15 (46.9)	
Yes	11	5 (45.5)	6 (54.5)	
UICC stage				0.36
I	9	3 (33.3)	6 (66.7)	
II	12	6 (50.0)	6 (50.0)	
III	2	2 (100)	0 (0)	
IV A, B	20	11 (55.0)	9 (45.0)	
Resection				1.00
Curative	41	21 (51.2)	20 (48.8)	
Non-curative	2	1 (50.0)	1 (50.0)	

HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C virus antibody; MF, mass forming type; IG, intraductal growth type; PI, periductal infiltrating type.

[a] One case was diagnosed with adenosquamous carcinoma.

2.5. Wound healing assay

Forty-eight hours after the transfection into HuCCT1 cells cultured in 6-well plates, the transfected cells were trypsinized and transferred to 35-mm culture dishes containing ibidi culture inserts (ibidi, Martinsried, Germany) which makes a "wound field" at a density of 4×10^4 cells in 70 μL of serum-free PRMI 1640, and were incubated until confluence. Culture inserts were removed carefully using sterile forceps, and the medium was aspirated from the well. After the well was washed with phosphate-buffered saline (PBS) to remove debris and unattached cells, serum-free RPMI1640 was added to avoid cell proliferation. Migration into the 0.5-mm open wound field was visualised under a light microscope. Wound closure was monitored and the percent closure rate (%) (migrated cell surface area/total surface area × 100) was measured at the indicated times.

2.6. Matrigel invasion assay

Forty-eight hours after the transfection into HuCCT1 cells cultured in 6-well plates, the transfected cells were harvested by trypsinization, and a suspension (7.5×10^4 cells) in 0.5 mL of serum-free PRMI 1640 was transferred to 24-well BD BioCoat[TM] Matrigel[TM] Invasion Chamber inserts (BD Biosciences, San Jose, CA). A 0.5-μL volume of RPMI 1640 medium supplemented with 10% FBS was added to each well. Two days after incubation at 37 °C under 5% CO_2, the medium was aspirated carefully from the inside of the insert, and the non-invading cells were removed from the interior of the insert using a cotton swab. The insert was transferred to a clean well containing 400 μL of Cell Stain Solution (Cell Biolabs, San Diego, CA) and incubated for 15 min at room temperature. After the stained insert was washed with water, the number of invaded cells were counted under a light microscope with four randomly chosen
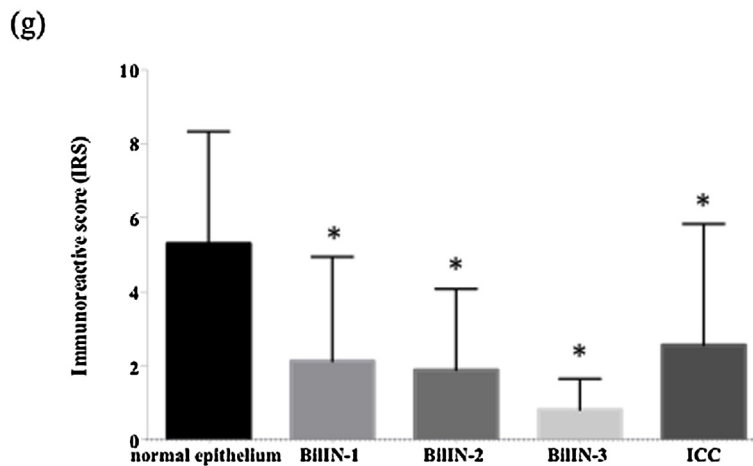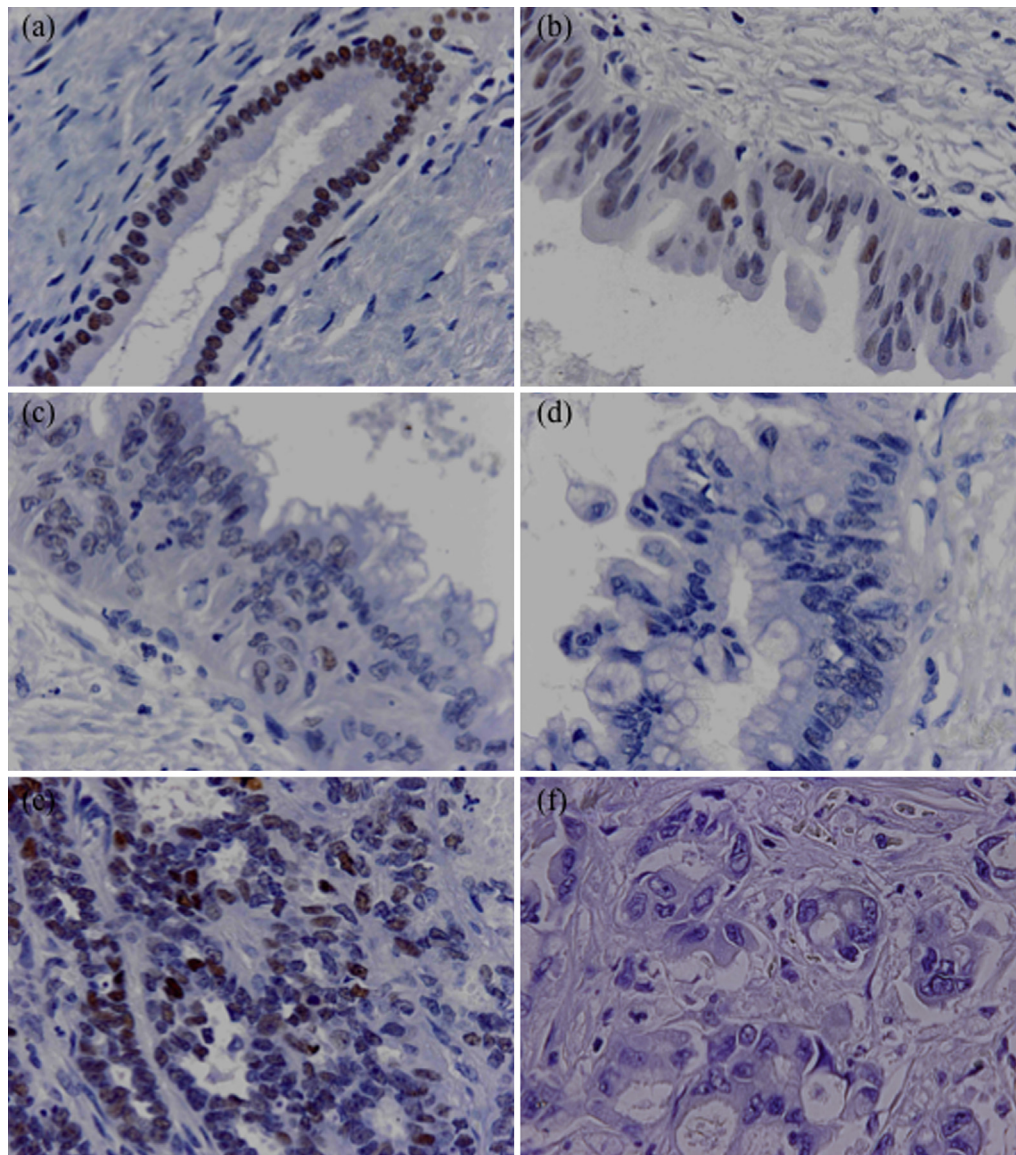
Fig. 1. Sox9 expression of normal epithelium, biliary intraepithelial neoplasia and intrahepatic cholangiocarcinoma. (a) The nuclei of normal epithelial cells were strongly positive for Sox9. (b, c, d) Sox9 expressions of biliary intraepithelial neoplasia-1 (b), -2 (c), and -3 (d) decreased in a stepwise fashion. (e, f) Although Sox9 expression was positive in 51.2% of the intrahepatic cholangiocarcinomas, the others were negative (original magnification ×400). (g) Immunoreactive score in normal epithelium, biliary intraepithelial neoplasia, and intrahepatic cholangiocarcinoma. The Sox9 expressions of biliary intraepithelial neoplasia and intrahepatic cholangiocarcinoma were significantly lower than that in the normal epithelium ($P < 0.0001$). The number of lesions investigated for the normal epithelium, biliary intraepithelial neoplasia-1, -2, -3, and intrahepatic cholangiocarcinoma were 43, 47, 27, 16, and 43, respectively. *$P < 0.0001$ (vs. normal epithelium). IRS, immunoreactive score; BilIN, biliary intraepithelial neoplasia; ICC, intrahepatic cholangiocarcinoma.
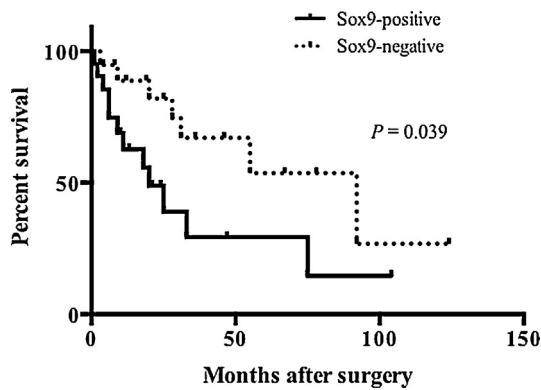
Fig. 2. Kaplan–Meier survival curve by Sox9 expression in the 41 patients with intrahepatic cholangiocarcinoma. The Sox9-positive patients ($n=21$) showed significantly poorer prognoses than the Sox9-negative patients ($n=20$) ($P=0.039$).

individual fields ($\times100$ magnification) per insert by two blinded investigators (T.K. and A.T.).

2.7. Statistical analysis

The statistical analysis was performed using GraphPad PRISM6 for Mac (GraphPad Software, San Diego, CA). The relationships between the Sox9 expression of ICC and clinicopathological features were analysed using Fisher's exact test or chi-square test. Overall survival was analysed using the Kaplan–Meier method and a log-lank test. All in vitro data were obtained from at least three individual experiments. Student's t-test was used to determine significant differences between two groups. All tests were two-sided, and P-values <0.05 were considered significant.

3. Results

3.1. Sox9 expressions in ICCs and BilINs compared to normal epithelium

The nuclei of cells in 90.7% (39/43) of the normal epithelium samples showed strong staining for Sox9 (Fig. 1a, Table 2). The cytoplasm of cells was not stained. The numbers of Sox9-positive cells in the BilIN-1, -2, and -3 lesions were lower than that in the normal epithelial cells (Fig. 1b–d). The percentages of Sox9-positivity in BilIN-1, -2, and -3 were 59.6% (28/47), 66.7% (18/27), and 56.3% (9/16), respectively (Table 2). On the other hand, 51.2% (22/43) of the ICC samples were positive for Sox9 expression (Fig. 1e, f, Table 2). The IRS in the BilINs and ICC were significantly lower than that in the normal epithelium ($P<0.0001$) (Fig. 1g).

3.2. Association between Sox9 expression in ICCs and biliary infiltration, overall survival

There was a significant difference in biliary infiltration between the Sox9-positive ICC patients and Sox9-negative ICC patients ($P=0.034$) (Table 1). The Kaplan–Meier curves by Sox9 expression are shown in Fig. 2 The Sox9-positive ICC patients showed significantly poor prognoses compared to the Sox9-negative ICC patients ($P=0.039$).

3.3. Sox9 expression effect on cell proliferation in HuCCT1 and HuH28 cells

To determine whether cell proliferation in HuCCT1 and HuH28 cells could be regulated by Sox9 expression, we assessed the cell proliferation using a WST-1 assay after the transfection of Sox9

cDNA. In both the HuCCT1 cells and HuH28 cells, there was no significant difference in the cell proliferation between the groups transfected with Sox9 cDNA and those transfected with empty vectors at 72 h following transfection (Fig. 3a).

3.4. Sox9 overexpression and migration in HuCCT1 cells

To examine the role of Sox9 in the migration of ICC cells, we performed wound healing assays. Migration was significantly promoted in the HuCCT1 cells transfected with Sox9 cDNA compared to the cells transfected with empty vector ($P<0.05$) (Fig. 3b). In contrast, the migration of the Sox9 siRNA-treated cells was significantly inhibited compared to the negative control ($P<0.05$) (Fig. 3c).

3.5. Sox9 overexpression and invasion in HuCCT1 cells

The transfection with Sox9 cDNA induced invasion of the HuCCT1 cells through the Matrigel chamber ($P<0.05$) (Fig. 4a). The invasion of the Sox9 siRNA-treated cells was significantly inhibited compared to the negative control ($P<0.05$) (Fig. 4b).

3.6. Sox9 overexpression and expression of E-cadherin, vimentin and α-SMA in ICC cell lines

To investigate the molecular mechanism mediated by Sox9, we determined the protein levels of epithelial-to-mesenchymal transition (EMT) markers including E-cadherin, vimentin and α-SMA by Western blotting (Fig. 4c). In both HuCCT1 and HuH28 cells, Sox9 overexpression significantly decreased the E-cadherin expression and increased the expressions of vimentin and α-SMA. These results suggested that Sox9 might promote migration and invasion in ICC at least in part by activating the EMT.

4. Discussion

Although ICCs are thought to arise from the intrahepatic bile duct through multi-step carcinogenesis, similar to the adenoma-carcinoma sequence in colon cancer [8,27,28], the mechanism has not been sufficiently elucidated. BilIN lesions are considered to be precursors of ICC, and to progress to invasive carcinoma through a multistep process [7,8]. To the best of our knowledge, we are the first to report Sox9 expression in BilINs and the effect of Sox9 expression on cell migration and invasion in ICC cell lines.

In recent years, mutations in several oncogenes and tumour suppressor genes have been reported. Mutations in the KRAS oncogene, the p16[INK4A] tumour suppressor gene and p53 inactivation in ICC have been reported [29,30]. Moreover, Nakanuma et al. reported that decreased p16[INK4A] expression was seen in BilIN-2, -3 and invasive ICCs [8]. Hsu et al. reported that p53 overexpression was not seen in BilIN lesions, and that the frequency of KRAS mutation was 25% in BilIN-1 and 30% in BilIN-3 [31].

Relationships between p53 activation, KRAS mutation and Sox9 expression have been reported in pancreatic ductal carcinoma and lung adenocarcinoma [32–34]. In the present study, the Sox9 expression in the BilIN-1 samples was significantly lower than that in the normal epithelium. Our results thus suggest that decreased Sox9 expression might be an earlier molecular event during the multi-step carcinogenesis compared to other genes and it might be related to ICC carcinogenesis. The molecular role of Sox9 in normal biliary tract and BilINs was not investigated in the present study, but Furuyama et al. reported that Sox9 is expressed and supplies cells continuously to maintain homeostasis or regeneration as progenitor cells in the bile duct of the adult liver [35]. Therefore, decreased Sox9 expression might contribute to the early carcinogenesis of ICCs based on the lack of the maintenance of normal biliary epithelial differentiation.

Table 2
Sox9-positivity in normal epithelium, biliary intraepithelial neoplasia, and intrahepatic cholangiocarcinoma.

Type of tissue	Number of lesions	Positive (n, %)	Negative (n, %)	P-value[*]
Normal epithelium	43	39 (90.7)	4 (9.3)	
BIN				
−1	47	28 (59.6)	19 (40.4)	<0.001
−2	27	18 (66.7)	9 (33.3)	0.02
−3	16	9 (56.3)	7 (43.7)	<0.01
ICC	43	22 (51.2)	21 (48.8)	<0.0001

BIN, biliary intraepithelial neoplasia; ICC, intrahepatic cholangiocarcinoma.

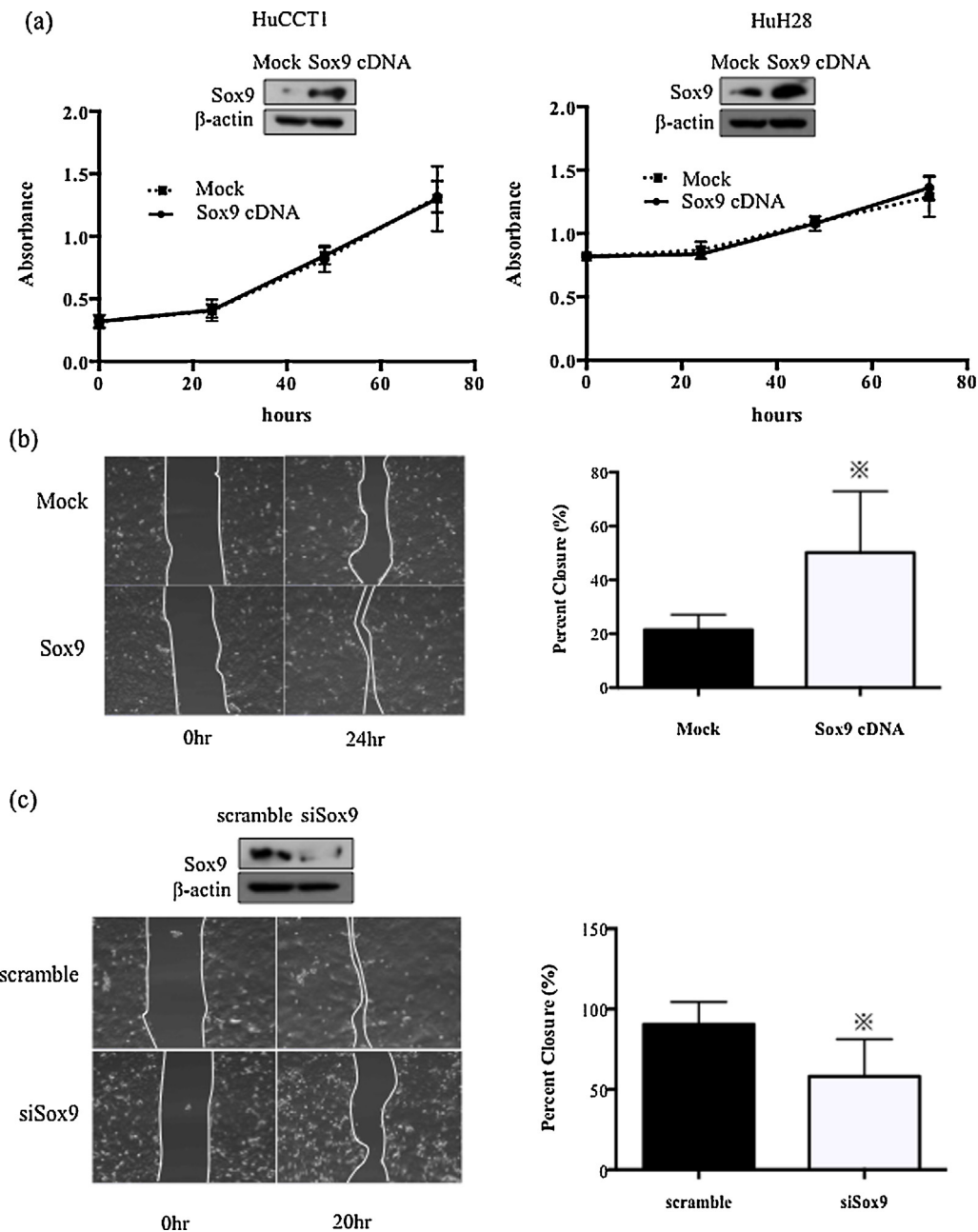[*] P-values compared with normal epithelium.

Fig. 3. Although Sox9 overexpression did not affect cell proliferation, the migration was promoted in the intrahepatic cholangiocarcinoma cell lines. (a) Proliferation of HuCCT1 and HuH28 cells transfected with empty vector (Mock) or Sox9 cDNA was measured using a WST-1 assay at 24, 48 and 72 h after the transfection. Sox9 overexpression did not affect cell proliferation. (b) Migration was significantly promoted in the HuCCT1 cells transfected with Sox9 cDNA compared to the cells transfected with empty vector (P < 0.05). Monitoring was conducted under a light microscope (×100 magnification). (c) The migration of the Sox9 siRNA-treated cells was significantly inhibited compared to the negative control (P < 0.05). The data were obtained from at least three independent experiments and are expressed as the mean ± SD. *P < 0.05.
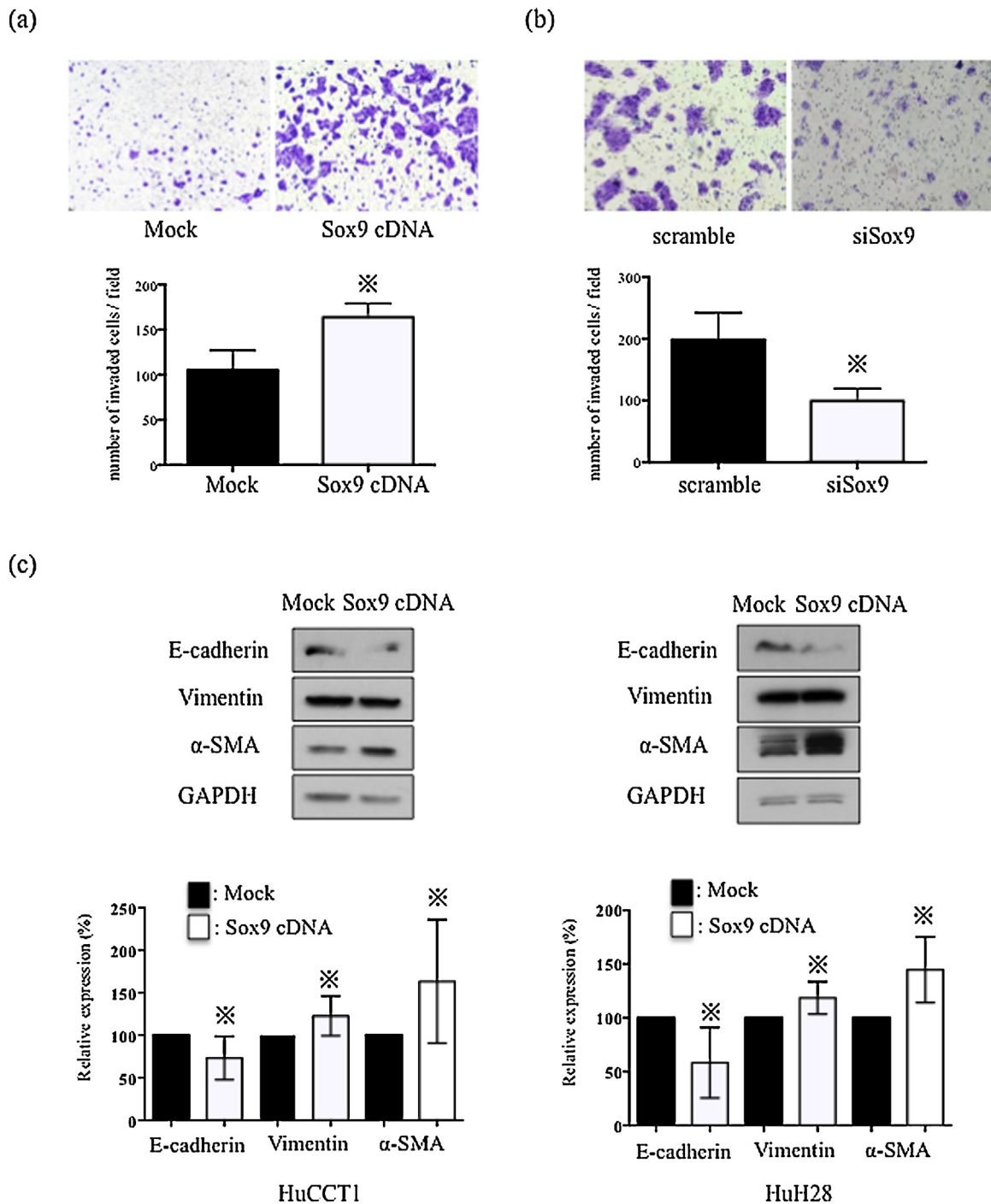
(a)

(b)

(c)

Fig. 4. The invasion of HuCCT1 cells was promoted by Sox9 overexpression and inhibited by Sox9 siRNA. (a) The Matrigel chamber assay showed that Sox9 overexpression increased the number of invaded cells. (b) Downregulated expression of Sox9 decreased the number of invaded cells in the Matrigel chamber assay. (c) Western blotting showed that Sox9 overexpression induced a decreased expression of E-cadherin and increased the expressions of vimentin and α-SMA in HuCCT1 and HuH28 cells. The data were obtained from at least three independent experiments and are expressed as the mean ± SD. *$P < 0.05$.

However, although the Sox9 expression in the present study's ICCs was significantly lower than that in the normal epithelium, the Sox9 expression of ICCs was significantly higher than that of the BilIN-3 lesions. These results suggest that Sox9 expression decreased in a stepwise fashion during the carcinogenesis and that it may increase in ICCs. A relationship between Sox9 expression and the carcinogenesis of GCs has been reported [20,36]. Sun et al. noted that the Sox9 expression increased during the normal-metaplasia-adenoma transition, and thereafter, the expression decreased during the progression to GC [36]. In the carcinogenesis

of pancreatic ductal adenocarcinoma (PDAC), it was reported that the Sox9 expression was decreased in pancreatic intraepithelial neoplasia and increased in PDAC [25,37]. These two-stage patterns of Sox9 expression in GC and PDAC carcinogenesis are thus similar to that of ICC carcinogenesis, and therefore the roles of Sox9 in the early stage of ICC carcinogenesis may be different from that in ICC progression. Further investigations are needed to elucidate the mechanism of Sox9 expression in ICC carcinogenesis.

In the present study, the Sox9 expression of the ICCs was associated with the biliary infiltration and poor prognosis. The molecular

effects of Sox9 that promote cell migration and invasion were confirmed by gene transfection using two ICC cell lines. The Western blot analysis showed that the upregulation of Sox9 expression induced the characteristic change of EMT. The EMT is one of the pivotal processes by which human cancers acquire their metastatic potential. During the EMT, the characteristics of gene expression of epithelial cells are altered including the down-regulation of epithelial markers and the up-regulation of the mesenchymal markers. These alternations induce epithelial cells to lose their polarity and acquire the migratory disposition [38,39]. We analysed E-cadherin as an epithelial marker in the present study, and vimentin and α-SMA were analysed as mesenchymal markers. Huang et al. reported that decreased E-cadherin and increased vimentin expression promote the invasion and metastasis of ICC and are correlated with poor prognosis [40]. Our present findings are thus consistent with their study. Although we observed that Sox9 expression was not associated with nodal status, distant metastasis, or venous invasion, the number of patients included in the present study ($n = 43$) is not large. Further investigations are necessary to determine the clinical value of Sox9 expression in ICC.

Notch signalling has been known to regulate the differentiation of cholangiocytes from hepatoblasts in embryonic formation, and Sox9 has been known as a downstream target gene of the Notch pathway [14,41]. According to previous reports, the persistent activation of Notch signalling is associated with hepatic tumours, and Notch1 activates ICC migration by causing EMT [42–44]. Moreover, Sox9 was reported as a mediator of Notch1-induced EMT in lung adenocarcinoma [35]. Our results therefore suggest that Sox9 might interact with Notch1 to induce EMT in ICC.

Mazur et al. reported that nuclear/cytoplasmic Sox9 was expressed strongly in 64%/17% of 111 cases of bile duct carcinomas and that cytoplasmic Sox9 expression was an independent prognostic factor for overall survival [45]. In the present study, cytoplasmic Sox9 was not expressed. The differing results between these two studies could be attributed to the differences of antibodies used for immunohistochemistry. Moreover, their study was comprised of 32.4% (36/111) gallbladder carcinomas and 52.8% (58/111) extrahepatic cholangiocarcinomas. The mechanism of differentiation or formation in the extrahepatic bile duct is considered to be different from that in the intrahepatic bile duct [46,47]. Therefore, in the present study, we excluded samples from patients diagnosed with gallbladder carcinomas and extrahepatic cholangiocarcinoma.

In summary, our findings suggest that Sox9 loss might be related to ICC carcinogenesis in the early stage, whereas increased Sox9 expression of ICC is related to tumour invasion, at least in part via EMT. Sox9 may be a preventive target for ICC carcinogenesis. In the near future, further studies should be performed to elucidate the molecular effects of Sox9 in ICC carcinogenesis and progression.

Conflict of interest
None declared.

Funding

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