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CXCL12 expression in intrahepatic cholangiocarcinoma is associated with metastasis and poor prognosis

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Abstract

Intrahepatic cholangiocarcinoma is a rare malignant biliary neoplasm that causes a poor prognosis even after curative hepatectomy. Liver metastasis is the major recurrence pattern of intrahepatic cholangiocarcinoma; therefore, the prevention of liver metastasis is a desirable objective. The aim of this study is to identify gene(s) related to liver metastasis of intrahepatic cholangiocarcinoma and to examine the inhibitory effects on metastasis of intrahepatic cholangiocarcinoma by controlling such gene(s). We collected 3 pairs of intrahepatic cholangiocarcinoma frozen samples, and 36 pairs (primary and metastatic lesions) of intrahepatic cholangiocarcinoma formalin-fixed paraffin-embedded samples, from patients who underwent surgical resection at hospitals related to the Kyushu Study Group of Liver Surgery between 2002 and 2016. We carried out cDNA microarray analyses and immunohistochemistry to identify candidate genes, and evaluated one of them as a therapeutic target using human cholangiocarcinoma cell lines. We identified 4 genes related to liver metastasis using cDNA microarray, and found that *CXCL12* was the only gene whose expression was significantly higher in liver metastasis than in primary intrahepatic cholangiocarcinoma by immunohistochemistry ($P = .003$). In prognosis, patients in the high *CXCL12* group showed a significantly poor prognosis in disease-free ($P < .0001$) and overall survival ($P = .0004$). By knockdown of *CXCL12*, we could significantly suppress the invasive and migratory capabilities of 2 human cholangiocarcinoma cell lines. Therefore, *CXCL12* might be associated with metastasis and poor prognosis in intrahepatic cholangiocarcinoma.

KEYWORDS
cDNA microarray, CXCL12, intrahepatic cholangiocarcinoma, metastasis, prognosis

Abbreviations: CA19-9, carbohydrate antigen 19-9; CAFs, cancer associated fibroblast; CXCL12, C-X-C motif chemokine 12; FFPE, formalin-fixed, paraffin-embedded; ICC, intrahepatic cholangiocarcinoma; IHC, immunohistochemistry; KRT83, keratin 83; LM, liver metastasis; OLFM4, olfactomedin 4; REG3G, regenerating islet-derived protein 3 gamma.

1 | INTRODUCTION

Intrahepatic cholangiocarcinoma is a malignant biliary cancer that generally has a poor prognosis. Even after curative hepatectomy, its 5-year overall survival rate remains 22%-24%.[1] One of the reasons for this is the high incidence of recurrence after curative hepatectomy. Therefore, patients with ICC need more effective and additional therapies, such as chemotherapy or radiotherapy before or after surgery, or both before and after surgery. Several clinical trials involving adjuvant or neoadjuvant therapy for ICC have been undertaken; however, there has been little evidence of their effectiveness to date.[2] Although reresection is an effective treatment for limited recurrence cases,[3] in order to improve patients' prognosis, developing new treatment methods to reduce ICC metastasis after curative hepatectomy is an urgent issue.

Several molecular and biological studies on ICC metastasis have already been reported.[4-8] As these studies were concerned with primary tumor or cholangiocarcinoma cell lines, they did not directly compare metastatic lesions to primary tumors using clinical samples. Therefore, how such proteins are expressed in metastatic lesions of ICC still remains unclear. In addition, in ICC, more than half of the instances of recurrence are LM[9]; therefore, being able to predict and prevent LM could lead to improved patient prognosis after curative hepatectomy.

The aim of this study is to find new therapeutic targets to suppress ICC metastasis using resected samples of primary and metastatic lesions of ICC.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

Intrahepatic cholangiocarcinoma frozen and FFPE samples were collected from patients who underwent surgical resection for ICC at hospitals affiliated with the Kyushu Study Group of Liver Surgery between 2002 and 2016. Three pairs of frozen samples and 127 patients' FFPE samples were finally included in this study. Among the 127 patients' samples, we could obtain 36 surgically resected metastatic FFPE samples in 30 primary samples. We obtained written informed consent from each patient, and the study procedure was approved by each institutional review board.

2.2 | cDNA microarray

cDNA microarray analysis was carried out according to the Oncomics protocol using RNA extracted from the 3 pairs of frozen samples of primary and metastatic lesions of ICC, using an RNeasy Kit from Qiagen according to the manufacturer's protocol.

2.3 | Cholangiocarcinoma cell lines

Human cholangiocarcinoma SSP-25 cells and HuH-28 cells were purchased from RIKEN Bioresource Center. SSP-25 cells were grown in RPMI-1640 medium supplemented with 10% FBS, and HuH-28 cells were grown in Eagle's minimum essential medium supplemented with 10% FBS. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.4 | Antibodies and siRNA

For IHC, mAbs against CXCL12 (MAB350) were obtained from R&D Systems, KRT83 (orb184603) from biorbyt, OLFM4 (#14369) from Cell Signaling Technology, and REG3G (ab198216) from Abcam. *CXCL12* expression was transiently downregulated using a predesigned Silencer Select siRNA directed against *CXCL12* from Thermo Fisher Scientific, and a nontargeting siRNA was used as a negative control. SSP-25 and HuH-28 were transfected with the annealed siRNA for 24 hours using Lipofectamine RNAimax from Thermo Fisher Scientific.

2.5 | Real-time RT-PCR

RNA was isolated from the cultured cells using an RNeasy Kit from Qiagen according to the manufacturer's protocol. mRNA expression levels were determined by quantitative RT-PCR using TaqMan probes (Roche), and the values were normalized to those of β-actin. All quantitative RT-PCR reactions were run using the LightCycler 480 System II (Roche Diagnostics). All data obtained using real-time RT-PCR were from experiments undertaken in triplicate, and the data are shown as the mean ± SE.

2.6 | Immunohistochemistry

Paraffin-embedded sections of tissues obtained from the ICC patients were deparaffinized and soaked in distilled water. Sample processing and IHC procedures were undertaken as described below. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. The sections were incubated with diluted antibodies (CXCL12, 1:50; KRT83, 1:200; OLFM4, 1:200; REG3G, 1:100) and detection was carried out with a biotin-free HRP enzyme-labeled polymer of the Envision Plus detection system (Dako). Positive reactions were visualized using diaminobenzidine solution, which was followed by counterstaining with Mayer's hematoxylin. All IHC staining was independently scored by 2 blinded pathologists, as follows: staining intensity was scored as 0 to 3 to indicate absent, weak, moderate, or strong expression, respectively. The percentage area of positive cells was scored as 0% to 100%. We calculated the IHC score according to the staining intensity score multiplied by the percentage area of positive cells, and then divided them into 2 groups by each median value of IHC scores.

2.7 | Growth assay

We evaluated cell growth using a CCK-8 Kit (Dojindo Molecular Technologies) according to the manufacturer's protocols. SSP-25 and HuH-28 cells were inoculated in a 96-well plate at 3.0×10^3 cells in 100 μL/well and the plate was incubated overnight in a humidified

incubator at 37°C with 5% CO_2. We used SSP-25 and HuH-28 cells 24 hours after transfection with siRNA when evaluating under conditions that knocked down *CXCL12*. Each well of the plate also received 10 μL CCK-8 solution at the indicated time points (0, 1, 2, and 3 days). Absorbance was measured at 450 nm using a microplate reader after incubating the plate for 1.5 hours. The absorbance of each sample was measured in triplicate.

2.8 | Invasion assay

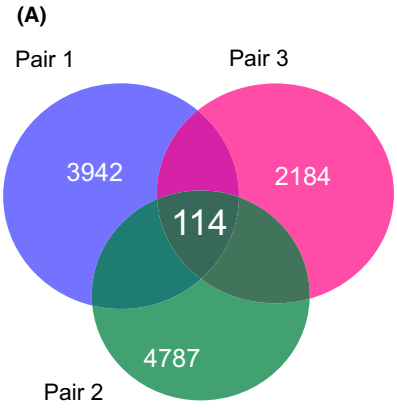
In vitro cell invasion assay was carried out using a BD BioCoat Matrigel Invasion Chamber (BD Biosciences) according to the manufacturer's protocol. Briefly, the invasion rate of tumor cells that migrated through Transwell inserts (8-μm pore size) with a uniform layer of BD Matrigel basement membrane matrix was assessed. The ICC cells were seeded (SSP-25, 2.0×10^4; HuH-28, 3.0×10^4) into the upper chamber of the insert in 500 μL medium, and 750 μL medium in the lower well. After 48 hours of incubation (37°C, 5% CO_2), the invading cells were fixed and stained. The number of invading cells in 3 predetermined fields (total magnification, ×100) was counted by independent investigators using a microscope. The mean of the number counted in each of the 3 fields was defined as the cell number. For each group, the cultures were carried out in triplicate.

2.9 | Migration assay

Six-well plates were imaged with a Keyence BZ-X700 All-in-one Fluorescence Microscope equipped with a CO_2- and temperature-controlled chamber and time-lapse tracking system (Keyence). Images were taken every 5 minutes for 24 hours and converted to movie files using a BZ-X Analyzer (Keyence). The movies were analyzed for cell migration with the video editing analysis software VW-H2MA (Keyence). We tracked 10 cells/well and the tracking data were subsequently processed with Microsoft Excel 2010 to create *x*-*y* coordinate plots and distance measurements.

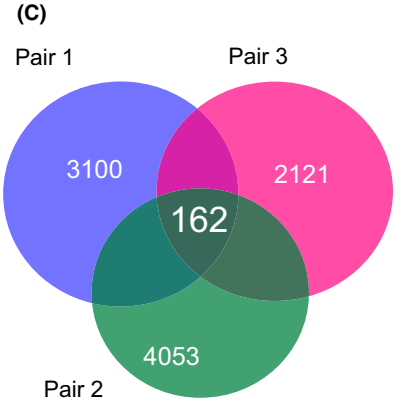
2.10 | Statistical analysis

All experiments were undertaken in triplicate, and the data shown are representative of consistently observed results. Data are



(B)

Gene	Pair 1	Pair 2	Pair 3
	Fold change		
KRT83	107.0	16.9	26.0
CXCL12	62.5	63.4	4.8
UGT2B15	65.7	46.9	3.1
CES1	8.0	78.9	3.0
ADD2	14.2	38.1	9.3
BCAT1	23.9	29.3	6.0
MT1G	3.9	51.9	2.0
GLT1D1	22.9	28.8	5.1
GCGR	2.4	34.6	8.8
LOC100132215	14.7	21.7	9.3



(D)

Gene	Pair 1	Pair 2	Pair 3
	Fold change		
REG3G	207.1	80.8	6.9
FAM3B	6.5	67.5	2.2
PTGDS	42.0	19.7	14.0
OLFM4	34.2	24.8	9.2
RASSF9	29.9	21.7	4.0
LOC101929412	27.1	19.7	7.6
DUOXA2	39.0	11.0	2.4
SCIN	2.7	37.7	2.2
MYO16	23.0	14.2	2.8
VWF	20.5	14.9	2.3

FIGURE 1 cDNA microarray using 3 resected intrahepatic cholangiocarcinoma frozen samples. A, A total of 114 genes were commonly more highly expressed in liver metastasis than in primary lesions. B, *CXCL12* and *KRT83* were most highly expressed among the 114 genes. C, 162 genes whose expression was commonly lower in liver metastasis than in primary lesions. D, *REG3G* was most weakly expressed among the 162 genes

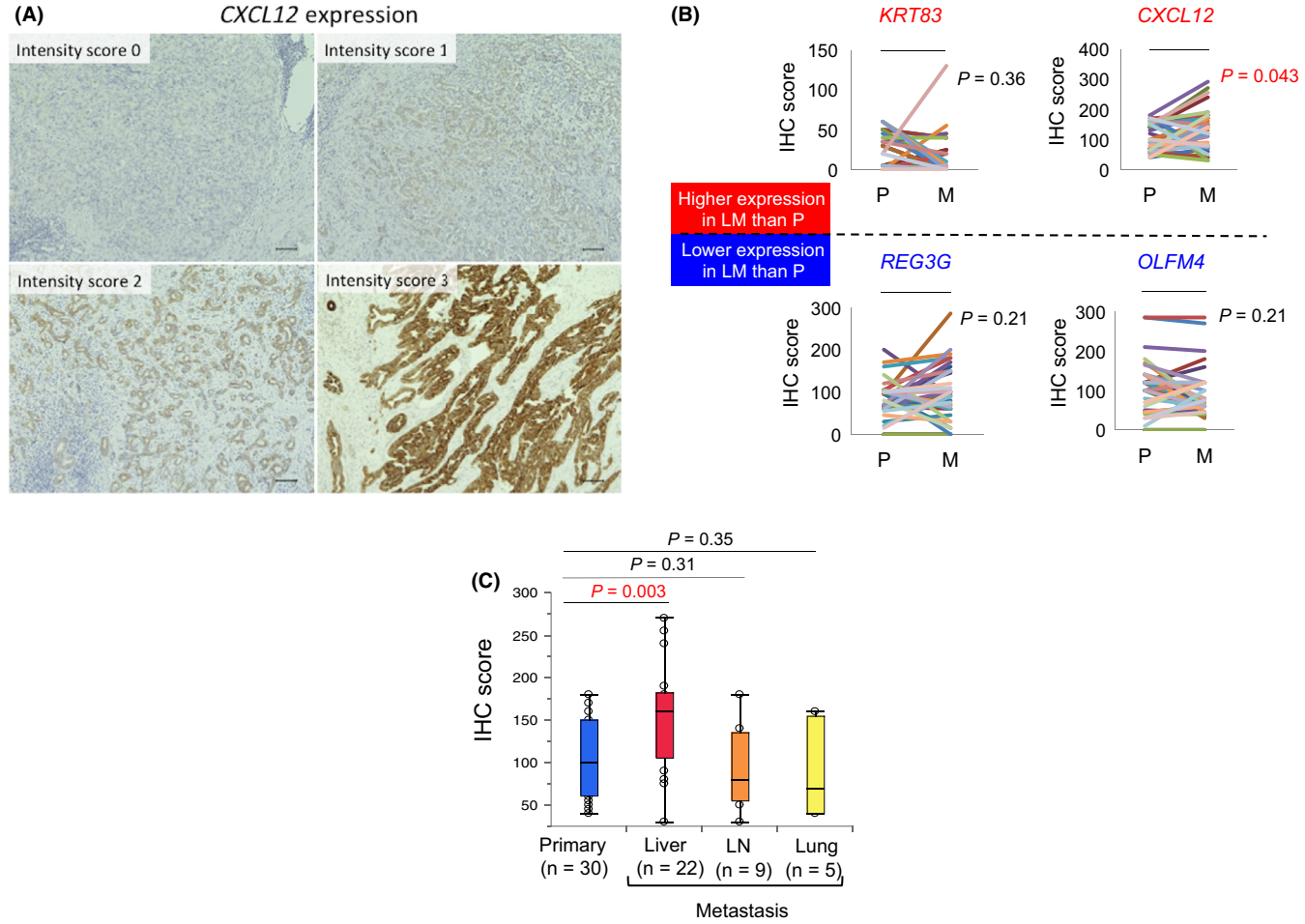


FIGURE 2 *CXCL12* is highly expressed in liver metastasis (LM) lesions of intrahepatic cholangiocarcinoma by immunohistochemistry. A, *CXCL12* expression according to intensity score 0 to 3. Scale bar = 500 μm. B, Only *CXCL12* was expressed significantly more highly in metastatic lesions (M) than in primary lesions (P) (P = .043). C, *CXCL12* expression was significantly higher in LM than primary lesions among several metastatic lesions (P = .003). LN, lymph node

presented as the mean ± SD. The Mann-Whitney *U* test was used to compare continuous variables between the 2 groups, and categorical variables were compared using the χ^2 test. Survival curves were constructed using the Kaplan-Meier method, and log-rank tests were used to evaluate the statistical significance of differences. For all statistical analyses, we used JMP software (version 12; SAS Institute) and considered P values less than .05 were statistically significant.

3 | RESULTS

3.1 | *CXCL12* identified as a key gene in LM of ICC by cDNA microarray and IHC

From the cDNA microarray, we extracted genes whose expression increased 2-fold or more in LM compared with the primary tumor among the 3 pairs of frozen samples. Notably, the *CXCL12* and *KRT83* genes were most highly expressed in metastatic lesions compared with primary among these 114 genes after sorting by total score of fold changes (Figure 1A,B). We also extracted 162 genes whose expression decreased 2-fold or more in LM compared with the primary tumor among the frozen samples (Figure 1C,D). *REG3G* was most weakly expressed in metastatic lesions compared with primary tumor. In addition, *OLFM4* was reported as highly expressed in cholangiocarcinoma.[10] Therefore, we chose *REG3G* and *OLFM4* of the lower expressed genes on metastatic lesions.

Next, we evaluated the expression of these 4 genes by IHC using FFPE samples including 30 primary and 36 metastatic lesions. Typical images by each Ab are shown in Figures 2A and S1. We confirmed that *CXCL12* was significantly highly expressed in metastatic lesions (P = .043); however, the other 3 genes were not significantly different (Figure 2B). In addition, we found that *CXCL12* expression was higher in LM than in lung or lymph node metastasis (P = .003) (Figure 2C). Moreover, we evaluated *CXCR4* and *CXCR7* expression, which are receptors to *CXCL12*, and found there were no significant differences in *CXCR4* and *CXCR7* expression between primary and metastatic lesions (data not shown).

TABLE 1 Comparison of clinicopathological characteristics and intrahepatic cholangiocarcinoma tumor-related factors according to *CXCL12* expression

Variable	*CXCL12* expression		*P* value
	Low (n = 61)	High (n = 66)	
Clinicopathological characteristic			
Age (years)	65.0 ± 9.8	66.3 ± 10.2	.48
Gender (M/F)	36/25	44/22	.46
BMI (kg/m^2)	23.2 ± 3.1	23.0 ± 3.8	.67
HBs-Ag (+/-)	7/54	6/60	.77
HCV-Ab (+/-)	13/48	9/57	.35
T-bil (mg/dL)	0.96 ± 1.7	0.86 ± 0.38	.22
Alb (g/dL)	4.1 ± 0.4	4.1 ± 0.4	.89
PT (%)	97.2 ± 14.2	95.5 ± 14.6	.33
ICG R15 (%)	10.7 ± 6.7	10.4 ± 5.9	.90
Child-Pugh (A/B)	60/1	63/3	.62
CEA (ng/mL)	2.9 ± 2.8	38.1 ± 258.4	.54
CA19-9 (U/mL)	190 ± 942	2684 ± 9893	<.01
Tumor-related factor			
Gross type			.14
Mass-forming	52	46	
Periductal infiltrating	3	8	
Mass-forming + periductal infiltrating	6	11	
Tumor size (mm)	34.5 ± 24.5	34.3 ± 25.2	.83
Tumor number (single/multiple)	53/8	57/9	.93
Tumor differentiation (well-mod/poorly)	38/21	44/18	.56
Vascular invasion (yes/no)	21/40	36/29	.02
Lymph node metastasis (yes/no)	6/55	15/51	.047
UICC pStage (I-II/III-IV)	43/18	38/27	.19
Adjuvant therapy (yes/no)	8/53	21/45	.02

Alb, albumin; BMI, body mass index; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; F, female; HBs-Ag, hepatitis B surface antigen; HCV-Ab, hepatitis C virus Ab; ICG R15, indocyanine green retention rate at 15 min; M, male; PT, prothrombin time; T-bil, total bilirubin.

3.2 | *CXCL12* expression in primary ICC correlates with poor prognosis and LM

We examined the association between *CXCL12* expression in primary ICC and patients' characteristics, prognosis, and LM. The following studies were carried out on *CXCL12* expression in 127 primary ICCs. Of 127 patients, 66 expressed a high level of *CXCL12* and 61 expressed a low level. In patients' characteristics, CA19-9 was significantly higher (P = .004) in the high *CXCL12* group than in the low group. In addition, in tumor-related factors, there were more patients with vascular invasion (P = .02), lymph node metastasis (P = .047), and adjuvant chemotherapy (P = .02) in the high *CXCL12* group (Table 1). In prognosis, the patients in the high *CXCL12* group had a significantly poor prognosis in both disease-free (P < .0001) and overall survival (P = .0004) (Figure 3). Furthermore, with regard to LM, patients in the high *CXCL12* group experienced significantly more frequent LM after resection (P = .0012) (Table 2).

3.3 | Knockdown of *CXCL12* leads to reduced invasion and migration of ICC cell lines

We examined the suppression of metastatic potential by knockdown of *CXCL12* using 2 human cholangiocarcinoma cell lines, SSP-25 and HuH-28. In the invasion assay, we confirmed that knockdown of *CXCL12* (Figure 4A), and it significantly inhibited the invasive capabilities of SSP-25 and HuH-28 cell lines (Figure 4B). In addition, in the migration assay, we also confirmed that knockdown of *CXCL12* significantly inhibited their migratory capabilities (Figure 4C). On the other hand, by knockdown of *CXCL12*, neither morphological change nor inhibitory effect on cell proliferation could be observed (data not shown).

4 | DISCUSSION

This is the first report to investigate key molecule(s) in LM of ICC by cDNA analysis using clinically resected samples. We carried out this experiment using paired samples of primary and metastatic lesions, and could identify that *CXCL12* was significantly highly expression in LM lesions compared to primary lesions by cDNA microarray and IHC. We also found that patients with high expression of *CXCL12* in primary lesions had a higher incidence of LM and poor prognosis. In addition, we could confirm the inhibitory effects on invasiveness and migration capabilities of 2 human ICC cell lines by suppressing *CXCL12* secretion by the cancer cell itself.

CXCL12, which is also known as stromal cell-derived factor-1, is a member of the C-X-C chemokine subfamily and a known ligand for the G protein-coupled receptors *CXCR4* and *CXCR7*.[11,12] Interactions between *CXCL12* and *CXCR4* or *CXCR7* comprise a biological axis that affects growth, angiogenesis, and metastasis of cancers. Cancer cells themselves secrete *CXCL12*, as do CAFs and several organs such as lung, liver, bone, and brain.[13] In other words, *CXCL12* plays a role not only in paracrine but also in autocrine signaling through *CXCR4* or *CXCR7*. There are several reports on paracrine function in cancer involving *CXCL12* and *CXCR4*/*CXCR7*. Izumi et al[14] found that *CXCL12*/*CXCR4* activation by CAFs promoted the invasiveness of gastric cancer cells. In ICC, Ohira et al[15] reported that the interaction of *CXCL12* released from fibroblasts and *CXCR4* expressed on ICC cells could be actively involved in ICC migration. Gentilini et al[16] showed that ICC cell migration and survival were modulated

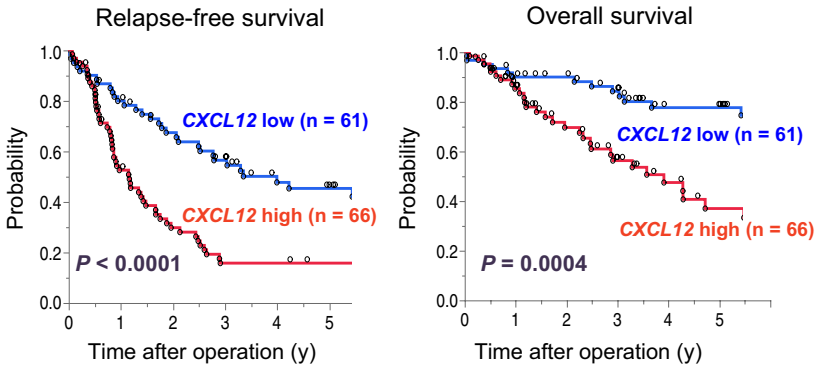


FIGURE 3 Association between prognosis and *CXCL12* expression in patients with intrahepatic cholangiocarcinoma. Patients with high *CXCL12* expression had significantly shorter relapse-free survival ($P < .0001$) and overall survival ($P = .0004$)

TABLE 2 Association between *CXCL12* expression and liver metastasis in intrahepatic cholangiocarcinoma

	All	*CXCL12* expression	
	n = 127	Low (n = 61)	High (n = 66)
Liver metastasis (+)	50 (39.4)	15 (24.6)	35 (53.0)
Liver metastasis (–)	77 (60.6)	46 (75.4)	31 (47.0)

Data shown as n (%).

by cross-talk between *CXCR4* and *CXCL12* released by human hepatic stellate cells. Zhao et al[17] also reported that interaction of *CXCR4-CXCL12* was associated with tumor formation, invasion, and migration of HuCCT-1 cells using *shCXCR4* and *CXCL12*. Thus, there are several reports concerning the paracrine interactions of *CXCL12* in ICC. However, although Calinescu et al[18] found that *CXCR4-CXCL12* autocrine positive feedback controlled glioblastoma progression, to our knowledge, there are no reports on autocrine signaling involving *CXCL12* in ICC. Our study therefore has novelty in that we could suppress invasive and migratory capabilities by suppressing the ICC cells' own *CXCL12*. As *CXCL12* can bind to *CXCR4* and *CXCR7*, if we target such receptors, we would need to block both receptors in order to suppress metastasis.

We also found that *CXCL12* expression was significantly higher in LM than in lymph node and lung metastatic lesions, and patients with high expression of *CXCL12* in the primary ICC were more likely to have significantly frequent LM. In addition, in our own experiments, *CXCL12* tended to be more highly expressed in cholangiocytes than in other organs (lungs and lymph nodes) in IHC (data not shown). It is conceivable that LM is increased due to differences in secretion of *CXCL12* in each organ, but to test this it will be necessary to further

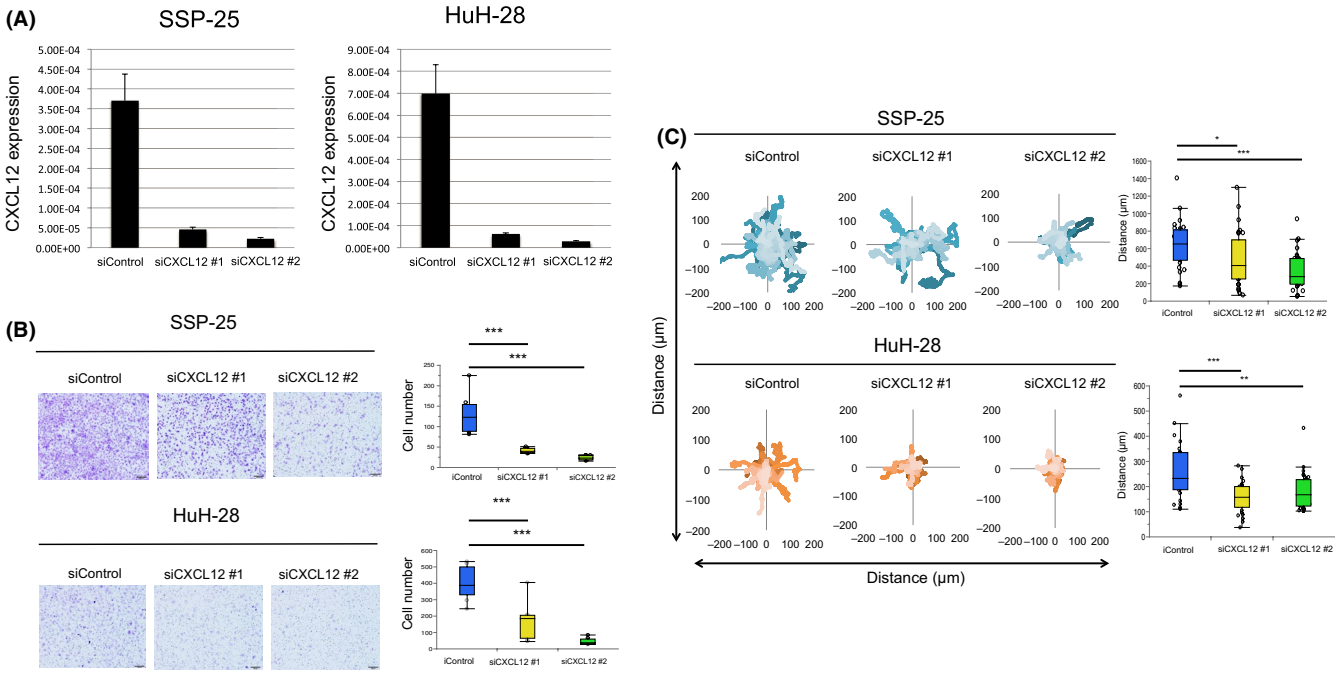


FIGURE 4 Knockdown of *CXCL12* suppresses invasion and motility of SSP-25 and HuH-28 cholangiocarcinoma cells. A, *CXCL12* was suppressed by siRNA in both cell lines. B, Invasiveness of SSP-25 and HuH-28 was significantly suppressed by *CXCL12* knockdown. C, Migration capabilities of SSP-25 and HuH-28 were significantly suppressed by *CXCL12* knockdown. *$P < .05$; **$P < .001$; ***$P < .0001$. siCntl, control

examine *CXCL12* in each organ by quantification and animal experiments. Patients with high *CXCL12*-expressing esophagogastric, lung, and pancreatic cancers had a poor prognosis compared those with low *CXCL12* expression[19]; however, in ICC, the effect of *CXCL12* expression on patients' prognosis remains unclear. In addition, there is also no information available on the association between *CXCL12* expression and metastatic lesions. Therefore, this study is the first to show an association between patients' prognosis and *CXCL12* expression in primary and metastatic lesions of ICC.

This study has 2 limitations. First, in the cDNA microarray, the number of pairs of clinical samples was small (n = 3); however, the pairs of primary and metastatic lesions of ICC frozen samples should be considered to be very valuable. Second, the study was undertaken using only clinical samples and cell lines. To confirm the inhibitory effect on LM by suppression of *CXCL12* in vivo, animal experiments will be required.

In summary, *CXCL12* was associated with invasion, migration, and metastasis in ICC, and might be a pivotal target that can improve prognosis in patients with ICC. Thus, additional studies are needed to evaluate this result as a potentially new therapeutic target or prognostic biomarker for ICC. In future, development of a multidisciplinary treatment strategy is expected to contribute to developing individualized therapeutic regimens in ICC.

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DISCLOSURE

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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