

RESEARCH ARTICLE

CD90 expression in human intrahepatic cholangiocarcinoma is associated with lymph node metastasis and poor prognosis

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Background and Objectives: Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer. However, its prognosis remains poor. Expression of cluster of differentiation 90 (CD90) has been identified as an indicator of poor prognosis in many cancers. Here, we examined the importance of CD90 expression in ICC.

Methods: We performed immunohistological assays for CD90 in human ICC surgical specimens and assessed its relationship with clinicopathological findings and prognosis. Moreover, we analyzed the characteristics of CD90⁺ cells, mainly with respect to metastatic potential, using human ICC cell lines.

Results: CD90 expression was significantly associated with lymph node metastasis and was revealed to be an independent prognostic factor. The CD90⁺ cells present in ICC specimens did not appear to be cancer-associated fibroblasts, as they did not express α -smooth muscle actin. In vitro, CD90⁺ cells exhibited greater migratory ability and higher expression of epithelial-mesenchymal transition (EMT)-related genes, including CXCR4 and MMP7, than CD90⁻ cells. Wnt/ β -catenin signaling pathway activation was also heightened in CD90⁺ cells. In such cells, EMT appeared to be induced by CXCR4 and MMP7 expression through activation of Wnt/ β -catenin signaling.

Conclusion: CD90⁺ cells demonstrate high metastatic potential owing to Wnt/ β -catenin signaling activation and are associated with poor prognosis in ICC.

KEYWORDS

cluster of differentiation 90, epithelial-mesenchymal transition, intrahepatic cholangiocarcinoma, lymph node metastasis, Wnt/ β -catenin signaling

1 | INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer after hepatocellular carcinoma.¹ Although effective new treatments for many other cancers have prolonged patient survival, ICC remains difficult to diagnose and treat, and its

prognosis remains poor.² Therefore, the establishment of novel therapeutic strategies is an urgent necessity.

Cluster of differentiation 90 (CD90), is a 25 to 37 kDa glycosylphosphatidylinositol-anchored glycoprotein that is expressed in many normal cells, including leukocytes, thymocytes, endothelial cells, and fibroblasts, and is involved in cell-cell and cell-matrix interactions.³

Moreover, hepatic stem/progenitor cells have been shown to express CD90 during normal fetal and adult liver development and regeneration.^{4,5} CD90 expression has been reported to be an indicator of poor prognosis in many malignancies, and has various effects in cancer development.⁶⁻⁸ For instance, in hepatocellular carcinoma, it is associated with tumor differentiation and metastasis.^{9,10} However, few studies on CD90 expression in ICC have been conducted.

The aims of this study were to clarify the significance of CD90 expression in ICC and examine its clinical utility. We tested CD90 expression in human ICC surgical specimens, and assessed its relationship with clinicopathological findings and prognosis. Moreover, we analyzed the characteristics of CD90^{+/−} cells, with a particular focus on metastatic potential, using human ICC cell lines to elucidate the role of CD90 and CD90 expression in this malignancy.

2 | MATERIALS AND METHODS

2.1 | Patients

This study included 77 consecutive patients with pathologically confirmed ICC diagnosed from January 2001 to December 2013 at Kyoto University Hospital. The clinicopathological characteristics of the subjects are summarized in Table 1. The observation period lasted until the patient's death or the end of this study (31 December 2014). Written informed consent for the use of resected tissue samples was obtained from all patients in accordance with the Declaration of Helsinki, and this study was approved by the institutional review committee of our hospital (R0921-1).

2.2 | Immunohistochemistry

We utilized thinly cut, paraffin-embedded sections of the surgically resected specimens for immunohistochemical analysis. Once deparaffinized and rehydrated, the sections were heated in Target Retrieval Solution (Dako, Santa Clara, CA) for 8 minutes in a microwave for antigen retrieval. The sections were then incubated in 3% hydrogen peroxide in methanol for 10 minutes, before being exposed to a mouse anti-human CD90 primary antibody (diluted 1:150; BioLegend, San Diego, CA) at 4°C for 16 hours. After thorough rinsing, the sections were incubated for 30 minutes with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (EnVision+ Kit, Dako) and subsequently for 5 minutes with 3,3'-diaminobenzidine substrate (Dako). The cells stained for CD90 were relatively rare even in the positive patients. The CD90-positivity of 5% or more was defined as a positive cut-off value to eliminate results from nonspecific staining. For immunofluorescence staining, sections were incubated in 2% bovine serum albumin for 30 minutes before being exposed to mouse anti-human CD90 and rabbit anti-human α -smooth muscle actin (α -SMA) primary antibodies (Abcam, Cambridge, UK) diluted 1:150 and 1:500, respectively. Alexa Fluor (Molecular Probes, Eugene, OR) 488-conjugated goat anti-mouse and Alexa Fluor 555-conjugated donkey anti-rabbit secondary antibodies (both diluted 1:500) were used to visualize CD90 and α -SMA staining, respectively. The stained tissues were covered with Vectashield mounting medium (Vector Laboratories

Inc., Burlingame, CA) containing 4',6-diamidino-2-phenylindole. The slides were reviewed and scored independently by two investigators (R Yamaoka and T Ishii) who were blinded to the patient's information.

2.3 | Cell culture

Three human ICC cell lines were tested in this study. RBE and SSP-25 cells were obtained from the RIKEN Bioresource Center (Tsukuba, Japan) and HuCCT-1 cells were obtained from the Cell Resource Center for Biochemical Research at Tohoku University (Sendai, Japan). All cells were authenticated by short tandem repeat profiling analysis before receipt and were propagated for less than 6 months after resuscitation. Cells were cultured at 37°C in 5% CO₂ in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Gibco, Waltham, MA).

2.4 | Flow cytometry and cell sorting

The cultured cells were prepared as described previously.^{11,12} Dead cells were eliminated by 7-amino-actinomycin D (Beckman Coulter, Brea, CA) staining. To determine the proportion of CD90⁺ cells, the cells were incubated on ice for 30 minutes with phycoerythrin (PE)-conjugated anti-human CD90 antibody (BioLegend), for which PE-conjugated mouse

TABLE 1 Clinicopathological findings of ICC patients

	CD90 expression		P value
	Positive, n = 25	Negative, n = 52	
Age, y (mean $\pm$ SD)	66.2 $\pm$ 13.1	64.4 $\pm$ 9.8	0.498
Gender (male/female)	13/12	33/19	0.457
Tumor number ($\leq$ 2)	7	10	0.392
Tumor size (>5 cm)	16	24	0.223
Lymph node metastasis	15	8	0.00002
Venous invasion	8	13	0.592
Portal invasion	14	19	0.145
Bile duct invasion	8	15	0.788
Intrahepatic metastasis	7	7	0.102
UICC stage (III-IV/I-II)	15/7	13/39	0.0007
CEA (>5 ng/mL)	6	10	0.765
CA 19-9 (>37 U/mL)	17	31	0.617
HBsAg positive	1	4	1
HCV Ab positive	5	7	0.502
Tumor differentiation			
Well + moderate	15	39	0.0784
Poorly	9	8	

Abbreviation: CD90, cluster of differentiation 90; UICC, union for international cancer control; CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9; HBsAg, hepatitis B virus surface antigen; HCV Ab, hepatitis C virus antibody; ICC, intrahepatic cholangiocarcinoma.

(BioLegend) was used as an isotype control. The cells were analyzed and isolated using a FACSria cell sorter (BD Biosciences, Franklin Lakes, NJ).

2.5 | Reverse-transcription polymerase chain reaction and reverse-transcription quantitative polymerase chain reaction

Reverse-transcription polymerase chain reaction (RT-PCR), and RT-quantitative PCR (RT-qPCR) were performed as described previously.¹³ An RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract total RNA, 1 µg of which was reverse-transcribed into cDNA, using ReverTra Ace qPCR MasterMix (Toyobo, Osaka, Japan) according to the manufacturer's protocol. Primers were synthesized for the following human genes: *CD90*, E-cadherin, vimentin, snail family transcriptional repressor 1, snail family transcriptional repressor 2 (*slug*), transforming growth factor β (*TGF β*), C-X-C motif chemokine receptor 4 (*CXCR4*), matrix metalloproteinase 7 (*MMP7*), *MMP9*, and β -actin. Their sequences are summarized in Supporting Information Table 1. RT-qPCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7500 system (Applied Biosystems). For each target, samples were tested in triplicate, and expression of target genes was normalized to that of β -actin.

2.6 | Wound-healing and migration assays

Wound-healing assays were used to assess cell motility. We seeded the isolated *CD90*⁺ or *CD90*⁻ cells at a density of 1×10^6 per well in 35-mm culture dishes. Once fully confluent, the cell layer was scratched with a plastic 200-µL pipette tip and cultured in low-serum (2% FBS) culture medium. Micrographs were taken 24 hours after scratching, and the distance between the migrating cells at the leading edges of the scratch was measured. For migration assays, 24-well cell culture plates (Corning Inc., Corning, NY) and 8-µm-pore inserts coated with type I collagen were used. We placed 2.5×10^4 *CD90*⁺ or *CD90*⁻ cells in serum-free medium in the upper chambers, and normal culture medium containing 10% FBS in the lower chambers. After incubation for 48 hours, the cells on the upper surface of the membrane were removed, and those on the lower surface were fixed with 4% paraformaldehyde and stained using a Diff-Quik staining kit (Sysmex, Kobe, Japan).

2.7 | CD90 knockdown by RNA interference

We transfected *CD90*-small interfering RNA (siRNA) (#4427037-s14125 or #4427037-s14126; Invitrogen) or negative control siRNA (#4390843; Invitrogen) at a final concentration of 10 nmol/L into ICC cells, using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol.

2.8 | Assessment of Wnt/ β -catenin signaling activation

To evaluate activation of the Wnt/ β -catenin signaling pathway in ICC cells, we stained nuclear active β -catenin by immunohistochemistry.

We fixed and stained the cultured cells as previously described.^{14,15} An anti-active β -catenin primary antibody (Merck Millipore, Burlington, MA) diluted 1:150 was used, and its binding was visualized with the EnVision system (Dako). For further evaluation of Wnt/ β -catenin signaling activation, we performed a T-cell factor/lymphoid enhancer factor (TCF/LEF) luciferase assay. A Signal TCF/LEF Reporter Assay Kit (Qiagen) and Dual-Glo Luciferase Assay System (Promega, Madison, WI) were used according to the manufacturers' protocols. The Wnt/ β -catenin signaling inhibitor ICG-001 was purchased from AdooQ (Irvine, CA), dissolved in 100% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO), and diluted with RPMI 1640 medium to the desired concentration, with the final DMSO concentration being less than 0.1%. *CD90*⁺ cells were cultured with 10 µM ICG-001 for 48 hours, after which RT-qPCR was performed to assess changes in gene expression due to inhibition of Wnt/ β -catenin signaling.

2.9 | Statistical analysis

Statistical analyses were performed using SPSS (IBM Corp., Armonk, NY) and GraphPad Prism Version 5.0 (GraphPad Software Inc., La Jolla, CA). The Student *t* test, F-test, Mann-Whitney *U* test, Fisher exact test, or χ^2 test, and analysis of variance were performed. Mean $\pm$ SD of three or more independent experiments are reported. Postoperative recurrence-free survival (RFS) and overall survival (OS) were calculated using the Kaplan-Meier method and analyzed with the log-rank test. Significant variables from the univariate analysis were included in a multivariate analysis using a Cox regression model with forward stepwise selection. *P* value less than 0.05 was defined as indicating a statistically significant difference.

3 | RESULTS

3.1 | CD90 expression in human ICC surgical specimens

To examine *CD90* expression in human ICC, 77 surgically resected ICC tumors were subjected to immunohistological assays. The follow-up period from surgery until death or the end of the study ranged from 0.34 to 11.7 years (median, 2.66 years). *CD90* expression was observed in 25 of the 77 patients. In one patient, *CD90*⁺ cells were found diffusely distributed within poorly differentiated ICC tissue (Figure 1A), whereas in another patient, they were seen maintaining an incomplete luminal structure in the tumor stroma (Figure 1B).

Lymph node metastasis was significantly associated with tumor *CD90* expression (Table 1). Furthermore, there were no significant correlations between *CD90* expression and the surgical factors (Table 2). Median RFS and OS were 0.52 and 2.00 years for *CD90*⁺ patients and 1.85 and 6.87 years for *CD90*⁻ patients, respectively. *CD90*⁺ patients exhibited significantly shorter RFS (Figure 1C; *P* < 0.01) and OS (Figure 1D; *P* < 0.01) than *CD90*⁻ patients. The log-rank test revealed that *CD90* expression, multiple tumors, lymph node metastasis, high serum carbohydrate antigen 19-9 (CA 19-9) level, and poorly differentiated tumor were associated with shorter

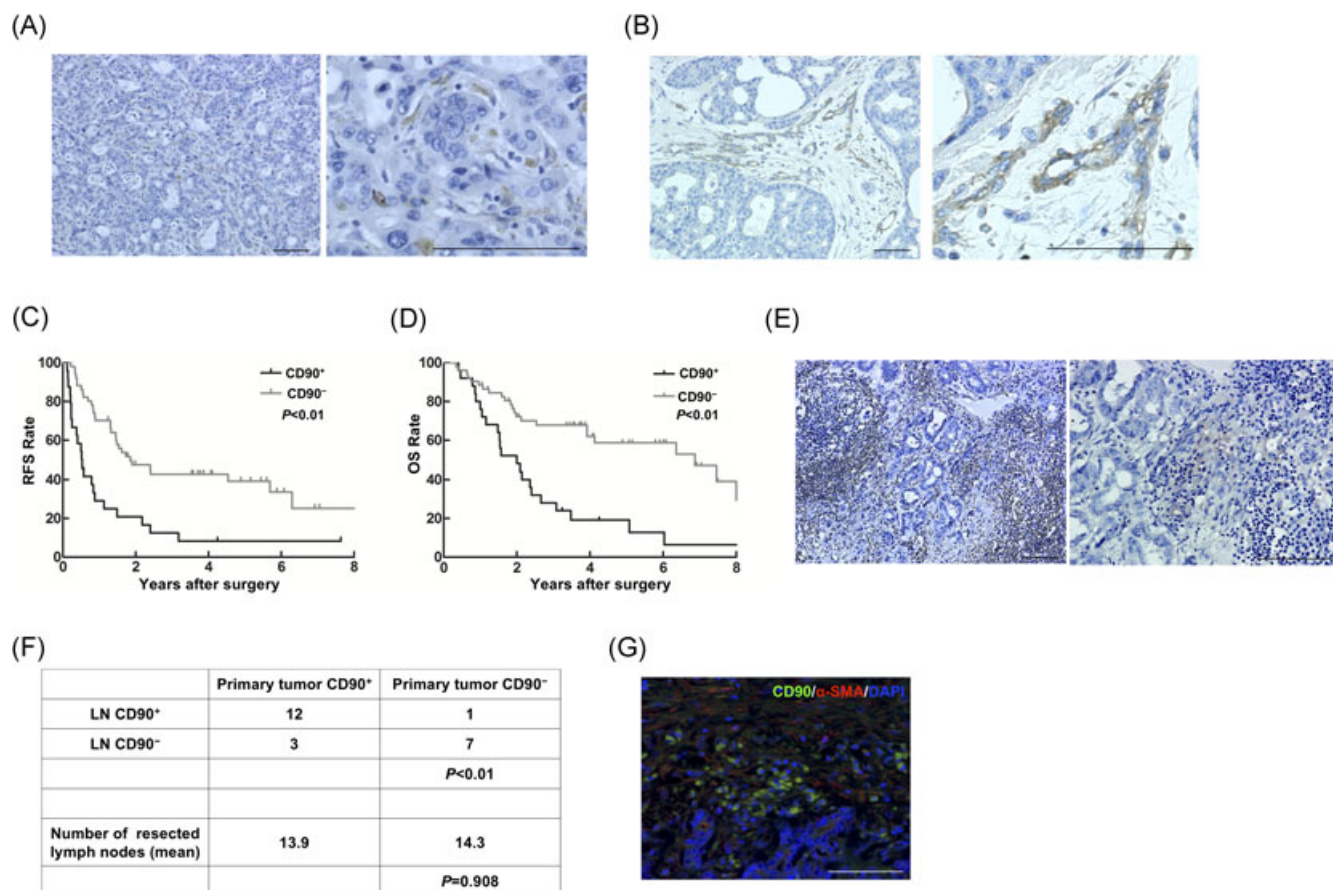


FIGURE 1 A, B, CD90 expression in human ICC tissues was assessed by immunohistochemistry. These images were obtained from tissues of two different patients. The scale bar represents 100 μ m. C, D, Kaplan-Meier curves and log-rank tests showed that patients with CD90⁺ tumors had worse prognoses in terms of RFS (C, $P < 0.01$) and OS (D, $P < 0.01$). E, Immunohistochemical analysis of CD90 expression in the metastatic lymph nodes of the ICC patients. F, CD90⁺ primary tumors were significantly associated with the presence of CD90⁺ cells in metastatic LN according to Fisher's exact test ($P < 0.01$). There were no significant differences in the total number of resected LN between the CD90⁺ and CD90⁻ primary tumors. G, Immunofluorescence staining of CD90 and α -SMA expression in human ICC specimens. CD90, cluster of differentiation 90; ICC, intrahepatic cholangiocarcinoma; LN, lymph nodes; OS, overall survival; RFS, recurrence-free survival; α -SMA, α -smooth muscle actin

RFS (Table 3) and OS (Table 4). Multivariate analysis demonstrated that tumor differentiation, CD90 expression, and CA 19-9 level were independent predictors of postoperative recurrence, and that the latter two were also independent predictors of OS (Table 5).

Immunohistochemical analysis was also performed to evaluate expression of CD90 in patients diagnosed with lymph node metastasis. CD90 expression was noted in the metastatic lymph nodes of 12 of the 23 patients in this group (Figure 1E). CD90 expression in metastatic lymph nodes was more frequent among patients with CD90⁺ primary tumors than among those with CD90⁻ primary tumors (Figure 1F; $P < 0.01$). There were no significant differences in the total number of resected lymph nodes between the CD90⁺ and CD90⁻ primary tumors (Figure 1F). These results suggest that CD90 expression in ICC is an independent indicator of poor prognosis, and is strongly associated with lymph node metastasis. To show that CD90 was expressed in only tumor cells and not stromal fibroblasts, we performed double immunofluorescence staining of CD90 and α -SMA, the latter of which is known to

TABLE 2 Postoperative findings of intrahepatic cholangiocarcinoma patients

	CD90 expression		P value
	Positive, n = 25	Negative, n = 52	
Postoperative mortality (90 d)	0	0	NA
Postoperative 90 d morbidity	14 (56%)	26 (50%)	0.636
Clavien-Dindo I-II complications	6 (24%)	17 (32%)	0.596
Clavien-Dindo III-IV complications	8 (32%)	9 (17%)	0.157
Reoperation	0	0	NA
Postoperative stay in days, median (range)	30 (10-111)	29 (12-141)	0.195

TABLE 3 Univariate analysis of recurrence-free survivals

Factors	Number of patients	Recurrence-free survival, y (median, 95% CI)	P value	Hazard ratio (median, 95% CI)
CD90 expression				
Positive	25	0.52 (0.23-0.88)	0.0001	2.906 (1.682-5.02)
Negative	52	1.85 (1.32-5.69)		
Age, y				
≥60	47	1.13 (0.56-2.40)	0.859	1.051 (0.5471-1.653)
<60	30	1.54 (0.88-2.40)		
Gender				
Male	46	1.48 (0.83-2.18)	0.312	1.318 (0.4435-1.298)
Female	31	1.32 (0.69-2.18)		
CEA, ng/mL				
≥5	16	1.62 (0.40-2.40)	0.879	1.053 (0.5421-2.0)
<5	61	1.45 (0.84-2.18)		
CA 19-9, U/mL				
≥37	48	0.88 (0.52-1.73)	0.00887	2.15 (1.195-3.868)
<37	29	2.41 (1.31-NA)		
Hepatitis C infection				
Present	12	1.97 (0.21-NA)	0.7	1.127 (0.4161-1.888)
Absent	64	1.45 (0.84-1.90)		
Hepatitis B infection				
Present	5	1.32 (0.51-NA)	0.804	1.138 (0.4088-3.167)
Absent	69	1.45 (0.83-1.90)		
Tumor size, cm				
≥5	40	1.45 (0.40-1.90)	0.222	1.399 (0.8135-2.405)
<5	36	1.32 (0.84-2.41)		
Tumor number				
Multiple	17	0.88 (0.23-1.90)	0.0355	1.923 (1.034-3.578)
Single	57	1.48 (0.84-2.41)		
Tumor differentiation				
Poor	17	0.40 (0.23-1.48)	0.01	3.211 (1.7176-007)
Others	54	1.85 (0.88-5.69)		
Portal invasion				
Positive	33	1.48 (0.69-2.40)	0.336	1.143 (0.6634-1.968)
Negative	43	1.45 (0.79-3.18)		
Venous invasion				
Positive	21	1.49 (0.51-6.30)	0.669	1.02 (0.5315-1.808)
Negative	55	1.32 (0.84-2.40)		
Bile duct invasion				
Positive	23	1.49 (0.88-2.40)	0.356	1.185 (0.663-2.119)
Negative	50	1.46 (0.56-4.54)		
Lymph node metastasis				
Positive	23	0.53 (0.25-0.88)	<0.0001	3.857 (2.146-6.934)
Negative	50	2.40 (1.32-6.30)		

Abbreviation: CD90, cluster of differentiation 90; CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9; NA, not available; CI, confidence interval.

TABLE 4 Univariate analysis of overall survivals

Factors	Number of patients	Overall survival, y (median, 95% CI)	P value	Hazard ratio (median, 95% CI)
CD90 expression				
Positive	25	2.00 (1.16-2.66)	<0.01	3.02 (1.66-5.464)
Negative	52	6.87 (3.92-NA)		
Age, y				
≥65	47	2.66 (1.93-8.99)	0.954	1.017 (0.5598-1.848)
< 65	30	3.92 (1.98-6.36)		
Gender				
Male	46	5.08 (1.98-NA)	0.404	1.285 (0.4306-1.405)
Female	31	2.66 (1.59-6.36)		
CEA, ng/mL				
≥5	16	2.03 (0.75-4.13)	0.166	1.593 (0.8193-3.095)
< 5	61	5.08 (2.40-7.46)		
CA 19-9, U/mL				
≥37	48	1.98 (1.53-3.58)	<0.01	3.306 (1.632-6.701)
< 37	29	6.36 (3.92-NA)		
Hepatitis C infection				
Present	12	3.50 (0.64-NA)	0.723	1.159 (0.511-2.628)
Absent	64	3.92 (2.09-6.87)		
Hepatitis B infection				
Present	5	5.08 (0.75-NA)	0.713	1.341 (0.3581-5.021)
Absent	69	3.92 (2.12-6.87)		
Tumor size, cm				
≥5	40	3.07 (1.57-6.87)	0.531	1.209 (0.667-2.191)
< 5	36	3.92 (2.00-NA)		
Tumor number				
Multiple	17	1.67 (1.05-3.08)	<0.01	2.712 (1.427-5.151)
Single	57	5.08 (2.40-NA)		
Tumor differentiation				
Poor	17	1.66 (1.16-2.09)	<0.01	2.684 (1.426-5.054)
Others	54	6.03 (2.66-8.99)		
Portal invasion				
Positive	33	2.36 (1.88-6.87)	0.336	1.335 (0.7411-2.405)
Negative	43	4.13 (2.00-8.99)		
Venous invasion				
Positive	21	2.66 (1.53-NA)	0.669	1.151 (0.6032-2.196)
Negative	55	4.13 (2.09-7.46)		
Bile duct invasion				
Positive	23	2.66 (1.59-7.46)	0.358	1.346 (0.7137-2.54)
Negative	50	6.03 (1.98-8.99)		
Lymph node metastasis				
Positive	23	1.59 (1.05-2.00)		5.627 (2.92-10.84)
Negative	50	7.46 (4.13-NA)		

Abbreviation: CD90, cluster of differentiation 90; CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9; NA, not available; CI, confidence interval.

TABLE 5 Multivariate analysis of factors predicting postoperative prognosis

Variable	Hazard ratio (95% CI)	P value
Recurrence-free survivals		
CD90 expression	1.92 (1.04-3.53)	0.03
Tumor differentiation	2.02 (1.03-2.49)	0.04
CA 19-9	2.01 (1.00-4.03)	0.05
Tumor number	1.28 (0.65-3.97)	0.47
Overall survival		
CA 19-9	2.94 (1.29-6.71)	0.01
CD90 expression	1.99 (1.04-3.79)	0.03
Tumor number	1.83 (0.93-3.60)	0.082
Tumor differentiation	1.67 (0.84-3.31)	0.14

Abbreviation: CD90, cluster of differentiation; CA 19-9, carbohydrate antigen 19-9; CI, confidence interval.

be expressed in fibroblasts. No cells coexpressing CD90 and α -SMA were identified (Figure 1G).

3.2 | CD90 expression in ICC cell lines

Expression of CD90 in ICC cells of the RBE, SSP-25, and HuCCT-1 lines was evaluated. RT-PCR revealed that CD90 was expressed in SSP-25 and RBE cells, but not in HuCCT-1 cells (Figure 2A), and flow cytometry analysis showed that $19.7\% \pm 1.8\%$ and $11.1\% \pm 1.5\%$ of the SSP-25 and RBE cells tested, respectively, expressed the

corresponding protein ($n = 3$; Figure 2B). Immunocytochemistry confirmed that some of the cultured RBE and SSP-25 cells expressed CD90, and demonstrated that the CD90⁺ cells were larger than the CD90⁻ cells (Figure 2C).

3.3 | Migratory ability of CD90^{+/−} ICC cells

To assess the effect of CD90 expression on cell migration in vitro, wound-healing and migration assays were performed. After 24 hours, wound widths were smaller in cultures of CD90⁺ cells than in those of CD90⁻ cells (Figure 3A). Moreover, in the migration assay, significantly more CD90⁺ than CD90⁻ cells were found to have migrated to the lower surface of the membranes (Figure 3B). Taken together, these results indicate that the migratory ability of CD90⁺ cells is greater than that of CD90⁻ cells.

Epithelial-mesenchymal transition (EMT) is one of the most important factors in tumor metastasis,¹⁶ conferring upon tumor cells migratory characteristics similar to those of mesenchymal cells. We therefore investigated the effect of CD90 expression on EMT-related genes, finding *CXCR4* and *MMP7* expression to be higher in CD90⁺ than in CD90⁻ cells (Figure 3C). These results indicate that CD90⁺ cells were involved in the EMT and that their high motility derived from expression of the genes *CXCR4* and *MMP7*.

3.4 | siRNA-mediated CD90 knockdown

CD90 expression was significantly downregulated by both of the siRNAs used (#4427037-s14125 or #4427037-s14126). As both

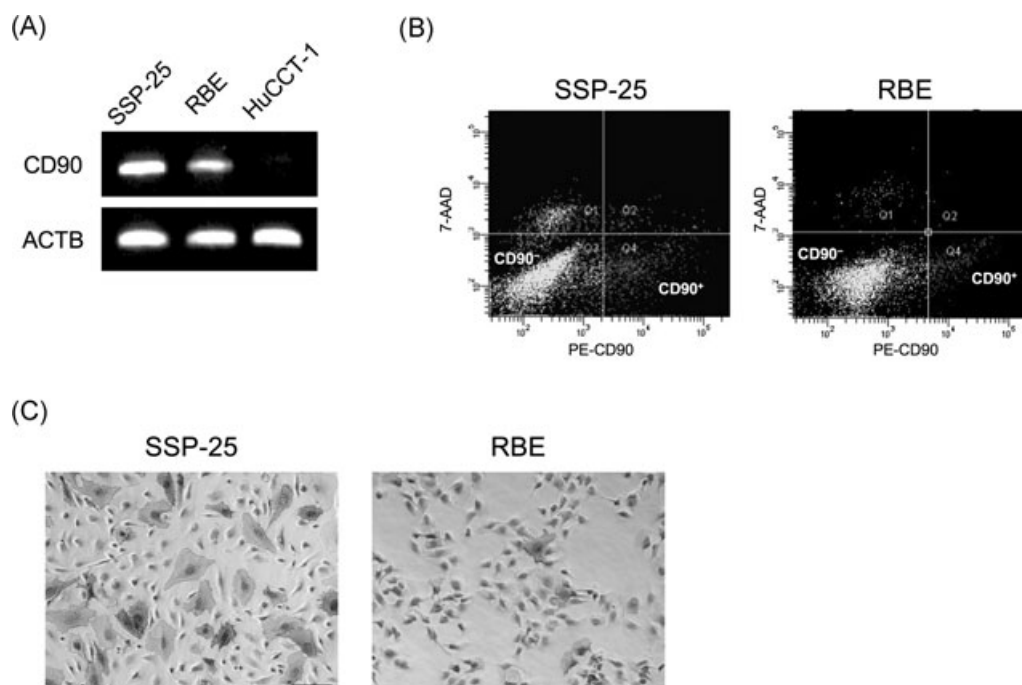


FIGURE 2 A, RT-PCR analysis of three human ICC cell lines. CD90 expression was observed in RBE and SSP-25 cells, but not in HuCCT-1 cells. B, Flow cytometric analyses of RBE (left panel) and SSP-25 (right panel) cells. C, Immunocytochemical staining of CD90 in RBE (left panel) and SSP-25 (right panel) cells visualized using DAB substrate. CD90⁺ cells were larger than CD90⁻ cells in both cell lines. CD90, cluster of differentiation 90; ICC, intrahepatic cholangiocarcinoma; RT-PCR, reverse-transcription polymerase chain reaction

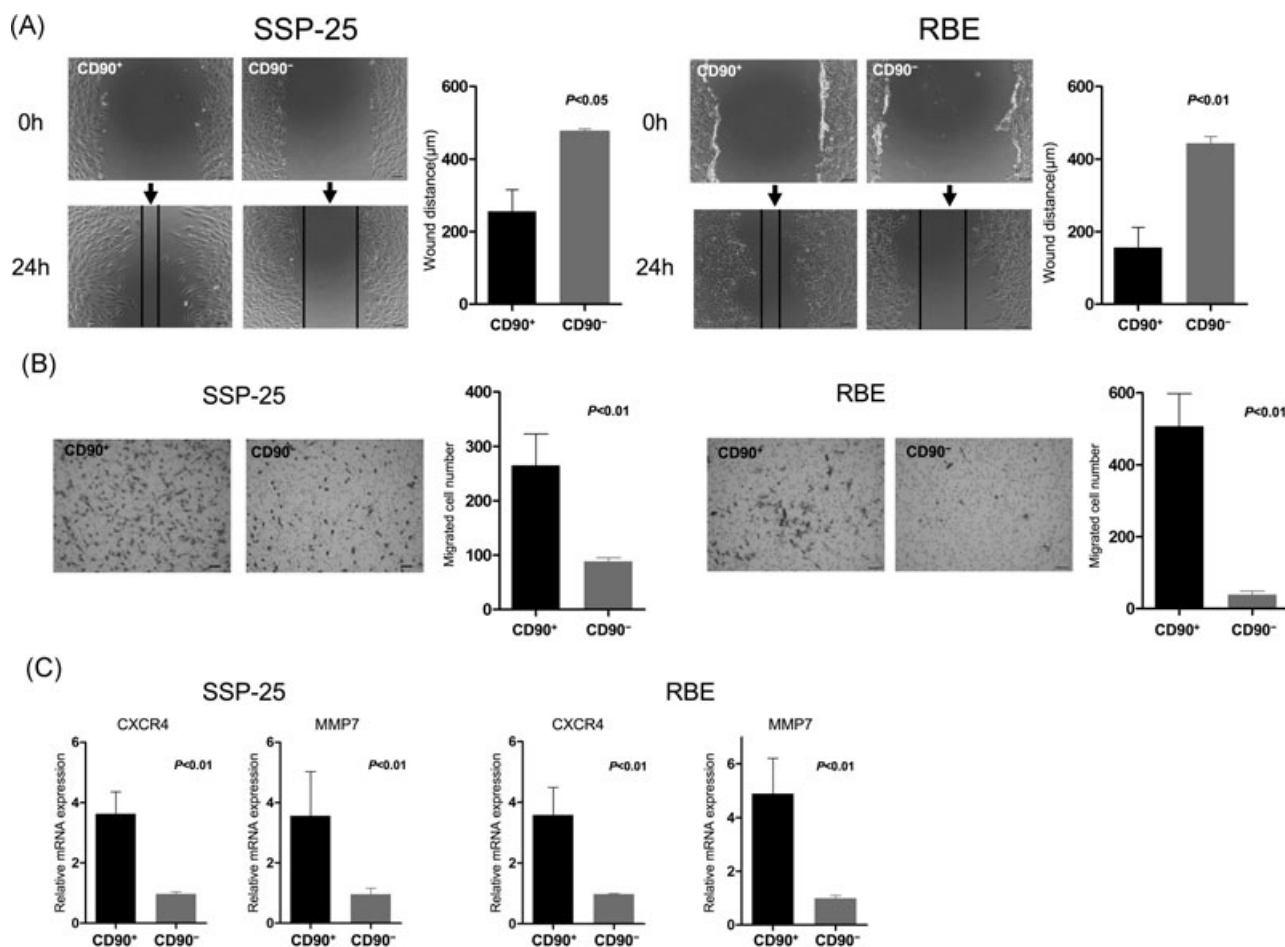


FIGURE 3 A, Wound-healing assay. The red lines indicate the leading edges of the wound formed by migrating cells. The distances between these lines were measured and statistically analyzed using the Student *t* test. The left panels show SSP-25 cells and the right panels show RBE cells. B, Migration assay. The number of cells having migrated from the upper to the lower surface of the membrane were counted, and the results were analyzed using the Student *t* test. The left panels show SSP-25 cells and the right panels show RBE cells. C, RT-qPCR analysis of EMT-related genes. CD90⁺ cells expressed CXCR4 and MMP7 at higher levels than CD90⁻ cells in both SSP-25 and RBE cells. EMT, epithelial-mesenchymal transition; RT-qPCR, reverse-transcription quantitative polymerase chain reaction

siRNAs yielded the same results, representative data obtained with one of them (#4427037-s14125) are shown. CD90 knockdown by siRNA in CD90⁺ cells resulted in decreased migratory ability and reduced CXCR4 and MMP7 expression (Figures 4A and 4B), suggesting that CD90 may positively regulate the expression of both of these genes.

3.5 | Activation of the Wnt/β-catenin signaling pathway in CD90⁺ cells

Since both CXCR4 and MMP7 were expressed at high levels in CD90⁺ cells and these genes have been reported to be targets of the Wnt/β-catenin signaling pathway,^{17,18} we investigated the activation of Wnt/β-catenin signaling in CD90⁺ cells. Immunocytochemical analysis indicated that significantly more CD90⁺ cells than CD90⁻ cells stained positively for active nuclear β-catenin (Figure 5A). In addition, in the TCF/LEF luciferase assay, CD90⁺ cells exhibited higher levels of relative luciferase activity than CD90⁻ cells (Figure 5B). Furthermore, inhibition of the Wnt/β-catenin signaling pathway in CD90⁺ cells using

ICG-001 led to decreased CXCR4 and MMP7 expression (Figure 5C). Our findings showed that the Wnt/β-catenin pathway was more strongly activated in CD90⁺ cells, and that this pathway regulated expression of CXCR4 and MMP7.

4 | DISCUSSION

In the current study, CD90 expression was examined in human ICC surgical specimens to clarify its association with clinicopathological features and prognosis. Immunohistochemical analyses of the 77 ICC patients included revealed CD90 expression to be an independent predictor of postoperative recurrence and survival, and significantly associated with lymph node metastasis. In addition, immunohistochemical assessment showed that patients with CD90⁺ cells in the primary tumor also tended to have CD90⁺ cells in metastatic lymph nodes. These findings suggest that primary tumor CD90⁺ cells may have a high malignant potential, as they are capable of spreading to lymph nodes, and may result in poor prognosis. In some ICC

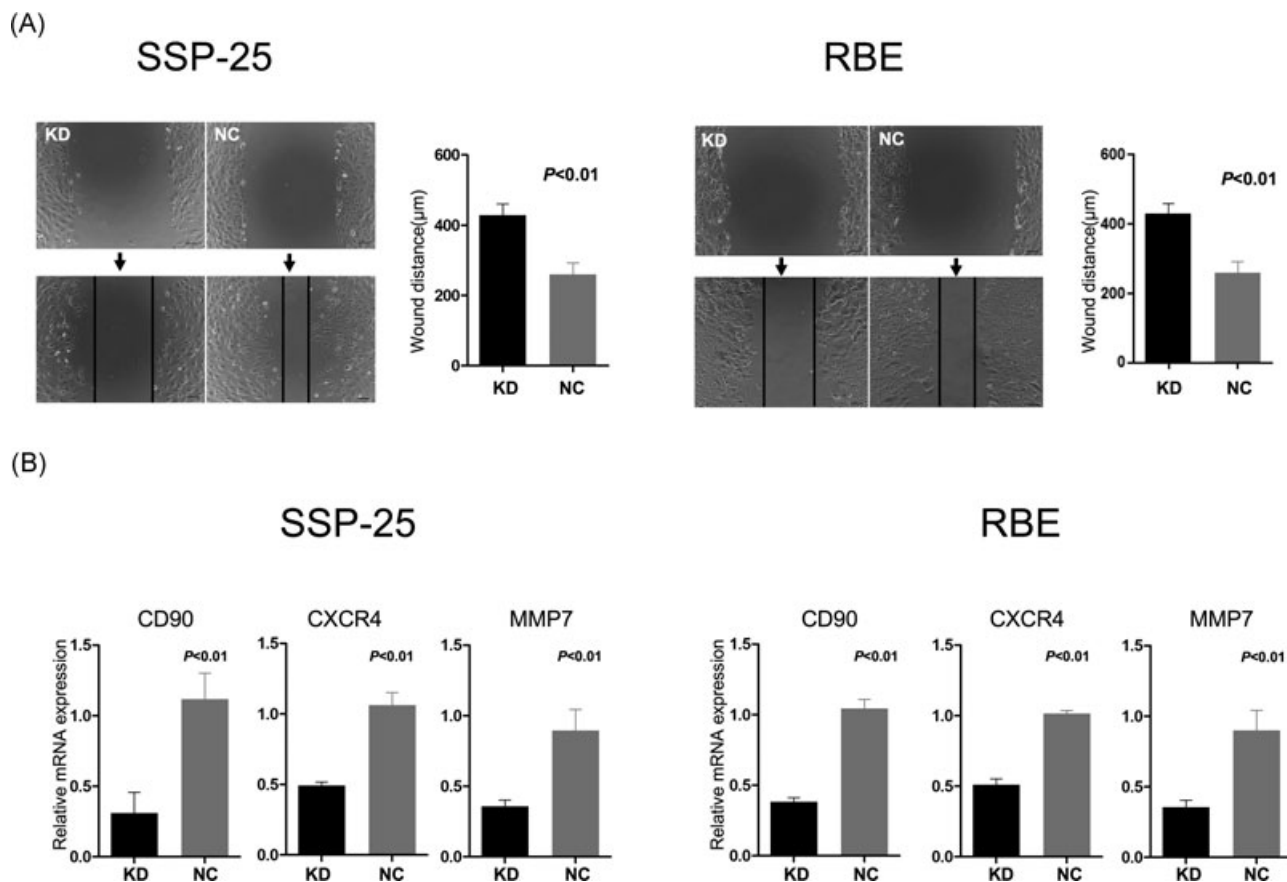


FIGURE 4 A, Wound-healing assay. CD90 KD reduced the wound-healing ability of CD90⁺ cells. B, RT-qPCR analyses of cells in the CD90 KD and NC groups. Expression of both CXCR4 and MMP7 was significantly reduced by CD90 KD according to the Student t test. CD90, cluster of differentiation 90; KD, knockdown; mRNA, messenger RNA; NC, negative control; RT-qPCR, reverse-transcription quantitative polymerase chain reaction

specimens, CD90⁺ cells were present in the tumor stroma. There have been a number of reports concerning the relationship between stromal CD90 expression and prognosis, and the significance of cancer-associated fibroblasts (CAFs) in the ICC stroma.^{19,20} Therefore, we examined CD90 and α -SMA expression by double immunofluorescence staining to explore the possibility that the CD90⁺ cells observed were CAFs. However, no cells coexpressing CD90 and α -SMA were noted, suggesting that this was not the case.

To further elucidate the characteristics of CD90⁺ ICC cells, human ICC cell lines were investigated in vitro, with a focus on EMT. Both the migration and wound-healing assay indicated that the motility of CD90⁺ cells was significantly greater than that of CD90⁻ cells. Moreover, RT-qPCR analysis showed that expression of the EMT-related genes CXCR4 and MMP7 was significantly higher in CD90⁺ than in CD90⁻ cells. CXCR4 has been reported to drive tumor cell EMT through various signaling pathways, and its overexpression is indicative of poor prognosis and associated with metastasis in various cancers, including ICC.²¹⁻²³ MMP7 also plays a role in triggering the EMT by degrading E-cadherin and is associated with poor prognosis in ICC.²⁴⁻²⁶ Our results suggest that the increased migratory ability of CD90⁺ cells was induced by initiation of EMT through expression of CXCR4 and MMP7. This was

supported by the results of siRNA knockdown of CD90, which reversed the increased motility of and CXCR4 and MMP7 expression in CD90⁺ cells.

The Wnt/ β -catenin signaling pathway has important functional roles in both normal and cancer cells,²⁷ and its activation leads to expression of Wnt target genes, including CXCR4 and MMP7. The elevated expression of these two genes evidenced by our RT-qPCR analysis suggested that Wnt/ β -catenin signaling was activated in CD90⁺ ICC cells. Subsequent immunocytochemical examination and TCF/LEF luciferase assays confirmed that this signaling pathway was indeed constitutively activated. ICG-001 is a Wnt/ β -catenin signaling inhibitor that binds cAMP-responsive element-binding protein-binding protein to disrupt its interaction with β -catenin and inhibit its function as a coactivator of Wnt/ β -catenin-mediated transcription.^{28,29} Here, ICG-001 suppressed expression of both MMP7 and CXCR4 in CD90⁺ cells. Taken together with our above results, these observations suggest that activation of the Wnt/ β -catenin pathway in CD90⁺ cells resulted in upregulation of MMP7 and CXCR4, and subsequent induction of EMT. Consequently, CD90 expression in human ICC might be associated with poor prognosis through its promotion of lymph node metastasis.

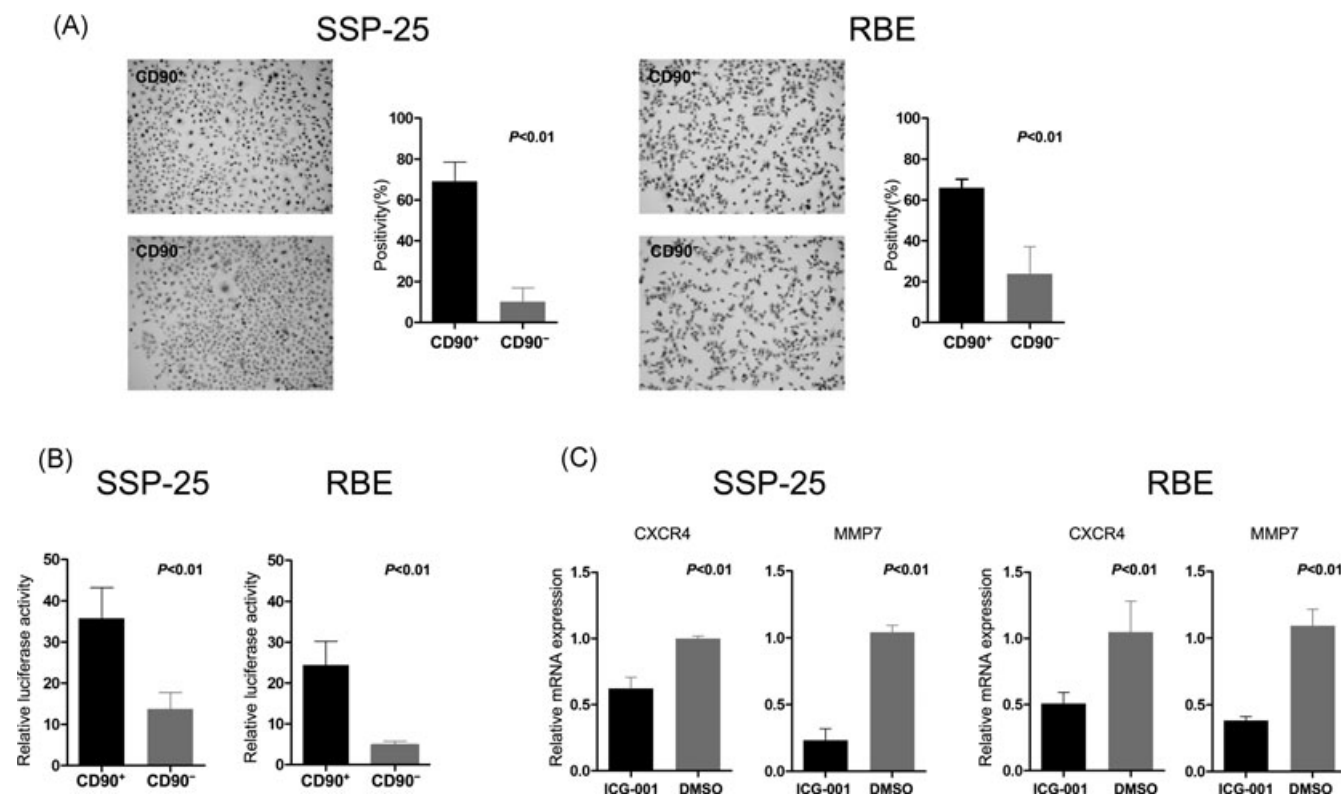


FIGURE 5 A, Immunocytochemical analysis of active β -catenin in the nuclei of CD90⁺ cells. B, TCF/LEF luciferase assay of CD90⁺ cells. C, RT-qPCR analysis of CD90⁺ cells treated with ICG-001. DMSO was used as a negative control. CD90, cluster of differentiation 90; DMSO, dimethyl sulfoxide; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; TCF/LEF, T-cell factor/lymphoid enhancer factor

There were a number of limitations to this study. First, the increased metastatic potential of CD90⁺ cells observed could not be reproduced in animal models. Although CD90⁺ or CD90⁻ tumor cells were injected into the abdominal cavities of nonobese diabetic/severe combined immunodeficiency mice, no tumors developed in either group. It is reported that the cell lines we used in this study have low tumorigenicity.³⁰ Therefore, there appear to be some discrepancies between animal models and clinical specimens with respect to CD90 expression in ICC. Second, the role of CD90 was not well defined in the present work. Although CD90 was shown to regulate CXCR4 and MMP7 expression in some manner, the mechanism underlying such regulation, including the relationship between CD90 and the Wnt/ β -catenin pathway, remains to be elucidated.

5 | CONCLUSION

Our study indicates that CD90⁺ ICC cells exhibit increased metastatic potential associated with EMT via activation of Wnt/ β -catenin signaling, thus resulting in a greater risk of lymph node metastasis.

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CONFLICTS OF INTEREST

All authors declare that there are no conflicts 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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