



OPEN Circulating galectin-9 as a novel prognosticator in patients with intrahepatic cholangiocarcinoma undergoing surgical resection

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Background Intrahepatic cholangiocarcinoma (ICC) is a rare but highly malignant liver cancer. Surgical resection provides the best long-term survival, yet poor prognosis requires improved treatments. Galectin-9 (GAL9) has gained attention for its role in tumor biology. This study investigated circulating galectin-9 (cGAL9) levels in ICC patients undergoing resection, along with tumor tissue characteristics. **Methods** GAL9 expression levels in circulating and tumor cells were measured using enzyme-linked immunosorbent assay and immunohistochemistry (IHC) in 91 ICC surgical patients. The mRNA expression levels of candidate genes were analyzed in 44 available frozen tissue samples.

Results The optimal cGAL9 cutoff was 12.0 ng/ml using minimum *P* value approach. Higher cGAL9 levels linked to multiple tumors ($P = 0.046$), poorer overall survival (OS), and recurrence-free survival (RFS). Tumor cell GAL9 expression by IHC did not correlate with OS or RFS. cGAL9 levels did not correlate with tumor cell expression in IHC analyses or GAL9 mRNA in resected specimens. However, cGAL9 levels correlated with mRNA levels of glycolysis markers (glucose transporter 1 and hypoxia inducible factor 1 alpha).

Conclusions Preoperative cGAL9 serves as a novel prognosticator for ICC patients after surgical resection. Its association with glycolysis highlights the potential for therapeutic guidance.

Keywords Liver cancer, Intrahepatic cholangiocarcinoma, Galectin 9, Hif 1 alpha, Glut1

Abbreviations

ICC	Intrahepatic cholangiocarcinoma
GAL9	Galectin-9
TIM3	T cell immunoglobulin and mucin domain 3
cGAL9	Circulating galectin-9
ELISA	Enzyme-linked immunosorbent assay
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
IHC	Immunohistochemistry
AJCC	American joint commission on cancer
DAB	Diaminobenzidine
OS	Overall survival
RFS	Recurrence-free survival
AIC	Akaike's information criterion
ROC	Receiver operating characteristic
AUC	Area under the curve
CEA	Carcinoembryonic antigen
CA19-9	Carbohydrate antigen 19-9
CRP	C-reactive protein
NLR	Neutrophil-to-lymphocyte ratio
GLUT1	Glucose transporter 1
HIF1 α	Hypoxia inducible factor 1 alpha

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Intrahepatic cholangiocarcinoma (ICC) is a rare but highly malignant form of liver cancer, with an increasing incidence and poor prognosis.¹ Surgical resection is the best option for achieving long-term survival in ICC patients, and a recent randomized controlled trial demonstrated that adjuvant chemotherapy can prolong survival in selected patients with biliary tract cancer, including ICC.^{2,3} However, it should be noted that outcomes with a 5-year survival rate ranging from 17 to 44%⁴ are unsatisfactory, and further improvement of the treatment strategy is required. In this context, identifying biomarkers to predict survival outcomes, guide therapeutic decisions, and evaluate drug resistance to adjuvant therapies is paramount for advancing the management of ICC.⁵

Galectin-9 (GAL9) is a tandem-repeat type galectin with two carbohydrate recognition domains and was first identified as an eosinophil chemoattractant and activator.⁶ GAL9 is a multifunctional protein involved in various aspects of tumor biology such as cell adhesion, survival, immune evasion, and angiogenesis.^{7,8} Importantly, GAL9 serves as a ligand for T cell immunoglobulin and mucin domain 3 (TIM3), and regulates immune homeostasis and tumor cell survival.⁹ This interaction promotes CD8 positive T cell exhaustion and expansion of myeloid-derived suppressor cells, contributing to tumor growth and immune evasion.¹⁰ GAL9 expression often differs between normal and cancer tissues, and given its functions and prognostic potential, GAL9 has emerged as a promising therapeutic target and biomarker in solid tumors.^{8,10,11} Especially, a notable advantage of GAL9 is its secreted nature, which allows noninvasive measurement through circulating levels. Recent studies have demonstrated the significant prognostic value of circulating galectin-9 (cGAL9) in various cancers,^{12–17} however, its role in ICC remains unexplored.

The aim of this study was to clarify the value of cGAL9 as a biomarker in ICC patients who underwent surgical resection. Additionally, to better understand its biological implications, we analyzed the tumor tissue expression of GAL9 and selected gene profiles, providing a comprehensive perspective on its role in ICC.

Methods

Study design

This study consisted of two parts: (i) a biomarker study to assess the prognostic value of cGAL9 using enzyme-linked immunosorbent assay (ELISA) on preserved blood samples, and (ii) an exploratory study to investigate the molecular associations between cGAL9 and tumor tissue. This study was approved by the Ethics Committee of Kyoto University Hospital, Graduate School and Faculty of Medicine (Approval No. R3844), and the Opt-out informed consent was obtained to use the participant data for research.

Study population

Biomarker study

This part of the study included 91 patients with pathologically diagnosed ICC who underwent surgical resection in Department of Surgery, Kyoto University Hospital between 2003 and 2019. Patients were included who were pathologically diagnosed with ICC after surgery, underwent curative (R0 or R1) resection, had no distant metastasis other than para-aortic lymph node metastasis, and had preoperative serum samples stored. Patients with recurrent ICC who had previously undergone liver resection were excluded (Supplementary Table 1). Operative procedures and surgical policy in our institution are described in Supplementary Methods. As a control subject, serum samples were collected from 16 healthy volunteers.

Exploratory study

GAL9 expression in tumor cells was evaluated by immunohistochemistry (IHC) using formalin-fixed paraffin-embedded resection specimens. In this assay, the same 91 patients in the biomarker study were included. Among this cohort, frozen tumor resection specimens were available for 44 patients. mRNA was extracted from the frozen specimens and used for bulk analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Study variables

Clinicopathological and survival data were obtained from a prospectively maintained institutional database. Clinicopathologic data, including age, sex, hepatitis virus markers, tumor makers, the Child–Pugh classification, primary tumor characteristics, and treatment-related variables, were collected. The tumor stage was assessed according to the 8th Edition of the AJCC Cancer Staging Manual: Pancreas and Hepatobiliary Cancers.¹⁸ Patients were followed up regularly after surgery: every 3 months during years 0–3, every 6 months between years 3 and 5, and annually from years 5 to 10. Follow-up included imaging examinations such as computed tomography and measurement of tumor markers (CEA: carcinoembryonic antigen, and CA19-9: carbohydrate antigen 19–9). Recurrence was defined as the detection of lesions on imaging indicative of tumor recurrence. Postoperative complications were assessed based on the Clavien–Dindo classification,¹⁹ and complications were defined as those classified as Grade II or higher. The follow-up data were updated in May 2024.

ELISA

The concentration of GAL9 in serum samples collected from patients and healthy volunteers was measured using the Human Galectin-9 Quantikine ELISA Kit (R&D Systems, #DGAL90), following the manufacturer's recommended protocol. Optical density was measured using a microplate reader (Molecular Devices, SpectraMax 340PC384) at a wavelength of 450 nm.

Immunohistochemistry

The expression of GAL9 in tumor tissues was evaluated by immunohistochemistry. Formalin-fixed paraffin-embedded 4 μ m-thick sections were deparaffinized and subjected to antigen retrieval by autoclaving. The sections were incubated overnight at 4 °C with an anti-GAL9 primary antibody (Cell Signaling Technology, #54,330). The antibody dilution condition is shown in Supplementary Table 2. EnVision + System- HRP Labelled Polymer Anti-Rabbit, a horseradish peroxidase-labelled polymer-conjugated anti-rabbit IgG antibody, was used as the secondary antibody, and the signal was developed using diaminobenzidine (DAB) substrate (Takara Bio). Hematoxylin counterstaining was performed for contrast. Human spleen tissue was used as the positive control for GAL9 staining. To quantify GAL9 expression, five randomly selected fields per case ($\times 100$ magnification) were processed using ImageJ to measure the DAB-positive areas, and the average percentage was calculated.

qRT-PCR

Total RNA was isolated from frozen tumor tissues using TRIzol reagent (Thermo Fisher Scientific), and purified using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the Omniscript RT Kit (Qiagen), and qRT-PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) and Fast SYBR Green Master Mix (Applied Biosystems). The expression levels of the target genes were normalized to those of *GAPDH* using the $\Delta\Delta C_t$ method. The ICC cell line (SSP-25, by the RIKEN BioResource Research Center, Tsukuba, Japan) was used as a reference sample (the value of SSP-25 cells = 1). For genes that were not expressed in SSP-25, the minimum value of the amplified samples was set to 1. Primers used in the experiments are listed in Supplementary Table 3.

Statistical analyses

Data from clinical samples were analyzed as follows: continuous variables were expressed as median values and interquartile ranges and compared using the Mann–Whitney U test. Categorical variables were expressed as numbers and percentages, and compared using the chi-square test or Fisher's exact test. The correlation between continuous variables was evaluated using the Spearman's correlation test. To label the strength of correlation, for absolute values of ρ , 0 to 0.19 is regarded as very weak, 0.2 to 0.39 as weak, 0.40 to 0.59 as moderate, 0.6 to 0.79 as strong, and 0.8 to 1 as very strong.²⁰ Overall survival (OS) was calculated from the date of surgery to the date of death or last follow-up, and recurrence-free survival (RFS) was calculated from the date of surgery to the date of death, recurrence, or last follow-up. Survival and recurrence rates were estimated using the Kaplan–Meier method and compared using the log-rank test. An optimal cut-off value of cGAL9 was determined based on the maximum significant OS differences that were calculated using the log-rank test (i.e., the minimum P value approach), and the value was rounded off to the nearest integer. A multivariate analysis was performed using the Cox regression model for variables with $P < 0.10$ in univariate analysis and selected variables on the basis of previous studies. When collinearity was encountered, a choice was made based on the P value and clinical reason. A P value < 0.05 was considered statistically significant. Receiver operating characteristic (ROC) analysis was conducted to assess prognostic predictive ability, and the ROC—area under the curve (AUC) was calculated. Statistical analyses were performed using JMP Pro Ver 17.0 software (SAS Institute), and a P value of less than 0.05 was considered statistically significant.

Results

Patient demographics

Patient demographics are shown in Table 1. Of these, 27 patients (29.7%) were classified as stage III or higher. Major hepatectomy was performed in 68 patients (74.7%), and adjuvant chemotherapy was administered to 47 patients (51.7%).

Comparison of cGAL9 levels between ICC patients and healthy controls

First, we evaluated cGAL9 levels in ICC patients and healthy controls. cGAL9 levels were significantly higher in ICC patients ($n = 91$) than in healthy controls ($n = 16$) [median; 9.2 ng/ml (range: 3.2–27.2) vs. 4.0 ng/mL (range: 3.2–8.8), $P < 0.001$, Fig. 1A]. ROC analysis of disease detection ability in a cohort of 91 patients and 16 healthy volunteers demonstrated an AUC of 0.936 (Fig. 1B).

Correlations between cGAL9 levels and tumor factors

Next, we evaluated cGAL9 levels in ICC patients according to the significant tumor factors based on the previous studies.²¹ The cGAL9 level comparison of the postoperative tumor factors is shown in Fig. 1C. The cGAL9 levels were significantly higher in multiple tumors (solitary vs. multiple, $n = 74$ vs. $n = 17$, $P = 0.046$). However, the cGAL9 levels did not differ significantly, according to AJCC stage (stage I–II vs. III–IV, $n = 64$ vs. $n = 27$, respectively, $P = 0.224$), microvascular invasion (no vs. yes, $n = 38$ vs. $n = 53$, respectively, $P = 0.847$), and N stage (N0/NX vs. N1, $n = 70$ vs. $n = 21$, respectively, $P = 0.504$). Furthermore, we assessed the relations between the known tumor markers, CEA or CA19-9, and cGAL9. As shown in Fig. 1D, no correlation was found between cGAL9 and either CEA or CA19-9 (Spearman's $\rho = -0.02$ and 0.13 , $P = 0.888$ and 0.219 , respectively). Given the previously reported association between cGAL9 and inflammation, we investigated the relationship between cGAL9 levels and commonly used inflammatory markers, including C-reactive protein (CRP) and neutrophil-to-lymphocyte ratio (NLR) (Supplementary Fig. 1). A weak but significant correlation was observed between cGAL9 and CRP (Spearman's $\rho = 0.27$, $P = 0.009$). In contrast, no significant correlation was found between cGAL9 and NLR (Spearman's $\rho = 0.07$, $P = 0.508$).

Variables	Overall (n = 91)	cGAL9 Low (n = 70)	cGAL9 High (n = 21)	P value
Clinical factors				
Age (≥ 70)	38 (41.8%)	27 (38.6%)	11 (52.4%)	0.260
Sex (male)	55 (60.4%)	46 (65.7%)	9 (42.9%)	0.060
Hepatitis B	6 (6.6%)	5 (7.1%)	1 (4.8%)	1.000 ^a
Hepatitis C	12 (13.2%)	7 (10.0%)	5 (23.8%)	0.139 ^a
Child–Pugh A	87 (95.6%)	67 (95.7%)	20 (95.2%)	1.000 ^a
CEA (ng/mL)	2.8 (1.9–4.6)	2.8 (1.9–4.7)	2.8 (1.3–4.4)	0.852
CA19-9 (IU/mL)	39.7 (18.5–198.4)	35.2 (17.2–145.0)	63.1 (24.6–236.3)	0.350
cGAL9 (ng/mL)	9.2 (6.8–11.8)	8.2 (6.1–10.1)	15.3 (13.7–17.7)	–
Pathological factors				
Tumor diameter (cm)	4 (2.7–6)	4 (2.5–5.5)	4 (2.9–10.3)	0.230
Multiple tumors	17 (18.7%)	10 (14.3%)	7 (33.3%)	0.050*
LN status				
N1	21 (23.1%)	14 (20.0%)	7 (33.3%)	0.397
N0	46 (50.1%)	36 (51.4%)	10 (47.6%)	
NX	24 (26.4%)	20 (28.6%)	4 (19.1%)	
Poor differentiation	10 (11.0%)	7 (10.0%)	3 (14.3%)	0.692 ^a
Microvascular invasion	53 (58.2%)	39 (55.7%)	14 (66.7%)	0.372
Biliary invasion	36 (39.6%)	25 (35.7%)	11 (52.4%)	0.171
Surgical outcomes				
R0 resection	71 (78.0%)	54 (77.1%)	17 (81.0%)	0.712
Major liver resection (≥ 3 segments)	68 (74.7%)	52 (74.3%)	16 (76.2%)	0.860
Neoadjuvant chemotherapy	4 (4.4%)	3 (4.3%)	1 (4.8%)	1.000 ^a
Adjuvant chemotherapy	47 (51.7%)	38 (54.3%)	9 (42.9%)	0.358
Gemcitabine	34 (37.4%)	27 (38.6%)	7 (33.3%)	0.663
S-1	13 (14.3%)	10 (14.3%)	3 (14.3%)	1.000
Others	3 (3.3%)	3 (4.3%)	0 (0.0%)	1.000 ^a
Morbidity (C-D grade $\geq$ IIa)	36 (39.6%)	26 (37.1%)	10 (47.6%)	0.389
Mortality (< 30 days)	1 (1.1%)	0 (0.0%)	1 (4.8%)	0.231 ^a

Table 1. Clinicopathological characteristics according to the cGAL9 levels. ^aFisher's exact test was used. cGAL9; Circulating galectin-9, CEA; Carcinoembryonic antigen, CA19-9; Carbohydrate antigen 19–9, LN; Lymph node, C-D: Clavien-Dindo classification. *Significant difference $P < 0.05$.

Impact of cGAL9 levels on survival outcomes following surgical resection in ICC patients

We then examined whether cGAL9 levels affected survival outcomes in ICC patients after surgical resection. The median follow-up period was 54.0 months (range: 0.1–209.3 months). During the observation period, 60 patients experienced tumor recurrence, and 66 patients died (54 from ICC-related causes and 12 not from ICC-related causes). The overall median OS was 54.0 months, and the 3- and 5-year OS rates were 60.4% and 46.2%, respectively. The median RFS was 17.7 months, and the 1- and 3-year RFS rates were 57.1% and 34.1%, respectively.

Using the minimum P value approach for the OS curve, the cutoff value of cGAL9 was determined to be 12.0 ng/ml by adjusting in 1 ng/ml increments (Supplementary Table 4). ICC patients were divided into the two groups according to this cutoff value: high cGAL9 ($n = 21$) and low cGAL9 groups ($n = 70$). Patient demographics according to this value are also shown in Table 1. High cGAL9 levels correlated with the presence of multiple tumors ($P = 0.050$; Table 1); however, there were no significant differences in the other variables.

In this setting, OS was significantly shorter in patients with high cGAL9 levels than in those with low cGAL9 levels ($P < 0.001$, Fig. 2A). The median OS and 3- and 5-year survival rates of the high and low cGAL9 groups were 18.8 months versus 71.8 months, 38.1% versus 67.1%, and 19.1% versus 54.3%, respectively. RFS was significantly shorter in patients with high cGAL9 levels than in those with low cGAL9 levels ($P = 0.004$, Fig. 2B). The median RFS and 1- and 3-year RFS rates of the high and low cGAL9 groups were 6.2 months versus 18.2 months, 38.1% versus 62.9%, and 14.3% versus 40.0%, respectively.

To assess the prognostic factors for OS and RFS, multivariate analyses were performed (Table 2). High cGAL9 levels and the presence of multiple tumors were identified as independent prognostic factors for both OS and RFS.

Furthermore, to evaluate the interaction between cGAL9 and therapeutic and tumor factors affecting prognosis, patients were stratified according to adjuvant chemotherapy and significant prognostic tumor factors (Table 3). Regardless of the use of adjuvant chemotherapy, OS was significantly shorter in patients with high cGAL9 levels than in those with low cGAL9 levels (adjuvant chemotherapy $-/+$, $P = 0.003/P = 0.011$, respectively). Similarly, patients with high cGAL9 levels had a significantly shorter OS regardless of the presence

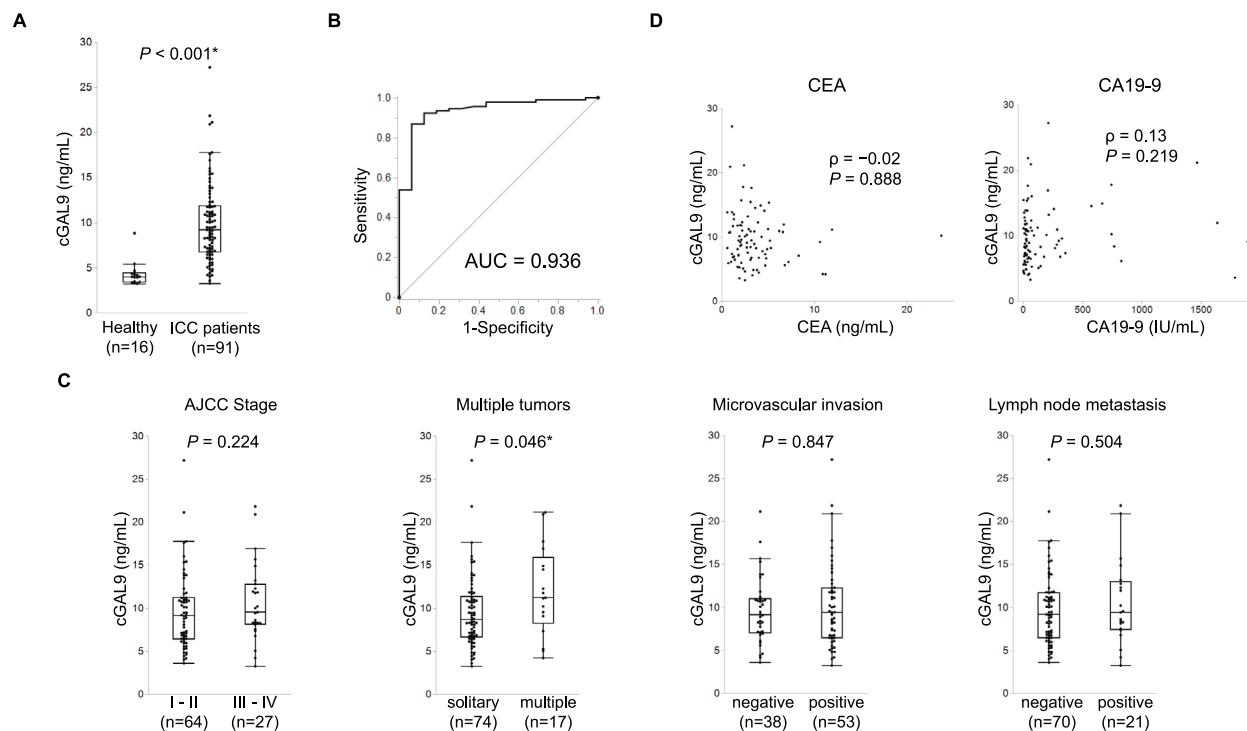


Fig. 1. Comparison of cGAL9 levels between ICC patients and healthy volunteers, and relations between cGAL9 levels and tumor factors. **A**, Comparison of cGAL9 levels between ICC patients (n = 91) and healthy volunteers (n = 16). **B**, ROC analysis of disease detection ability in the cohort of 91 ICC patients and 16 healthy volunteers. **C**, cGAL9 levels according to the significant tumor factors in ICC patients (n = 91). **D**, Relations between known tumor makers and cGAL9 in ICC patients (n = 91). * Significant difference $P < 0.05$. Abbreviations: cGAL9; circulating galectin-9, ICC; intrahepatic cholangiocarcinoma, ROC; receiver operating characteristic, CEA; carcinoembryonic antigen, CA19-9; carbohydrate antigen 19–9, AJCC; American Joint Commission on Cancer.

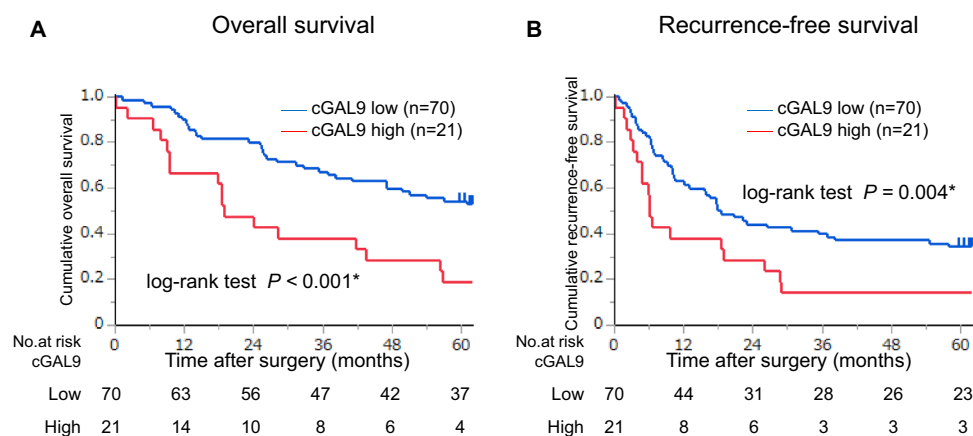


Fig. 2. Prognostic impact of cGAL9 levels in ICC patients after surgical resection. **A** and **B**, Kaplan–Meier analyses for OS (**A**) and RFS (**B**) according to the cGAL9 levels. *Significant difference $P < 0.05$. Abbreviations: cGAL9; circulating galectin-9, ICC; intrahepatic cholangiocarcinoma, OS; overall survival, RFS; recurrence-free survival.

of microvascular invasion (microvascular invasion $-/+$, $P = 0.047/P < 0.001$, respectively). In the group of solitary tumors or negative lymph nodes (N0 or NX), patients with high cGAL9 levels had significantly worse OS than those with low cGAL9 levels ($P < 0.001$ and $P < 0.001$, respectively). In the group of multiple tumors or positive lymph nodes, no significant difference in OS was observed according to the cGAL9 levels ($P = 0.526$ and $P = 0.737$, respectively).

Variables	Univariate analysis	Multivariate analysis	P value
	P value	Hazard ratio (95% confidence interval)	
Overall survival			
Age ≥ 70 years (vs. < 70 years)	0.312		
Male (vs. female)	0.798		
High cGAL9 (vs. low)	< 0.001*	2.106 (1.145–3.874)	0.017*
Multiple tumors (vs. solitary tumor)	< 0.001*	2.379 (1.191–4.751)	0.014*
Lymph node metastasis (vs. N0/Nx)	< 0.001*	1.412 (0.720–2.771)	0.316
Biliary invasion (vs. negative)	0.435		
Microvascular invasion (vs. negative)	0.035*	1.217 (0.685–2.163)	0.502
Poor differentiation (vs. well/moderate)	0.580		
Tumor size > 5 cm (vs. ≤ 5 cm)	0.342		
R1 resection (vs. R0 resection)	0.045*	1.759 (1.312–4.105)	0.056
Recurrence-free survival			
Age ≥ 70 years (vs. < 70 years)	0.094	0.760 (0.430–1.342)	0.343
Male (vs. female)	0.671		
High cGAL9 (vs. low)	0.008*	1841 (1.0127–3.301)	0.041*
Multiple tumors (vs. solitary tumor)	< 0.001*	3.905 (1.826–8.350)	< 0.001*
Lymph node metastasis (vs. N0/Nx)	0.003*	1.325 (0.681–2.578)	0.408
Biliary invasion (vs. negative)	0.551		
Microvascular invasion (vs. negative)	0.051	1.139 (0.656–1.976)	0.645
Poor differentiation (vs. well/moderate)	0.060	0.748 (0.320–1.752)	0.503
Tumor size > 5 cm (vs. ≤ 5 cm)	0.093	0.943 (0.525–1.697)	0.847
R1 resection (vs. R0 resection)	0.119		

Table 2. Univariate and multivariate analyses for survival outcomes. * Significant difference $P < 0.05$. cGAL9: Circulating galectin-9.

Variables		Median overall survival (months)		P value ^a
		cGAL9 low	cGAL9 high	
Adjuvant chemotherapy	– (n = 44)	89.1 (n = 32)	13.6 (n = 12)	0.003*
	+ (n = 47)	55.5 (n = 38)	28.3 (n = 9)	0.011*
Tumor number	Solitary (n = 74)	84.4 (n = 60)	35.0 (n = 14)	< 0.001*
	Multiple (n = 17)	13.2 (n = 10)	9.4 (n = 7)	0.526
Microvascular invasion	– (n = 38)	101.9 (n = 31)	56.9 (n = 7)	0.047*
	+ (n = 53)	51.0 (n = 39)	18.1 (n = 14)	< 0.001*
Lymph node status	N0/X (n = 70)	101.9 (n = 56)	18.3 (n = 14)	< 0.001*
	N1 (n = 21)	25.6 (n = 14)	24.0 (n = 7)	0.737

Table 3. Comparison of median overall survival (months) between high and low cGAL9 levels in patients with resected intrahepatic cholangiocarcinoma, stratified according to the presence or absence of adjuvant chemotherapy and significant prognostic factors. ^alog-rank test. cGAL9: Circulating galectin-9. *Significant difference $P < 0.05$.

Tissue expression levels and prognostic value of GAL9 in resected specimens

Next, we evaluated the tumor cell expression levels of GAL9 in resected specimens of ICC patients. The validity of immunohistochemical detection of GAL9 was confirmed using a validated anti-GAL9 antibody (Cell Signaling Technology, #54,330; Supplementary Fig. 2). IHC analyses revealed that positive expression of GAL9 in tumor cells was found in only 9 patients out of the 91 patients (9.9%, Fig. 3A). On the basis of IHC positivity of tumor cells, survival outcomes were analyzed. Figure 3B shows the OS and RFS between the positive and negative groups of GAL9 expression by IHC. There were no significant differences both in OS or RFS between the two groups ($P = 0.892$ and $P = 0.250$, respectively). We evaluated the association between cGAL9 and the tissue expression levels of GAL9. According to the tumor cell positivity by IHC, there was no significant difference in cGAL9 levels ($P = 0.671$, Fig. 3C).

Meanwhile, GAL9-positive cells were also observed in the stroma; however, their presence was sporadic (representative image are shown in Supplementary Fig. 3). To estimate the potential expression levels of GAL9 in stromal cells, mRNA expression was analyzed using frozen tissue samples. Positive expression of GAL9 (LGALS9) mRNA was detected in 27 of 44 patients (61.4%). Among patients whose tumor cells were negative for GAL9

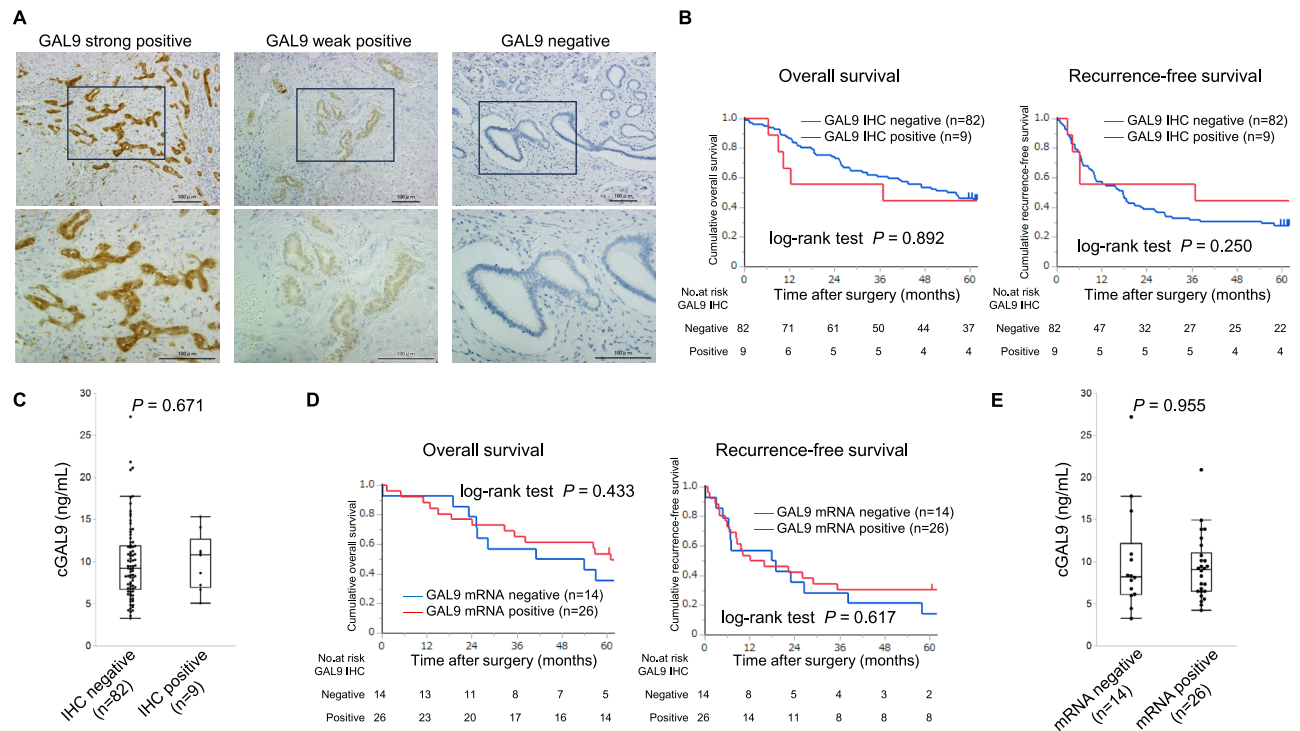


Fig. 3. Tissue expression levels by IHC and prognostic value of GAL9 in resected specimens. **A.** IHC staining of GAL9 in surgical specimens of ICC. **B.** OS and RFS between the positive and negative groups of GAL9 IHC expression. **C.** OS and RFS between the positive and negative groups of GAL9 (*LGALS9*) mRNA expression in the GAL9 IHC negative cohort (n = 40). **D.** Comparison of cGAL9 levels according to GAL9 mRNA positivity in the GAL9 IHC negative cohort. * Significant difference $P < 0.05$. Abbreviations: GAL9; galectin-9, IHC; immunohistochemistry, cGAL9; circulating galectin-9, ICC; intrahepatic cholangiocarcinoma, OS; overall survival, RFS; recurrence-free survival.

by IHC (n = 40), no significant differences in OS or RFS were observed between those with positive (n = 26) and negative (n = 14) GAL9 mRNA expression ($P = 0.433$ and $P = 0.617$, respectively; Fig. 3C). Additionally, no significant difference in cGAL9 levels was found between these two groups ($P = 0.955$; Fig. 3D), and there was no significant correlation between cGAL9 and GAL9 mRNA levels (Spearman's $\rho = -0.10$, $P = 0.540$; Supplementary Fig. 4).

Relations between mRNA expression in tumor tissue and cGAL9 Levels in ICC patients

To further explore the molecular characteristics associated with high cGAL9 levels, we examined the relations between cGAL9 levels and mRNA expression in tumor tissues.

From the clinical aspects, as shown in Table 1, high cGAL9 levels were associated with the presence of multiple tumors. Thus, we explored the correlations between cGAL9 levels and various genes associated with multiple tumors. Based on previous studies, we assessed eight genes associated with tumor glycolysis [glucose transporter 1 (GLUT1, *SLC2A1*) and hypoxia inducible factor 1 alpha (HIF1 α , *HIF1A*)]²³ and epithelial-mesenchymal transition (EMT; *TGFB1*, *ZEB1*, *TWIST1*, *SNAIL*, *CDH1*, and *VIM*).²⁴ As shown in Fig. 4A, correlation analyses revealed significant positive correlations between cGAL9 levels and the mRNA expression of GLUT1 and HIF1 α , with Spearman's correlation coefficient of 0.41 and 0.40 ($P = 0.005$ and $P = 0.007$, respectively), indicating moderate correlations. Conversely, no significant correlation was observed between the cGAL9 levels and EMT-related gene expression (Fig. 4B).

Discussion

In this study, we investigated the prognostic role of cGAL9 levels in ICC patients after surgical resection, and the associated molecular features using the tumor tissue. This study found that (i) cGAL9 levels were associated with tumor multiplicity, (ii) cGAL9 predicted OS and RFS, and was identified as a robust prognostic factor; (iii) cGAL9 levels were not correlated with tumor tissue expression levels of GAL9 but were associated with mRNA expression levels of tumor glycolysis (i.e., GLUT1 and HIF1 α). Collectively, cGAL9 levels may represent clinical relevance in these patients, and could potentially serve as a surrogate marker for tumor glycolysis.

In this study, we aimed to explore novel biomarkers that could predict the prognosis of ICC patients after surgical resection. At the same time, there has been growing interest in immune checkpoint inhibitors that have led to a paradigm shift in biliary tract cancer, including ICC.^{25,26} Currently, interest in immune pathways, such as the programmed cell death 1/programmed cell death 1—ligand 1 axis, has increased,²⁷ however, the GAL9-TIM3 pathway remains underexplored in ICC. Therefore, we focused on the prognostic impact of GAL9, which can act

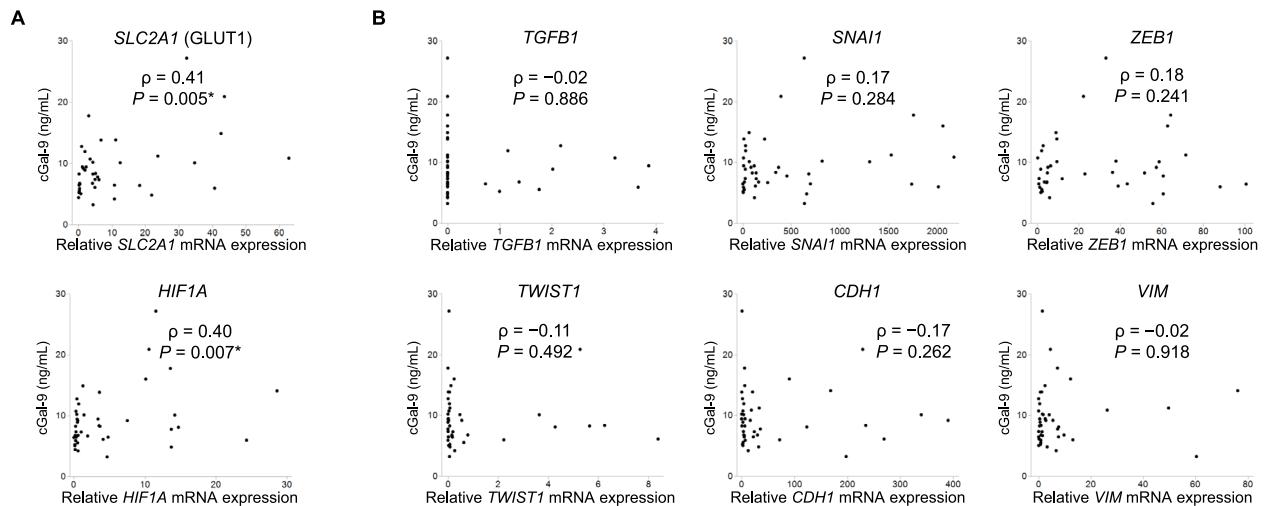


Fig. 4. Relations between cGAL9 and mRNA expression of genes related to tumor glycolysis and EMT. * Significant difference $P < 0.05$. Abbreviations: cGAL9; circulating galectin-9, EMT; epithelial-mesenchymal transition.

as a ligand for TIM3, and prioritized its circulating levels due to the noninvasive nature of blood measurements. The presence of ICC appeared to upregulate cGAL9 levels, as shown in Fig. 1A, indicating that cGAL9 seemed to be tumor-associated. Specifically, cGAL9 levels were associated with tumor multiplicity and correlated with both OS and RFS. Additionally, an independent cohort study using plasma samples reaffirmed its relevance regarding survival and recurrence, supporting the potential utility of cGAL9 as a clinical prognosticator for ICC. Previous reports on the prognostic value of cGAL9 in solid tumors have shown inconsistent significance. For example, high levels of cGAL9 predict poor prognosis in pancreatic and ovarian cancers^{12,14} but favorable prognosis in hepatocellular carcinoma and non-small cell lung cancer.^{13,17} This study is the first to report the prognostic significance of cGAL9 in ICC, and that high levels of cGAL9 were associated with poor prognosis.

GAL9 is known to be expressed in various tissues throughout the body, mainly in bone marrow and lymphatic tissues,⁷ and any organ may be a source of cGAL9. GAL9 is released extracellularly via vesicles or exosomes through a non-classical pathway, but blood levels may also increase due to increased production in cancer cells, tissue damage, and responses to stress such as infection and inflammation.²² In this study as well, cGAL9 showed a weak correlation with CRP, but no significant correlation with NLR. The presence of inflammation due to hilar invasion or cholangitis has been reported as a poor prognostic factor in intrahepatic cholangiocarcinoma,²⁸ and this may partially explain why cases with high cGAL9 levels have a worse prognosis, but it is unlikely to account for the entire mechanism. In vitro study has reported that cell lines derived from breast cancer, Wilms tumor, kidney rhabdoid tumor, and glioblastoma secrete little or no GAL9 when cultured alone, but secrete GAL9 when co-cultured with T cells.²⁹ Considering the possibility that cGAL9 in ICC patients may be tumor-derived, we proceeded subsequent analyses to evaluate the expression of GAL9 in cancer tissues.

To investigate the mechanism by which cGAL9 levels impacted survival outcomes, we first performed IHC analyses using the resected specimens with the hypothesis that GAL9 release was originated from tumor cells. Unexpectedly, GAL9 expression in ICC tumor cells was found in only approximately 10% of the study population. Moreover, GAL9 expression in ICC tumor cells did not show an impact on survival outcomes. In contrast to other malignancies,⁷ tumor cell expression of GAL9 may not affect the prognosis of ICC patients. Furthermore, no significant correlation between GAL9 expression in tumor cells and cGAL9 levels suggested that mechanisms other than direct surrogacy might be involved (Fig. 3C).

To explore another possible influence on cGAL9 levels, we analyzed the mRNA levels of GAL9 in tumor tissues. The mRNA levels of GAL9 could be derived from stromal cells, because limited expression of GAL9 was found in tumor cells based on IHC analyses, which is consistent with another study that evaluated the localization of GAL9 in tissues of hepatocellular carcinoma.³⁰ However, a limited correlation was observed between tissue mRNA levels and cGAL9 (Supplementary Fig. 4). Additionally, no prognostic differences were observed based on tissue GAL9 expression in IHC or mRNA analysis. One hypothesis to explain this is that tumors may promote GAL9 release from other organs. Collectively, tissue expression and blood concentration of GAL9 should be considered separately.

To date, there have been few reports regarding the molecular features of tumors associated with cGAL9, including in ICC patients. To resolve this issue, we explored candidate genes from the clinical perspective, especially focusing on tumor multiplicity. Previously, we reported that tumor multiplicity is associated with glucose metabolism,³¹ and EMT,²⁴ therefore, the correlations between cGAL9 and these genes were evaluated. Although EMT-related genes were not directly correlated with cGAL9 in this study, the association between HIF-1 α and EMT,³² as demonstrated in our previous study,²⁴ suggested that tumor glycolysis associated genes may impact the metastatic potential in ICC patients. Consequently, tumor glycolysis associated genes (GLUT1 and HIF1 α) showed significant and moderate correlations with cGAL9. One possible hypothesis for this explanation

is that tumors with high glucose metabolic activity may stimulate several organs, such as T cells, releasing soluble GAL9 into the blood.¹⁴ Alternatively, cGAL9 itself may contribute to the enhancement of tumor glucose metabolism. However, the precise mechanisms underlying the formation and function of soluble GAL9 remain incompletely understood. Distinguishing the roles of tissue-expressed versus circulating GAL9 will be essential, and further mechanistic studies are warranted to clarify their respective contributions to tumor biology.

In addition, cGAL9 is known to be associated with inflammatory processes,²² and in this study, its levels showed a weak correlation with CRP. Poor prognosis has been reported in ICC patients with hilar invasion, cholangitis, or systemic inflammation,²⁸ suggesting that inflammatory status may partially explain the prognostic relevance of cGAL9. Given that inflammation is closely linked to immune regulation,³³ further investigation into the relationship between cGAL9 and the tumor immune microenvironment may be warranted. These findings raise the possibility that cGAL9 reflects more complex tumor–host interactions that influences disease progression and survival.

How to use cGAL9 should be discussed in the context of clinical practice. Recently, a Japanese study demonstrated that adjuvant S-1 chemotherapy provides benefits for biliary tract cancer,² and adjuvant chemotherapy after surgical resection for ICC is becoming the standard strategy. In addition, the additive effect of immune checkpoint inhibitors on conventional chemotherapy has been demonstrated in biliary tract cancer,^{25,26} and immunotherapy is expected to expand to ICC. In order to further improve the prognosis of ICC, it is necessary to develop more optimal adjuvant drug therapy regimens, consider additional treatment for cases predicted to have poor prognosis, or explore the possibilities of neoadjuvant chemotherapy. In this context, cGAL9 may serve as a useful tool to guide treatment selection, including the choice of adjuvant or neoadjuvant therapy. Furthermore, given its non-invasive nature, cGAL9 also holds promise for use in post-treatment surveillance. However, further research is needed to establish its clinical utility in these settings.

The limitations of this study include its retrospective design, single-center setting, and relatively small sample size, which may have introduced inherent biases. The prognostic factors identified differ partly from those in previous studies,^{34,35} possibly due to institutional practice or sample characteristics. Although this is a biomarker study, external validation has not been performed; in particular, the cut-off value and prognostic role of cGAL9 require confirmation in independent cohorts. Further large-scale, multi-institutional prospective studies are needed to validate and generalize these findings. Frozen section specimens were not stored for approximately half of the patients in the cohort and could not be analyzed. Additionally, regarding the expression of GAL9 in tumor tissues, we interpreted that IHC expression mainly reflects expression in tumor cells, and mRNA expression mainly reflects expression in stromal tissues in accordance with the previous study,¹³ but the validity of this interpretation has not been verified. Furthermore, the detailed molecular mechanisms underlying the involvement of cGAL9 in tumor glycolysis, as well as its unexplained associations (e.g., with gender), have not yet been elucidated. Evaluating the relation between GAL9 and the tumor immune microenvironment is considered an important issue; however, it remains unclear whether cGAL9 actually functions locally within the tumor, and it should be examined in the future. Furthermore, the detailed molecular mechanism by which GAL9 is involved in tumor glycolysis has not yet been explored. Nevertheless, this study is the first to demonstrate the significance and molecular features of GAL9 in ICC. The measurement of cGAL9 may be useful for predicting the prognosis and glycolysis characteristics of ICC patients.

Conclusion

In conclusion, preoperative cGAL9 serves a novel prognosticator in ICC patients after surgical resection. The observed molecular associations between cGAL9 and tumor glycolysis, rather than its tumor expression, provide some implications regarding the prognosis and treatment strategy of ICC.

Data availability

The data that support the findings of this study are available from the corresponding author.

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Study conception and design: M.K. and T.Y.; material preparation and data collection: M.K., T.Y., Y.H., H.N., K.Y., S.K., K.O., Y.T., M.S., T.N., and T.I.; analysis and interpretation of data: M.K., T.Y., Y.H., H.N., K.Y., S.K., K.O., Y.T., M.S., T.N., and K.I.; writing and revision of the manuscript: M.K. and T.Y.; study supervision: E.H. All authors read and commented on the manuscript, and approved the final version.

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Declarations

Ethics approval and consent to participate

The Opt-out informed consent was obtained to use the participant data for research. Informed consent was obtained from all participants and/or their legal guardians. This study was approved by the Ethics Committee

of Kyoto University Hospital, Graduate School and Faculty of Medicine (Approval No. R3844), and performed in accordance with the Declaration of Helsinki.

Competing interests

The authors declare no competing interests.

Additional information

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